Expression and Activity Evaluation of Reteplase in Escherichia coli TOP10

Fatemeh Shafiee, Fatemeh Moazen, Mahammad Rabbani, Hamid Mir Mohammad Sadeghi*

Department of Pharmaceutical Biotechnology and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran

*Corresponding Author: email address: h_sadeghi@pharm.mui.ac.ir (H. Mir Mohammad Sadeghi)

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-Arabinose at 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 proteins is 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 DH5α with 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 performed using T4 DNA ligase for 30 min at 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/gIII 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 ml of 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) at 37°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%). The final OD600 of inoculum was read and the samples were centrifuged at 7000 xg, 4°C for 15 min and 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 samples and incubated for 30 min on ice. Subsequently, centrifugation was carried out for 30 min at 4°C (13000 xg). 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/v Triton X-100 solution and homogenized. The samples were then centrifuged for 30 min at 4°C and 13000 xg and the pellets were resuspended in 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/L Tris (pH=10.5), 0.5 M L-arginine, 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 polyvinylidenedifluoride 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. Transferring of proteins onto this membrane was performed for 1 hour 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 was added (incubated at room temperature for 1 hour). The membrane was washed three times with 10 mL TB-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, H2O2 30% was added. The bands were detected after 24 hours.

**Measurement of Retepase 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 Retepase (Retelies®) as positive control for total volume of 120 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°C in a humid incubator and the absorbance at 405 nm was measured after 1 hour for each sample.

**RESULTS**

**Cloning of Retepase**

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

![Figure 1](image1.png)

*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](image2.png)

*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 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.
Expression of Reteplase in bioreactor

After the transformation of pBADgIIIa plasmid containing Reteplase gene to *E. coli* TOPO10, the effect of the concentration of L-Arabinose (0.02%) on protein expression was evaluated for 2 hours incubation 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 attributed to the t-PA enzymatic activity.

Furthermore digestion of this recombinant vector with EcoR1 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.
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 was measured 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**

Retelies 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. Retelies 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 scales using astirred-tank bioreactor. Previously, the recombinant Retelies was produced in *Pichia Pastoris* in shaking flask and optimized [22]; however, because the Retelies 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 pBADgII 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 Retelies cDNA insert and Neol 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. pBADgII 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 6x His amino acids (His-tag) in C-terminal of our protein that facilitates the purification of Retelies by nickel-affinity chromatography. In this study, the amount of the expressed protein in periplasmic space was low and most of it 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 proteins is 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. Different refolding 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 bond so that it’s molecular weight shows 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.

The activity of our product is lower than the positive control and this may be due to the 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 parameters such as rpm, temperature, pH, and foam formation. Because of the lack of floor during the process, anti-foaming agent was not used. pH did 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.

REFERENCES
16. Shen T, Zhang AH, Bi L, Shi LR. Construction, expression and identification of a single chain anti-body variable against human 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 reteplase will be performed.