Brief Report

Molecular cloning of Reteplase and its expression in *E. coli* using tac promoter

Safieh Aghaabdollahian, Mohammad Rabbani, Kamran Ghaedi[1], Hamid Mir Mohammad Sadeghi

Departments of Pharmaceutical Biotechnology, Isfahan Pharmaceutical Science Research Center, School of Pharmacy and Pharmaceutical Science, Isfahan University of Medical Sciences, [1]Biology, Faculty of Sciences, University of Isfahan, Isfahan, I R Iran

Abstract

Background and Aims: This study aimed to clone and express the reteplase cDNA, a thrombolytic agent used for the treatment of acute myocardial infarction and stroke, in *E. coli*, utilizing tac promoter for its expression.

Materials and Methods: Reteplase cDNA was amplified by polymerase chain reaction (PCR) with designed primers. The product was then cloned into pTZ57R plasmid. The cloned cDNA was digested out and ligated into pGEX-5x-1 expression vector. The presence of the insert was confirmed by restriction digestion. By using 0.2, 0.5 and 1 mM isopropyl beta-D thiogalactopyranoside (IPTG), expression of reteplase was induced in *E. coli TOP10* cells and analyzed by SDS-PAGE.

Results: Electrophoresis of PCR product and also double digested recombinant pTZ57R plasmid, also, pGEX-5x-1 vector, showed a 1068bp band of reteplase. SDS-PAGE analysis showed a 60 KDa band of protein product induced with different concentrations of IPTG.

Conclusion: In the present study, reteplase cDNA was successfully cloned and expressed using tac promoter. This vector will be used for the optimization of the expression of reteplase in *E. coli*.

Key words: Cloning, reteplase, t-PA, tac promoter

Address for correspondence:
Dr. Mir Mohammad Sadeghi, Department of Pharmaceutical Biotechnology and Isfahan Pharmaceutical Science Research Center, School of Pharmacy and Pharmaceutical Science, Isfahan University of Medical Sciences, Isfahan, I.R. Iran. E-mail: h_sadeghi@pharm.mui.ac.ir
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INTRODUCTION

Reteplase (recombinant plasminogen activator; r-PA) is one of the third generation thrombolytic agents that activates fibrin-bound plasminogen.[1,2] It is used for the treatment of occlusive disorders, especially myocardial infarction which is responsible for more than 50% of cardiovascular events deaths.[3-6] One of the important clinical advantages of t-PA is that it produces no side effects such as systemic hemorrhage and fibrinogen depletion and therefore many researchers are working towards the reduction of the cost of its production.[1,2,7] In comparison to tissue plasminogen activator (t-PA), reteplase is nonglycosylated and has longer half life. Reteplase is expressed in various bacterial, fungal, mammalian and insect systems.[4,8,9] However due to the variability of expression levels of r-PA in these systems, problems of protein instability and downstream processing, each expression system differs for its capabilities for the production of

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reteplase. Although the expression of r-PA in bacterial systems like *E. coli* has been investigated by some researchers, various cloning and expression plasmids and conditions can affect the yield and production characteristics of reteplase. One of the strongest promoters in *E. coli* is tac promoter, a hybrid of trp and lac promoters,[10,11] which its efficacy is 2 to 3 times more than trp promoter and 7 times more than the lac UV5 promoter. It can be repressed by the lac repressor and can be induced with IPTG.[10,12] Therefore, in this study we cloned and expressed r-PA gene in *E. coli* using tac promoter.[13]

MATERIALS AND METHODS

Strains

E. coli XL1-blue, *BL21 (DE3)* and *TOP 10* were obtained from Cinnagen, Iran.

Enzymes, primers, chemicals and plasmids

Restriction enzymes, modifying enzymes, SDS-PAGE reagents and other molecular related reagents were purchased from Fermentas, Poland. Primers for PCR reaction were purchased from Fanavarie Kowsar, Iran.

