Expression and Activity Evaluation of Reteplase in *Escherichia coli TOP10*

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ABSTRACT

Reteplase is a part of tissue plasminogen activator (t-PA) used for the removal of thrombi in blood vessels. In the present study we express the Reteplase gene in *Escherichia coli TOP10* and then its thrombolytic activity was measured. The recombinant plasmid pBADgIIIA was transformed into the competent *Escherichia coli TOP10* and then transformed bacteria was seeded into bioreactor containing 1.5 L LB medium and induced by 0.02% L-Arabinoseat 37°C, pH 7, and 180 rpm until OD 600 of 0.6 was reached. Samples were analyzed by SDS-PAGE and western blotting and the expression of Reteplase was examined. Finally the activity of this recombinant protein was evaluated using Chromogenic Activity Assay Kit. The presence of Reteplase in transformed *Escherichia coli TOP10* was examined by western blotting which revealed that the target protein in form inclusion body was expressed as a unique band at 39 and the refolded Reteplase was 66 KDa. The amount of protein produced was 90.5 µg/mL and its activity was determined as 0.8 units. In this study, the expression of Reteplase in *Escherichia coli TOP10* was scaled up under optimum condition. Furthermore we earned Reteplase with partially suitable thrombolytic activity.

Key words: *Escherichia coli TOP10*; Expression; Reteplase; Activity

INTRODUCTION

Tissue plasminogen activator (t-PA) is a protein involved in the breakdown of blood clots [1,2]. Each year, about 2 million patients through the world, are hospitalized because of acute myocardial infarction [3] and providing a suitable drug for the removal of thrombi is essential. t-PA is a selective thrombolytic agent that is a choice for the treatment of acute myocardial infarction [4]. t-PA converts plasminogen into plasmid to dissolve clot and restore blood flow. It also acts on other molecular targets, such as matrix metalloproteinase and low-density lipoprotein receptor-related proteins that induce vascular remodeling, angiogenesis, neurogenesis and axonal regeneration [5]. The advantage of this protein is that this has no side effects such as systemic hemorrhage and fibrinogen depletion [4]. Reteplase is a recombinant non-glycosylated form of human t-PA, which has been modified and contains 357 of the 527 amino acids of the original protein [6]. It is produced in the bacterium *Escherichia coli*. Reteplase is similar to the recombinant human t-PA (Alteplase), but the modifications in this protein, give reteplase a longer half-life of 13–16 minutes [7]. Reteplase also binds fibrin with lower affinity than Alteplase, and can penetrate into clots [7]. t-PA has five domains: F, EGF, P, Kringle 1 and Kringle 2 domains and the latter has 355 amino acids which is the active part of t-PA. Kringle 2 domain plus the first three amino acids of t-PA has been named reteplase which is available as a thrombolytic agent in the market [8].

*E. coli*is one of the most widely used prokaryotic hosts for the production of recombinant heterologous proteins and its genetic specifications are better characterized than those of any other microorganism [9]. The many advantages of *E. coli*have ensured that it remains a valuable organism for high-level production of recombinant proteins [10]. Reteplase can be targeted to the periplasmic space with less reducing environment compared to cytoplasm [11]. In this study, the pBAD/gIIIA vector that carries arapBAD promoter was used for the transformation of *E. coliTOP10*. Production of recombinant proteinis induced by adding L-Arabinose to the medium.In this study, the expression of reteplase was performed in a stirred-tank bioreactor at optimum conditions and its ability for converting the plasminogen to its active form (plasmin) was evaluated.

MATERIAL AND METHODS

Materials

Recombinant pET15b/reteplase was previously prepared at the School of Pharmacy of Isfahan University of Medical Sciences [12]. The pBAD/gIII plasmid and bacterial strain *E.coli TOP10* were purchased from Pasteur Institute, Iran. Luria-Bertani (LB) media was prepared according to the guidelines in the laboratory manual offered by Sambrook and Russell [13]. Screening based on antibiotic resistance was performed on LB agar plates containing 100 µg/Ml ampicillin obtained from Sigma, Germany, and plasmid minipreparation kit was purchased from Fermentas Co., Poland.Finally, for comparing our recombinant Reteplase, the standard reteplase (Retelies®), was purchased from Osveh, Iran.

