Original Article

Improvement of Soluble Production of Reteplase in *Escherichia coli* by Optimization of Chemical Chaperones in Lysis Buffer

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Abstract

Background: Reteplase is a nonglycosylated derivative of recombinant tissue plasminogen activator, a thrombolytic agent, which can be easily expressed in *Escherichia coli*. However, overexpression of reteplase in *E. coli* usually leads to accumulation of insoluble and inactive aggregates and inclusion bodies. In the present study, we aimed to optimize chemical additives of lysis buffer to avoid the initial aggregation and formation of inclusion bodies of reteplase at cell disruption step. **Materials and Methods:** After protein expression in *E. coli* BL21 (DE3), the bacterial cells were disrupted in different lysis buffers using microsmashing. Eleven chemical additives at two concentration levels were combined based on a Plackett–Burman design to prepare 12 different lysis buffers used at cell disruption stage. Then, three additives with the most positive effect on improvement of solubility of reteplase were chosen and used for the second screening based on Box–Behnken model. **Results:** The primary screening results showed that among 11 additives, arginine, K_2PO_4, and cetyltrimethylammonium bromide (CTAB) had the most positive effect on solubility of reteplase. Our final results based on 14 runs of Box–Behnken design showed that the optimum buffer additive condition is 0.005 mg/ml CTAB, 0.065 mg/ml arginine, and 0.026 mg/ml K_2PO_4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and Western blotting of soluble and total fraction of samples confirmed that these additives significantly improved soluble production of reteplase compared with control. **Conclusion:** Our study indicates that the application of chemical additives in cell lysis can improve the solubility of reteplase. Further studies are still required to understand the exact mechanism of chemical additives as a chemical chaperone during cell lysis.

Keywords: *Cell lysis, chemical chaperone, optimization, reteplase*

Introduction

Tissue plasminogen activator (t-PA) is a proteolytic enzyme which can lead to the lysis of blood clots (i.e., thrombolysis) through the conversion of plasminogen to plasmin. Recombinant tissue plasminogen activator (rt-PA), t-PA produced by recombinant DNA technology, is mainly used for the treatment of embolic or thrombotic stroke.[1-4] One of the most challenging factors in the production of this thrombolytic agent is its glycosylation which makes it difficult to produce the protein in prokaryotic systems (e.g., *Escherichia coli*). However, other derivatives of rt-PA such as reteplase with no glycosylated domain can be easily expressed in *E. coli*. There are some reports on successful expression of reteplase in *E. coli*,[5,6] although overexpression of protein usually leads to accumulation of insoluble and inactive aggregates and inclusion bodies.[7] There are different approaches for improvement of soluble expression of proteins i*n E. coli* including construction of recombinant fusion proteins such as maltose-binding protein,[8-10] alternative expression approaches such as the use of cell-free extracts for expression of protein[11] or baculovirus expression system,[12] the use of carboxyl- or amino-terminal deletion mutants,[13] co-expression of molecular chaperone proteins with the target protein,[14] and modification of culture conditions such as reduction of culture temperature[15] Furthermore, structural proteomic approaches such as the use of soluble derivative of a protein[16] or deletion of flexible regions or residues that interfere with protein solubility[17] can be applied to enhance the expression of

How to cite this article: Fazeli B, Akbari V, Barkhordari A, Mir Mohammad Sadeghi H. Improvement of soluble production of reteplase in *Escherichia coli* by optimization of chemical chaperones in lysis buffer. Adv Biomed Res 2019;8:65.

Received: 14-10-2018; **Revised:** 27-06-2019; **Accepted:** 09-07-2019; **Published:** 31-10-2019.