PCR Amplification and Cloning of r-PA cDNA

pETret, a recombinant plasmid containing reteplase cDNA (previously cloned in our laboratory), was used as template for PCR amplification. PCR was carried out with forward primer: 5´CGG CGC GGA TCC CCA TGT CTT ACC AAG GAA ACA GTG ACT GCT AC 3´ and reverse primer: 5´GAA CCG CTC GAG TCA CGG TCG CAT GTT GTC ACG AAT CCA G 3´ in a total volume of 50 µl containing 1.4 µl MgCl2, 1 µl of dNTP, 1 µl of each PCR primer, 5 µl 10x-PCR buffer, 0.4 µl of Taq polymerase enzyme and 1 µl of DNA template. Thermal cycles included initial denaturation at 94°C for 5 min followed by 30 cycles of 94° C for 1 min, 55°C for 2 min, and 72°C for 3 min; and one cycle of 72°C for 15 min. The purified PCR product was ligated into pTZ57R plasmid using Ins T/A clone kit (Fermentas, Poland) and transformed into *E. coli XL1-blue* cells using heat shock method.[14] Afterward, plasmid preparation was performed from the obtained colonies using Aurum plasmid mini kit (Roche, Germany) according to the manufacturer's instructions.

Construction of the recombinant expression vector

Recombinant pTZ57R plasmid (PTZ57R/ r-PA), and expression vector, pGEX-5x-1, were digested with *BamH*I *and Xho*I restriction enzymes and the obtained insert and pGEX-5x-1 vector were gel purified. Subsequently, reteplase cDNA was ligated to the pGEX-5x-1. Then this product was used for transformation of *E. coli XL1-blue* cells. The recombinant plasmids were then identified by restriction analysis and DNA sequencing.

Expression of reteplase in *E. coli*

The recombinant plasmid, pGEX-5x-1/ r-PA (rpGEX), was transformed in to competent *E. coli* strain *TOP10* cells prepared using calcium chloride method. For negative control, pGEX-5x-1 plasmid was used for the transformation of *TOP10* competent cells. Transformed cells were grown on LB agar plates supplemented with 100 mg/ml ampicillin (Roche, Germany) at 37°C. A single colony was inoculated into 5 ml LB medium. After overnight growth, 200 µl of the culture was transferred to the flasks containing 15 ml fresh LB medium and ampicillin. Additionally, 200 µl of the overnight culture of *TOP10*/ pGEX-5x-1 and *TOP10* without recombinant plasmid were transferred to the flasks containing 15 ml fresh LB medium containing antibiotic. Cells were shaken at 180 rpm to an OD_{600} of 0.4-0.5, then over-expression of recombinant protein was induced by the addition of IPTG to the final concentrations of 0.2, 0.5, and 1 mM. The cultures were incubated further at 37°C. The OD_{600} of these cultures were measured at intervals of 1 h for 4 h. Following each step, 1.5 ml of sample was taken and centrifuged at 4000 rpm. Pellets were resuspended in 100 µl of PBS (0.75 mMNa2HPO4, 0.1 mM NaCl, 1.7 mM KH2PO4, pH = 7.2) and 5 µl of SDS sample buffer (0.5

M Tris-HCl, pH=6.8, Glycerol, 10% SDS, 2.9 mM β-mercaptoethanol and 0.5% bromo-phenol blue) was added and then samples were heated at 90°C for 10 min. 20 µl of each sample was electrophoresed on a 12% SDS-polyacrylamide gel.

RESULTS

The size of the PCR product was about 1068 bp which is the length of reteplase cDNA [Figure 1]. The reteplase cDNA was ligated into pTZ57R vector and was used for the transformation of *E. coli XL1 blue* cells. The obtained recombinant plasmids were then subjected to digestion with *Sac*I restriction enzyme for the determination of the presence of reteplase cDNA in these plasmids. The observed 750 bp and 2850 bp bands confirmed the presence of reteplase [Figure 2].