Transformation of E. coli DH5αwith recombinant pET15b plasmids

Recombinant pET15b plasmid containing Reteplase gene were digested using *NcoI* and *BamHI* restriction enzymes to obtain Reteplase cDNA insert. pBADgIIIA vector, on the other hand, was digested with *NcoI* and *BglII* enzymes. Ligation process between vector and insert (molar ratio of 3:1, vector to insert) was performedusing T4 DNA ligase for 30 minat room temperature. This recombinant pBADgIIIA vector was transformed to *E. coli TOP10* using heat shock method (42°C, 1.5 min), spread on LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. Finally, the obtained recombinant pBAD/gIIIA plasmids containing reteplase gene were sequenced (Gene Fanavaran, Iran) using the Analyzer Genetic Device and Capillary Base.

Expression of Reteplase in stirred-tank bioreactor

One colony of *E. coli TOP10* containing recombinant plasmid was cultured in 5 ml LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) at 37°C and 180 rpm overnight. This culture was inoculated into 150 mlof LB medium containing ampicillin (100 µM) and finally the culture was inoculated into 1500 ml of LB medium supplemented with ampicillin and was injected to stirred-tank bioreactor (BioTron, Korea) at37°C, pH 7, and 180 rpm until OD600 nm of 0.4-0.6 was reached. L-Arabinose was then added (final concentration: 0.02%). Thefinal OD600 of inoculum was readand the samples were centrifuged at 7000 ×g, 4°Cfor 15 minand the final product was stored at -20°C [14,15].

Preparation, solubilization and refolding of inclusion bodies

All pellets were resuspended in 150 ml of buffer containing 0.1 M Tris and 20 mM EDTA and homogenized using a shearing rod, Micro Smash (Tomy, Japan).Lysozyme 0.25 mg/ml was then added to the samplesand incubated for 30 minon ice. Subsequently, centrifugation was carried out for 30 min at 4°C (13000 ×g). The pellets were resuspended in 90 ml buffer containing 0.1 M Tris, 20 mM EDTA and 2.5% V/V Triton X-100 and homogenized again. Then the samples were centrifuged, and resuspended in 90 ml of 0.1M Tris, 20 mM EDTA and 0.5% v/vTriton X-100 solution and homogenized. The samples were thencentrifuged for 30 minat 4°C and 13000×g and the pellets were resuspendedin 75 ml of 0.1 M of Tris and 20 mM EDTA [16, 17]. The prepared inclusion bodies were stored at -20°C.

After preparation the inclusion bodies, proteins were extracted by resuspension in Tris 25 mM, EDTA 10 mM, containing 1% Triton X-100 and urea 8M [18]. The reducing agent and buffer components were separated by dialysis (pH=7) at 4°C [19,20]. The solubilized samples were incubated in a mixture of Tris 0.1 mol/L (pH=8.5), urea 8mol/L, EDTA 2 mmol/L, and 2-mercaptoethanol 1%. Subsequently, the pH of the solution was adjusted to 7 with concentrated hydrochloric acid (12 N). Refolding of the protein took place by dilution with 0.1 mol/LTris (pH=10.5), 0.5 M L-argenine, 1 mM EDTA, 6 M urea, 1mM reduced glutathione, 0.1 mM oxidized glutathione, and 1 mg/ml bovine serum albumin. Then, the samples were incubated for 24 hours at 20°C in 180 rpm [19,20].

Dialysis of refolded proteins

The reducing agent and buffer components were separated by dialysis of the protein samples using 0.1 M Tris and 1mM EDTA (pH=8) buffer, and this stage was repeated three times for 1 hour and final repeat was performed for 24 hours [19,20].

SDS-PAGE analysis

One sample of inclusion bodies was dissolved in PBS buffer (pH=7.4). The refolded proteins were also prepared and Retelies® dissolved in PBS buffer too. subsequently all samples were boiled for 5 min at 85°C and electrophoresed on a 12% (v/v) non-continues SDS-PAGE with 5% stacking gel.

Subsequently, the gels were stained with Coomassie blue G250.

Western blot analysis

The protein samples on the SDS-PAGE were transferred onto an immobilon-P polyvinylidenediflouride membrane, 0.45 micron pore size, 10 ×10 (Sigma, Germany). For preparation of the membrane, it was put on methanol for 15 sec and then transferred to TB (transfer buffer) for additional 3 min. transferringof proteins onto this membrane was performed for 1hour with 400 mA of electric current. After incubation of the blot with blocking buffer (5% non-fat dry milk in TB) overnight at 4°C, anti His-tag antibody (anti t-PA, 1:250 to 1:1000 in TB-Tween buffer) was added and incubated for 1 hour at room temperature. Subsequently, three washes with TB-Tween buffer were performed and anti-rabbit IgG-HRP conjugate (Roche, Germany) as secondary antibody (diluted 1:5000) was added (incubated at room temperature for 1 hour). The membrane was washed three times with 10 mlTB-Tween for 10 min. The TB-Tween solution was then removed, and for detection of bands, 9 mg of 3,3-Diaminobenzidine (DAB, Sigma, Germany) was used and after dissolution, H_2O_2 30% was added. The bands were detected after 24 hours.