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Access this article online

Website: www.advbiores.net

DOI: 10.4103/abr.abr_212_18

Quick Response Code:

soluble protein in some instances. Refolding of solubilized inclusion bodies can be also used to recover soluble protein.[18] The mentioned approaches involve additional time, effort, and cost and do not always succeed. There is a conventional belief that it may not be worth attempting to solubilize proteins from inclusion bodies. Some researchers hypothesized that the main fraction of protein was firstly expressed as a soluble protein and accumulates as insoluble aggregates after cell lysis.[19] They proposed that adjustment of cell lysis buffer conditions such as pH, ionic strength, and presence of chemical additives could result in increase of soluble protein production. In the present study, we aimed to optimize chemical additives of lysis buffer to avoid the initial aggregation of reteplase at cell disruption step.

Materials and Methods

Expression of reteplase

E. coli BL21 (DE3) cells were made competent using $CaCl_2$ treatment, and recombinant plasmid (pDset-527-Ret) was introduced into competent cells by heat shock method. A single recombinant colony was inoculated into 50 ml Luria-Bertani (LB) broth and incubated at 37°C overnight. This culture was used to inoculate 500 ml of fresh LB broth and incubated at 37°C and 180 rpm until reach to OD_{600} of 0.4–0.6. Then, protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside, and the culture was incubated at 37°C for 2 h. Finally, the culture was aliquoted in 1 ml volumes and centrifuged at 5000 g for 5 min, and the bacterial pellet was stored at −70°C for further analysis.

Design of experiments

The different lysis buffers and the concentration of 11 additives [Table 1] in each buffer have been designed by Design-Expert software (version 8.0.7.1, Stat-Ease Inc., Minneapolis, USA). A Plackett–Burman design with 11 factors and 12 runs [Table 2] was used to choose three most important factors. Based on Box–Behnken model, these three factors at three levels were combined [Table 3].

Table 1: Eleven additives and their maximum and minimum concentrations used for primary screen

Additives	Maximum (mg/ml)	Minimum (mg/ml)
L-arginine	0.065	0.013
K_2PO_4	0.026	0.008
Xylitol	0.152	0.005
$CuCl_2$	0.002	0.001
Trehalose	0.239	0.049
Sodium selenite	0.002	0.001
Proline	0.575	0.011
Mannitol	0.910	0.019
CTAB	0.005	0.001
Sodium citrate	0.038	0.012
Glycine betaine	0.117	0.001

CTAB: Cetyltrimethylammonium bromide

Preparation of different lysis buffers

The standard lysis buffer consisted of 500 mM NaCl, 10% w/v glycerol, 0.025% w/v NaAzide, 10 mM $MgCl_2$, and 25 Mm Tris (pH 8). This standard buffer was used for preparation of all different lysis buffers used in this study. The different additives were added to this standard buffer, and after dissolving, the pH was adjusted to eight.

Cell disruption

Five hundred microliters of different lysis buffers was added to the bacterial pellet. After resuspending, the samples were subjected to cell lysis using a Micro Smash MS-100 (Tomy, Japan). To do this, an appropriate amount of glass beads (0.1 mm) was added to cell suspension and microsmashed at 4500 rpm for 1 min and then kept on ice for 2 min; this procedure was repeated five times. The sample was centrifuged at 7500 g for 10 min to separate the soluble and insoluble fractions. The obtained samples were stored at −70°C for further analysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Western blotting, and biological activity

The same amount (20 µl) of the protein samples was loaded into each sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) well and separated by applying an electric field (80 V for 5% gel and 150 V for 12% gel). Then, the gel was subjected to Coomassie staining and destaining to visualize protein bands. To confirm protein expression and estimate size of expressed protein, the Western blotting technique was used. After electrophoresis, separated proteins by SDS-PAGE were transferred from the gel to a nitrocellulose paper. Nonspecific binding was blocked by incubation of the membrane in 3% skim milk overnight. Then, the paper was incubated with Anti-6X His tag (HRP) antibody (Sigma-Aldrich, Abcam, USA, 1:10,000) at room temperature for 2 h. The paper was washed three times, and the blots were visualized via incubation with a chromogenic substrate, 3,3'-diaminobenzidine.