The recombinant pTZ57R plasmid was double digested with *Xho*I and *BamH*I restriction enzymes and the obtained insert was gel purified [Figure 3]. Additionally, pGEX-5x-1 plasmid was double digested with these enzymes. These were then ligated and used for the transformation of *E. coli BL21* cells. Subsequently, the obtained recombinant pGEX-5x-1 plasmids were digested with *Xho*I and *BamH*I

restriction enzymes to confirm the presence of the insert. Reteplase is a 39 KDa protein, but because of the presence of glutathione s-teransferase (*gst)* protein (25 KDa), an approximately 60 KDa protein was expressed. SDS-PAGE analysis of samples showed that target protein was induced with all IPTG concentrations [Figure 4].

DISCUSSION

In this study we followed the aim of cloning and expression of reteplase in *E. coli* using tac promoter. In comparison to t-PA, reteplase is a single chain deletion mutant of t-PA[2,4] containing 355 amino acid residues with molecular mass of 39 kDa. The Kringle II and Protease domains of t-PA is kept in reteplase and it retains enzymatic activity that is comparable to the native t-PA *in vitro*.[15] Reteplase has higher half life, lower adverse effects and easier administration.[2,7] It is used for treating occlusive disorders of the lungs, heart, and brain.[3-6] t-PA has been expressed in different systems such as bacteria, fungi, insect, yeasts, Chinese Hamster Ovary (CHO) and mammalian cell lines.[4,8,9,16,17] A problem occurring when mammalian cell lines are used is glycosylation of the product which leads to the rapid clearance of this drug.[1] Also because of wrong folding, low secretion to the culture media and high level of glycosylation, yeasts like *Saccharomyces cervisiae* and insect cells are unsuitable expression systems.[18,19] Also, expression in CHO system could result in extraction complexity, and risk of viral infection.[20] Bacterial systems have many advantages such as lack of glycosylation, impaired receptor binding potential, fast cultivation,

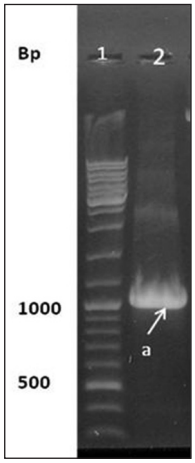

Figure 1: PCR amplification of reteplase cDNA (Lane 2, a = 1068 bp). Lane 1 shows 100 bp molecular weight marker. Lane 2: Arrow shows the band of the expected PCR product

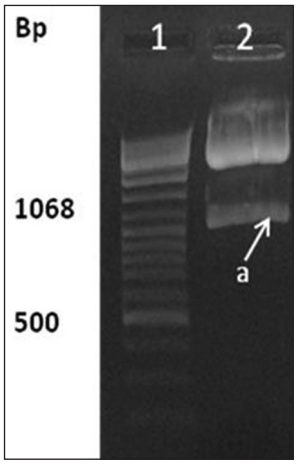

Figure 3: Digestion of pTZ57R/ reteplase with BamHI and XhoI restriction enzymes. Lane 1: Molecular weight marker, Lane 2: Digested recombinant plasmid containing reteplase cDNA (1068 bp)

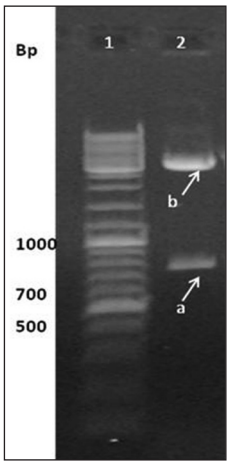

Figure 2: Digestion of reteplase cDNA with SacI restriction enzyme. Lane 1 shows 100 bp molecular weight marker, lane 2: Digested PCR product with SacI (a: 750 bp, b: 2850 bp)

