

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Cloning and Expression of a Human Tissue Plasminogen Activator Variant: K2S in *Escherichia coli*

¹Kianoush Dormiani and ¹Yahya Khazaie, ^{1,2}Hamid Mir Mohammad Sadeghi,
^{1,2}Mohammad Rabbani and ²Fatemeh Moazen

¹Isfahan Pharmaceutical Sciences Research Center and ²Department of Pharmaceutical Biotechnology,
School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract: The DNA sequence of Kringle-2 and serine protease domains of the human tissue plasminogen activator (reteplase, K2S) was PCR amplified. This product was then cloned into the expression vector pET1 5b plasmid. The presence of the insert was confirmed by restriction digestion, PCR and determination of the nucleotide sequence. By using isopropyl β -D thiogalactopyranoside (IPTG), reteplase was induced in *E. coli* BL21 cells and analyzed using polyacrylamide gel electrophoresis (PAGE).

Key words: Tissue plasminogen activator, reteplase, K2S, cloning, expression, *Escherichia coli*

INTRODUCTION

Defect in blood circulation due to clot formation is one of the most prominent causes of death in humans. The most serious form of this disorder is the blockage of the blood flow to the heart, brain and lungs which result in heart attack, brain infarction and lung embolism, respectively.

Tissue-plasminogen activator (t-PA) would facilitate the destruction of blood clot through the conversion of plasminogen to plasmin. The latter is an enzyme which digests the fibrin network in the blood clots. Therefore this substance and its derivatives can be used as a drug for the treatment of thrombotic disorders especially acute myocardial infarction which is named thrombolytic therapy (Dowdle, 1990; Rao and Insly, 2003). The aim of this therapy is removing blood clots from the arteries and thus reperfusion of the ischemic organ as rapidly as possible.

In this study, reteplase has been selected as an effective fibrinolytic agent which is produced by recombinant DNA technology in *E. coli*. Reteplase has no oligosaccharid side chains in its structure and has only 355 amino acids out of 527 amino acids in t-PA (amino acid 1 to 3 and 176 to 527). It has two domains; serine protease and Kringle 2 from five domains (finger, epidermal growth, Kringle 1, Kringle 2 and Serine protease) in t-PA (Kohnert *et al.*, 1992; Martin *et al.*, 1993; Manosroi *et al.*, 2001). The advantages of using reteplase instead of tissue plasminogen activator are as follows:

- It has weaker affinity to fibrin due to missing fibrin domain (Martin *et al.*, 1993). This can reduce the possibility of intra-cranial bleeding (Smalling *et al.*, 1995).
- Absence of Kringle 1, epidermal growth domains and oligosaccharide side chains which reduce the affinity of reteplase for its hepatic receptors and reduce its clearance, therefore its half life is about 4 fold longer than t-PA (Martin *et al.*, 1999).
- Stronger thrombolytic effects and longer half life of reteplase produce faster, more complete and sustained thrombolytic effects.

Therefore, the purpose of the present study was cloning of reteplase and its expression in *E. coli* using a proper expression vector so that in future studies this enzyme could be produced in large quantities.

MATERIALS AND METHODS

Bacterial strains and plasmids: The bacterial strains used in this study were *E. coli* HB101 and *E. coli* BL21 (from Cinagen Co.).

The pET1 5b cloning/expression vector obtained from Novagen Co.

Media and chemicals: Luria-Bertani (LB) media was prepared according to Sambrook and Russell (2001). Screening based on antibiotic resistance was performed on LB agar plates containing ampicillin ($100 \mu\text{g mL}^{-1}$) which was obtained from Sigma Co. 0.1 and 0.5 mM IPTG

were also used for the induction of reteplase gene (purchased from Fermentas Co.). Restriction enzymes were purchased from Fermentas, Roche and Invitrogen companies.