The biological activity of obtained protein was evaluated using AssaySense Human tPA Chromogenic Activity Kit (Assaypro, USA) as described previously.[19] The standard reteplase (Retelies®) and the buffer were used as a positive control and a negative control, respectively.

Results

Primary screening of chemical additives

Eleven different additives that had been previously proven to increase the solubility of recombinant proteins at cell lysis step and avoid initial aggregation of the expressed protein were chosen including L-arginine, K_2PO_4, xylitol, $CuCl_2$, trehalose, sodium selenite, proline, mannitol, cetyltrimethylammonium bromide (CTAB), sodium citrate, and glycine betaine. Maximum and minimum

Table 2: Plackett–Burman design of 11 cell lysis buffer additives

Runs	L-arginine (mg/ml)	K_2PO_4 (mg/ml)	Xylitol (mg/ml)	$CuCl_2$ (mg/ml)	Trehalose (mg/ml)	Sodium selenite (mg/ml)	Proline (mg/ml)	Mannitol (mg/ml)	CTAB (mg/ml)	Sodium citrate (mg/ml)	Glycine betaine (mg/ml)
1	0.065	0	0	0.001	0.256	0	0.576	0	0	0.026	0.117
2	0	0.017	0.152	0.001	0.256	0	0	0.91	0	0	0.117
3	0.065	0.017	0	0	0.256	0.001	0	0	0.005	0	0.117
4	0	0	0.152	0	0	0.001	0.576	0	0	0.026	0.117
5	0	0	0	0	0	0	0	0	0	0	0
6	0.065	0	0.152	0	0.256	0.001	0	0.91	0	0.026	0
7	0.065	0.017	0.152	0.001	0	0	0	0	0.005	0.026	0
8	0	0.017	0	0	0.256	0	0.576	0.91	0.005	0.026	0
9	0	0	0	0.001	0	0.001	0	0.91	0.005	0.026	0.117
10	0	0	0.152	0.001	0.256	0.001	0.576	0	0.005	0	0
11	0.065	0	0.152	0	0	0	0.576	0.91	0.005	0	0.117
12	0.065	0.017	0	0.001	0	0.001	0.576	0.91	0	0	0

CTAB: Cetyltrimethylammonium bromide

Table 3: Box–Behnken experimental design of three additives at three levels for preparation of lysis buffers

Runs	L-arginine (mg/ml)	K_2PO_4 (mg/ml)	CTAB (mg/ml)
1	0.013	0.0175	0.001
2	0.013	0.0175	0.005
3	0.065	0.0175	0.001
4	0.065	0.0175	0.005
5	0.039	0.009	0.001
6	0.013	0.0175	0.005
7	0.039	0.0175	0.003
8	0.039	0.026	0.001
9	0.065	0.0175	0.001
10	0.065	0.009	0.003
11	0.013	0.026	0.003
12	0.065	0.026	0.003
13	0.039	0.0175	0.003
14	0.039	0.0175	0.003

CTAB: Cetyltrimethylammonium bromide

concentrations of each additive [Table 1] were selected based on the previous investigations.[19] To primary screen these additives, a Plackett–Burman design with 12 runs [Table 2] was applied. These lysis buffers were used at cell disruption stage, and after centrifugation, soluble and total protein fractions were analyzed by SDS-PAGE [Figure 1]. The gels were analyzed by TL120 software (Nonlinear Inc, Durham NC, USA) to estimate soluble protein concentrations in each run, and then, the results were analyzed by Design-Expert software. It was shown that three best additives for improvement of reteplase solubility during cell lysis were L-arginine, CTAB, and K_2PO_4.