Measurement of Reteplase activity

Activating of plasminogen by reteplase was detected by Chromogenic Activity Assay Kit. Assay diluent (50 µL), plasminogen (10 µL) and plasmin substrate (20 µL) was added to 20 µL of tPA standard Reteplase (Retelies®) as positive control for total volume of120 µL. This stage was repeated for 20 µL of the samples and 20 µL of water as blank and these materials were added to the supplied 96-well plate. The plate was incubated at 37°Cin a humid incubator and the absorbance at 405 nmwas measuredafter 1 hour for each sample.

RESULTS

Cloning of Reteplase

Digestion of recombinant pBADgIIIA plasmid containing Reteplase with *NcoI* and *HindIII* created a band in about 4145 bp which is equal to the molecular size of pBADgIIIA and another in 1128bp which is equal to the size of Reteplase gene (Figure 1).

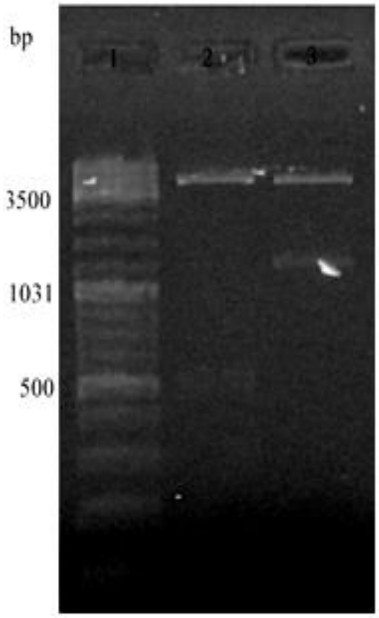

Figure 1. Digestion of the recombinant pBAD/gIII plasmid with *EcoRI, NcoI* and *HindIII*. Lane 1: Standard molecular weight marker. Lane 2: Obtained vector (4145 bp) after digestion with *EcoRI*, Lane 3 Obtained insert (1128 bp) and vector (4145 bp) after digestion with the above mentioned enzymes.

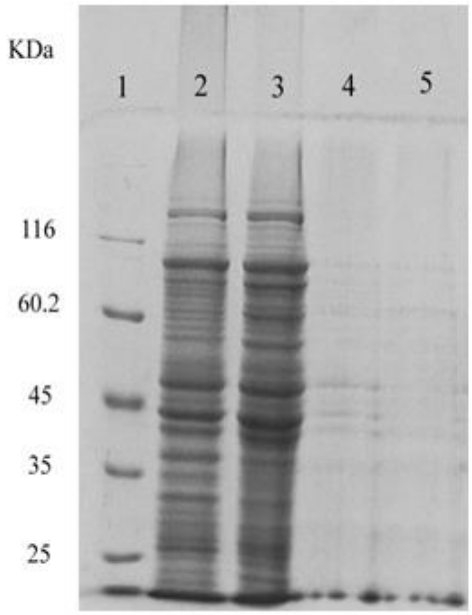

Figure 2. SDS-PAGE of the periplasmic and inclusion bodies proteins. Lane 1: Standard marker. Lane 2: Inclusion body from uninduced cells containing recombinant pBAD/gIII. Lane 3: inclusion body from cells induced induced with 0.02% L-Arabinose (2 hrs). Lane 4: periplasmic protein from induced sample with 0.02% L-Arabinose (2 hrs) Lane 5: periplasmic protein from uninduced sample.