Cloning of K2S cDNA: K2S gene was obtained from a source that was expected to contain its complete cDNA. Using this template and specific primers (RETF5: 5'-GGGAATCCATATGTCTTACCAAGGAAACAGTGACTCCTACTTTG-3' and RETR5: 5'-GAACGCGGATCCTCACGGTTCGCATGTTGTCACGAA TCCAG-3'), the desired sequence was amplified. These primers were designed to have these features: *Nde*I and *Bam*HI sites at the ends, the code of the three first amino acids of t-PA and two oligonucleotide tails at each end for the efficient digestion by the desired endonucleases. The PCR amplification was conducted according to parameters that are given in Table 1. To remove 5'-terminal phosphate and to prevent recircularization of the plasmid before ligation, the vector was dephosphorylated by treatment with calf intestinal alkaline phosphatase (from Roche Co.). The amplified K2S DNA was digested by appropriate restriction endonucleases and purified by QIAquick Gel Extraction Kit from agarose (0.7%) gel. Subsequently, this product was ligated into a pET15b vector digested with the same enzymes using T4 DNA Ligase (from Fermentas Co.) at 16°C overnight. The ligated DNA was then transformed into *E. coli* HB101 competent cells using heat shock method (Sambrook and Russell, 2001).

The obtained colonies were used for plasmid preparation using High Pure Plasmid Isolation Kit (Roche Co., Germany). The presence and orientation of the insert in these plasmids was confirmed by digestion using proper endonucleases.

Expression of K2S gene: *E. coli* BL21 (DE3) was transformed by the above mentioned plasmid (K2S/pET15b) and cells were spread over agar plates containing Luria-Bertani/ampicillin medium and incubated at 37°C overnight. Subsequently, a colony was grown in 5 mL LB (100 µg mL⁻¹ ampicillin) and 100 µL of the overnight culture was used to inoculate 5 mL of prewarmed LB/ampicillin medium. The culture was grown in a shaking incubator at 35°C until OD₆₀₀ reached 0.5-1. Protein expression was induced by the addition of IPTG

at mid-log phase with final concentration of 0.1 mM and the culture was grown for 4 h. 1.5 mL of culture was harvested separately every two hours after induction. Cells were then harvested by centrifugation at 13000×g for 1 min at room temperature and after removing the supernatant, the bacterial pellet was resuspended in 15 µL of 6×SDS gel-loading buffer. The samples were heated at 100°C for 3 min. The tubes were then centrifuged at 13000×g for 1 min at room temperature and were stored on ice until loading in a gel. Protein expression was analyzed using sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) according to Sambrook and Russell (2001). For this purpose, a 12% polyacrylamide gel was utilized (100 V). Proteins were stained with coomassie blue for 20 min and individual proteins were visualized by destaining with methanol: acetic acid solution (Sambrook and Russell, 2001).

RESULTS

Construction of recombinant plasmid: Tissue plasminogen activator was PCR amplified. Gel electrophoresis of this product demonstrated the expected DNA band of 1581 bp (Fig. 1). Digestion with *Eco*RI restriction endonuclease produced two bands of approximately 1200 and 400 bp. Digestion with *Sac*I resulted in two bands of approximately 650 and 500 bp. The 500 bp band is possibly the product of two overlapping bands since *Rsa*I digests tissue plasminogen activator at 613 and 1085 sites which should result in three bands of 613, 496 and 482 bp.

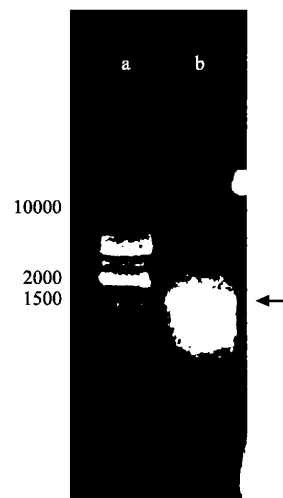


Fig. 1: Agarose gel electrophoresis of tissue plasminogen activator which was amplified by PCR. This figure demonstrates the expected DNA band of 1581 bp. Lane a: ladder, Lane b: the PCR product. The arrow shows the position of the band of t-PA

Table 1: The PCR amplification according to parameters

Amplifying steps	Denaturation	Annealing	Extension		
First heat shock	5 min at 94°C	-	-	-	-
35 cycles	-	1 min at 94°C	2 min at 55°C	3 min at 72°C	-
Final extension	-	-	-	-	20 min at 72°C