Secondary screening of chemical additives

A Box–Behnken design gave 12 runs with duplicates at center points. As a result, a total of 14 runs were performed [Table 3]. These lysis buffers were used at cell disruption stage, and after centrifugation, concentrations

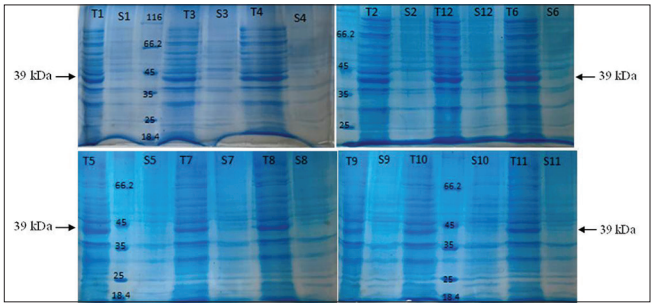

Figure 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of soluble (s) and total (t) fractions from 1 to 12 runs after cell disruption using lysis buffer proposed by Plackett–Burman design. The arrows indicate reteplase

of protein in soluble and total protein fractions were determined by SDS-PAGE [Figure 2]. Based on SDS-PAGE analysis, the optimum lysis buffer was determined as for 0.065 mg/ml L-arginine, CTAB, and 0.026 mg/ml K_2PO_4. Western blotting analysis also confirmed that these additives significantly improved soluble production of reteplase compared with control [Figures 3 and 4]. Biological activity analysis revealed that obtained soluble reteplase had a specific activity of 0.42 IU per 1 mg which was comparable with positive control.

Discussion

The purpose of this study was to improve the soluble expression of reteplase in *E. coli* by adding different chemical chaperones at cell lysis stage. In the study performed by Leibly *et al.*, the effect of 144 additive conditions for cell lysis on the enhancement of solubility of 41 target proteins expressed in *E. coli* was evaluated.[20] Based on their results, we used 11 of the best additives as chemical chaperone to increase the solubility of reteplase during cell lysis. These additives could be categorized into four groups: (1) additives that could act as a ligand being necessary for the maintenance of soluble conformation of protein such as $CuCl_2$ and sodium selenite;[21,22] (2) additives

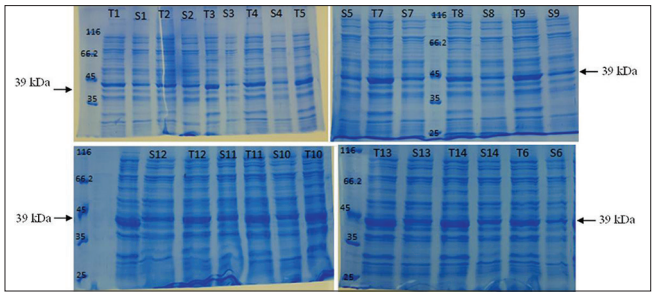

Figure 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of soluble (s) and total (t) fractions from 1 to 12 runs after cell disruption using lysis buffer proposed by Box–Behnken design. The arrows indicate reteplase

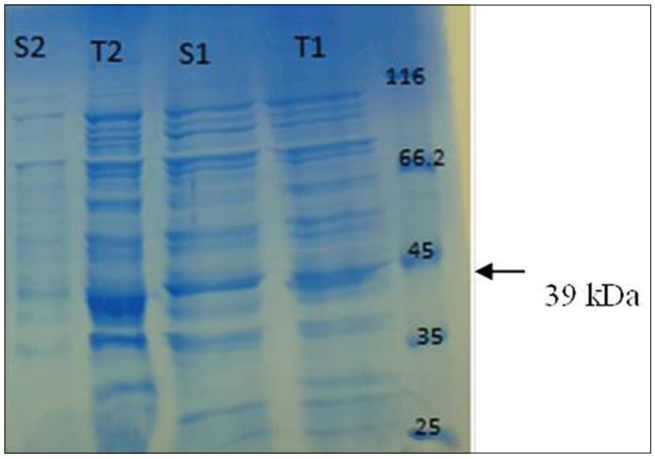

Figure 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of soluble (s) and total (t) fractions after cell disruption using optimum lysis buffer (1) and control lysis buffer (2). The arrow indicates reteplase