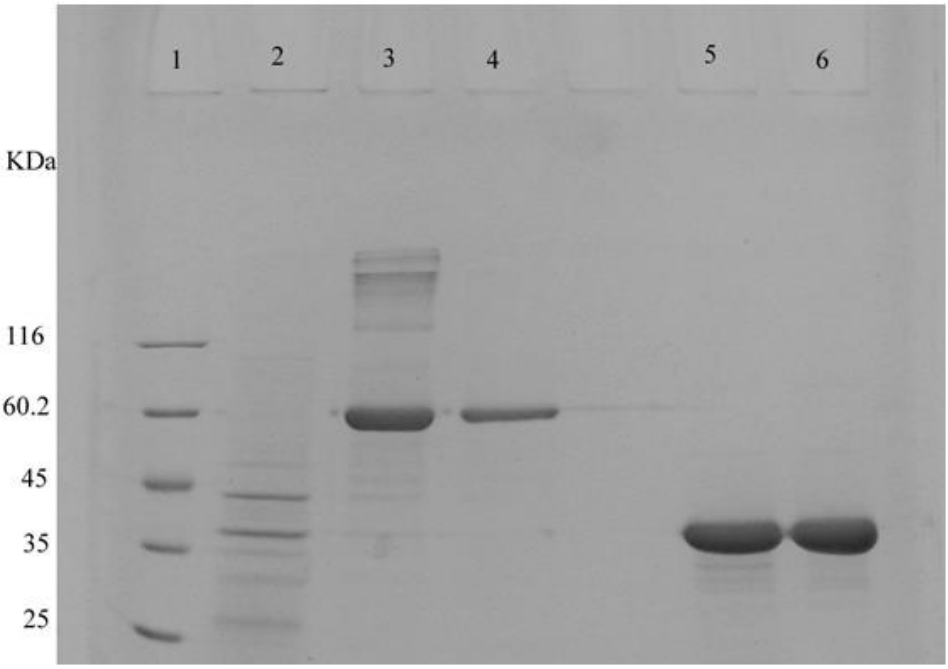

Figure 3. SDS-PAGE of the proteins extracted from inclusion bodies before and after refolding. Lane 1: Standard molecular weight marker. Lane 2: inclusion body of recombinant pBAD/gIII plasmids containing Reteplase. Lanes 3 and 4: Obtained proteins after refolding .and Lane 5, 6: commercially available Reteplase.

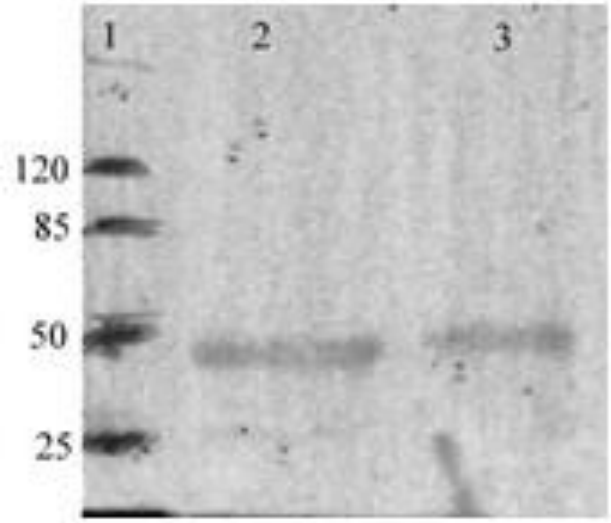

Figure 4. Western blot analysis by DAB staining method. Lane 1: Standard molecular weight marker, Lane 2: inclusion body of recombinant Reteplase (39 KDa), Lane 3: refolded recombinant Reteplase (66 KDa).

Furthermore digestion of this recombinant vector with *Eco*R1 created a band at about 478bp (Figure 1) and this confirm the existence of insert in vector. Sequencing of this plasmid also confirmed the presence and correct orientation of Reteplase cDNA in the pBADgIIIA plasmid.

Expression of Reteplase in bioreactor

After the transformation of pBADgIIIA plasmid containing Reteplase gene to *E. coli TOP10*, the effect of theconcentrationof L-Arabinose (0.02%) onprotein expression was evaluated for2 hoursincubation time at 37°C. The final OD600 was determined as 1.3. The expressed proteins were electrophoresed using SDS-PAGE. A protein with an estimated size of ~39 KDa was observed in SDS-PAGE for samples as inclusion body (Figure 2). After solubilization of inclusion bodies, refolding and dialysis of reteplase, it was analyzed using SDS-PAGE and compared with the Retelies® which showed bands at ~66 and 39KDa, respectively (Figure 3).

Western blotting

The obtained Reteplase was confirmed by Western Blotting (Figure 4).

Activity of Reteplase

The activity of the obtained recombinant protein was analyzed using standard t-PA enzyme activity kit and commercially available Reteplase (Retelies®). The differences in the absorbance (405 nm) in the reaction solution was directly attributedto the t-PA enzymatic activity.