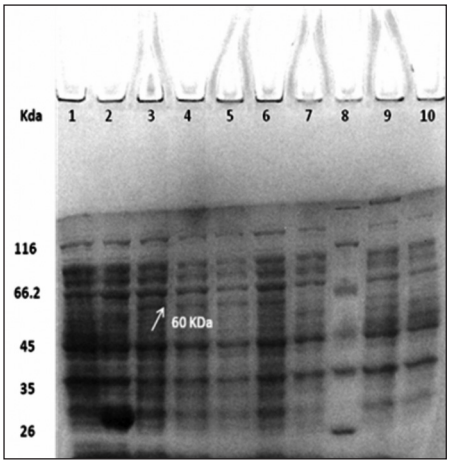

Figure 4: Induction of the expression of reteplase with different concentrations of IPTG 37°C. Proteins in the whole cell lysates were separated using SDS-PAGE. Lane 1: pGEX-5x-1 uninduced, Lane 2: pGEX-5x-1 induced for 2 h, Lane 3-6: rpGEX-5x-1- TOP10 induced with 1, 0.5, and 0.2 mM IPTG for 2 h respectively, Lane 7: rpGEX-5x-1-TOP10 induced with 1 mM IPTG for 4 h, Lane 8: Protein marker, Lane 9: TOP 10 bacteria at 37°C, Lane 10: rpGEX-5x-1- TOP10 uninduced. Arrow shows the 60kDa reteplase plus gst protein band

and low cost of downstream processing.[4] Liao *et al*., also have expressed reteplase in *E. coli*[21] but they have used a mutant sequence and a different cloning and expression vector as compared to our study (PMD18, pTZ57R cloning vectors, respectively). pTZ57R plasmid has an excess thymidine nucleotide at its 3'end and could be used for T/A cloning.[22,23] This method is optional specially when we could not achieve suitable inserts for ligation.[23]

In the present study, we used pGEX-5x-1 expression vector which contains tac promoter. Tac promoter is a functional hybrid of the trp and the lac UV5 promoters. In this promoter the DNA upstream of position -20 with respect to the transcriptional start site is derived from the trp promoter and the DNA downstream of position -20 is derived from the lac UV5 promoter. Also it has a consensus pribnow box sequence in addition to a consensus -35 sequence.[10,12] tac promoter is one of the strongest promoters in *E. coli* that have been characterized *in vitro* up to now.[11] Its efficacy is more than its parentral promoters and we could induce this promoter with IPTG. So this hybrid promoter is useful for the controlled expression of foreign genes at high levels in *E. coli*.[10,24] pGEX-5x-1 expression vector includes GST sequence at the beginning of the insert. The pGEX GST fusion protein system is widely used for high level expression and rapid and efficient purification of fusion proteins expressed in bacterial lysates.[25-27] Also, this system has specific protease cleavage sites to facilitate proteolysis of the bacterial fusion proteins.[25] In a similar study, reteplase was expressed in *E. coli TOP10* using pBAD/gIII expression vector.[28] This vector allows the product being transferred into the preplasmic space. However, the persiplasmic expression of reteplase was low and thus it seems that high levels of cytoplasmic expression of this enzyme would result in higher yields. Therefore, pGEX-5x-1 can be a better selection for expressing reteplase.

The results of our study showed expression of an approximately 60 KDa protein but in the other studies,[13,21] a 39 KDa band was observed. This disagreement is because of the fusion of GST protein. Also, in the Gao *et al,* study,[15] an approximately 40 KDa protein band was expressed. This was due to the expression of a fusion protein consisting of hirudin-PA and reteplase. Also, Aflakiyan and etc observed a 45 KDa protein band because of post translational modifications occurred in the insect systems.[29]

CONCLUSION

In the present study we successfully cloned and expressed reteplase cDNA utilizing tac promoter. The induced vector can be used for future analysis of reteplase expression in *E. coli*.

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