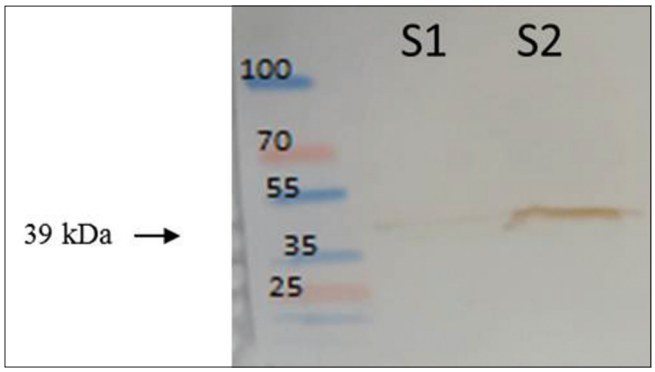

Figure 4: Western blot analysis soluble fraction after cell disruption using control lysis buffer (1) and optimum lysis buffer (2), and the arrow indicates reteplase

which could decrease protein–protein interactions or aggregations such as CTAB;[23] (3) additives which known as protein stabilizers such as arginine, xylitol, trehalose, proline, mannitol, and glycine betaine;[24-28] and (4) additives that could change the buffering capacity or ion strength of the solution such as K_2PO_4 and sodium citrate.

Among 11 additives tested in this study, L-arginine, CTAB, and K_2PO_4 were the best chemical additives which could significantly increase the solubility and stability of protein during cell disruption.

Arginine is an amino acid which widely used as an additive in formulation of many therapeutic proteins. It has been demonstrated that arginine could increase the stability and solubility of protein probably through inhibition of protein aggregation.[29] It was also reported that addition of arginine into growth medium could enhance soluble expression of proteins.[30] Furthermore, arginine could improve the recovery of soluble protein from inclusion bodies.[31] CTAB, a cationic surfactant, is an amphipathic molecule which could reduce the aggregation of protein and increase the protein stability during cell disruption. There are also some reports about the application of surfactants in protein refolding studies.[32] K_2PO_4 can affect pH and ion strength of the lysis buffer and therefore influence the conformational stability of protein. Different types of buffers have various effects on solubility and stability of a protein even in the same pH.[33] Therefore, it is important to optimize the buffer condition for each individual protein.

Two main mechanisms, enhancement of protein stability at folded state and inhibition of protein aggregation, have been proposed for improvement of protein solubility by chemical additives at cell lysis stage. It has been demonstrated that proteins can be equilibrated between unfolded (higher energy state or ΔG) and folded (lower energy state or ΔG) forms. The lower energy required for unfolding of a protein, the more susceptibility of the protein to be in unfolded state. Stabilizing of protein by additive during lysis could increase the energy needed for unfolding of proteins.[20] Second, chemical additives can act as refolding aids and improve native folding of proteins.[34,35] It was suggested that some of recombinant proteins exist in partially folded state in cytosol of bacteria, and when cell lysis taken place in the presence of additives or chemical chaperones, proteins could be in its fully and correct folded states.[36]

Although it was not the aim of the present study, addition of chemical additives in both the growth medium and cell lysis buffer could improve the overall production of soluble protein. Further studies are required to evaluate this idea and to understand the exact mechanism of chemical additives as a chemical chaperone during cell lysis.

Conclusion

In this study, we tried to optimize the expression of reteplase in *E. coli* using chemical chaperones. We evaluated the effect of adding different additives with different concentrations at lysis stage. According to our observation, the best additives and their best concentration were as follows: 0.005 mg/ml for CTAB, 0.065 mg/ml for L-arginine, and 0.026 mg/ml for K_2PO_4. The methodology explained in the present study could also be applied to optimize buffer conditions of other similar proteins.

Acknowledgment

We thank Mrs. Fatemeh Moazen for her excellent technical assistance.

Financial support and sponsorship

This work was financially supported by the Research Council of Isfahan University of Medical Sciences (No: 396813).

Conflicts of interest

There are no conflicts of interest.

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