The concentrations of t-PA standard kit and Retelies® with 1 U/mL activity were both 20.6 µg/mL. The enzymatic activity of samples wasmeasured as 0.8 U/mL compared to Retelies®. The concentration of samples determined using Bradford test according to the standard curve of BSA (albumin serum), was 90.5 µg/mL.

DISCUSSION

Reteplase is a potential recombinant thrombolytic drug that may offer an appropriate alternative to currently employed plasminogen activators. Furthermore, this agent appears to be an acceptable alternative thrombolytic agent with a satisfactory safety and efficacy profile in the setting of peripheral arterial and venous occlusion. Reteplase may provide an attractive alternative for the treatment of peripheral arterial and venous thrombotic occlusions [21].Therefore, the present research was conducted to produce recombinant reteplase in *E. coli TOP10*in large scalesusing astirred-tank bioreactor. Previously, the recombinant Reteplase was produced in *Pichia Pastoris* in shaking flask and optimized [22]; however, because the Reteplase is a non-glycosilated protein, it is not necessary to produce in eukaryotic systems and using of a prokaryotic host is simpler and cheaper for production of such recombinant proteins. The usage of pBADgIII vector for producing recombinant proteins has been reported by many investigators [10,19,18,23], although none of them have studied the expression of reteplase in this system. In order to cloning this insert, we used *NcoI* and *BamHI* restriction enzymes to obtain Reteplase cDNA insert and *NcoI* and *BglII* for digesting pBADgIIIA vector. Although *BamHI* and *BglII* have different restriction sites but after the digestion, the sticky ends created by two these enzymes can to adhere and create a recombinant vector containing insert. pBADgIII vector adds a signal sequence at the N-terminal of reteplase gene, enabling it to be secreted into the periplasmic space, and this provides a better environment for proper folding of the enzyme and formation the disulfide bond become easier [23]. Furthermore the effect of host destructive protease on the protein is decreased [23]. This vector also adds a 6× His amino acids (His-tag) in C-terminal of our protein that facilitates the purification of Reteplase by nickel-affinity chromatography. In this study, the amount of the expressed protein in periplasmic space was low and most ofit was present inside the cell as inclusion bodies. This might be due to high expression of proteins disturbing the mechanisms of cells for exporting recombinant proteins to the periplasmic space. Similar results have been reported by other investigators [12,15]. The main advantage of inclusion bodies is that they are mostly composed of recombinant proteins (25% of total protein), can be easily isolated from the cell debris, furthermore this form of protein is inactive and thus it can be produced in large amounts for cell toxic protein and finally, the inclusion bodies are resistant to the host protease and don't require to protease free hosts [2,14,24].

However, inclusion body form of proteinsis insoluble and inactive [9,12,13,24,25]. Thus, the main problem in purification process of inclusion bodies is optimizing the refolding and renaturation conditions by preventing the formation of inactive aggregates. Differentrefolding strategies have been used for refolding the protein and in this study we used arginine and oxidizing/reducing glutathione [26]. There are several reports about the use of oxidizing/reducing glutathione in refolding of recombinant proteins such as prochymosin, growth hormone and alkaline phosphatase [27].

The process of refolding reteplase may result in the dimerization of the protein or change of its disulfide bandsso that it's molecular weightshows higher in the SDS-PAGE although it is active. According to the SDS-PAGE analyze, the molecular weight of the Retelies® is 39 KDa and this form has more activity than our product. Therefore, it seems that changing refolding protocol is necessary.

Theactivity of our product is lower than the positive control and this may be due tothe presence of His-tag in the sequence of the gene that facilitates the purification of reteplase using affinity chromatography with nickel in stationary phase. But decrease the thrombolytic activity of this protein.

Qiu *et al* and Lee and Im, have also produced tissue plasminogen activators and measured their activities [2,28]. However, the unit activity of reteplase is defined differently as compared to

other types of t-PA and therefore the results of these investigations cannot be compared with our study. In this study, we controlled key parameterssuch as rpm, temperature, pH, and foam formation.Because of the lack of floor during the process, anti-foaming agent was not used.pHdid not change significantly during the growth of the bacteria and therefore acid or base was not added. An important aspect of our study is large-scale production of reteplase in a bioreactor, since no other study has been published for mass production of this drug.

On the other hand, it seems that production of a protein with 0.8 U/ml activity is an acceptable result.

CONCLUSIONS

In this study, the expression of reteplase in *Escherichia coli TOP10* was performed in optimum conditions and scaled up.

In future studies, purification and removing the His-tag from the sequence of reteplasewill be performed.

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