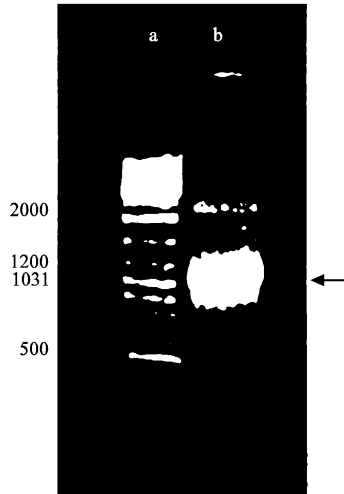


Fig. 2: Reteplase (K2S) DNA sequence was amplified by PCR. Tissue plasminogen activator sequence was used as the template. Lane a: ladder, Lane b: the PCR product. The arrow shows the position of the band of K2S that is between the region of 1031 and 1200 bp that corresponds with the desired size: 1068 bp

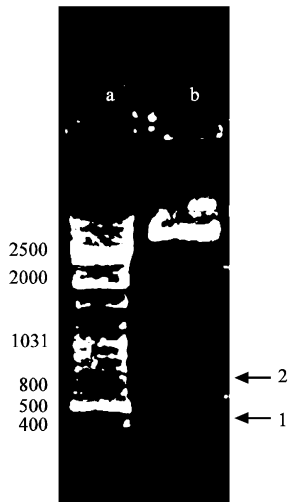


Fig. 3: Digestion of the recombinant plasmid with *EcoRI* would result in two bands of 400 and 800 bp. Lane a: ladder, Lane b: the products of digestion. Arrow 1 and 2 show the regions of 400 and 800 bp, respectively

With the use of tissue plasminogen activator as template and specific primers, reteplase (K2S) DNA sequence was amplified. As shown in Fig. 2, a single band of approx. 1100 bp was obtained. Digestion with *EcoRI* restriction enzyme produced one band of 100 bp and a wide band of ~500 bp (data not shown). The PCR product

```
5'atgtcttccaaggaacagtgactcctactttgggaatgggtcagcctaccgtggcacgcacagcct
caccgagtcgggtgcctcctcctcccgtggaattccatgatcctgataggcaaggttacacagcacag
aaccacagtgcccagcactggcctgggcaacataaactcctcgggaatcctgatggggatgccaa
gccctgggtcccagtgctgaagaaccgaggctgacgtgggagtagctgtgatgtgccctcctgtccac
ctggcctgagacagtagaccaccctcagtttcgcatcaaaaggagggtcctctcggccacatgcctc
ccaccctggcaggctgcatcttggccaagcacaggaggtcgcgccggagagcgggtcctgtcgggg
gcatactatcagctcctgtggattctctcctcggccactgctccaggagagggttccgcccccaccac
ctgacgggtgatcttggcagaacataccgggtgtcctggcgaggaggagcagaattgaaagtcga
aaaatacatgtccataaggaattcgatgatgacacttacgacaatgacattgcgctgctgcagctgaaatc
ggattctccctgtgccaggagagcagcgtgtgtcgcactgtgtgcctccccggcgacctgca
gctgccggactggacggagtgtagctctccggctacggcaagcatgaggcctgtctcctttctattcg
gagcggctgaaggaggctcatgtcagactgtaccctccagccgctgcacatcacaacattacttaaca
gaacagtcaccgacaacatcgtgtgtgtgtggagacactcggagcggggcccccaggcaacttgca
cgacgctgccaggcggattcggggaggccccctgtgtgtgtgaacgatggccgcatgactttgggtgg
gcatcatcagctgggctggctgtggacagaaggatgccgggtgtgtacaccaagggttaccacct
acctagactggattgtgacaacatgcgaccg 3' (1068)
```

Fig. 4: The result of sequencing of the cloned K2S in pET15b

was digested with *NdeI* and *BamHI* restriction enzymes and then ligated into pET15b plasmid. Determination of the orientation of the insert was performed using *EcoRI*. The correct orientation should result in two bands of 400 and 800 bp (Fig. 3). Also the presence of the insert was confirmed using specific primers and PCR amplification of the insert. The entire insert was also sequenced (Fig. 4). This experiment also demonstrated that reteplase was properly inserted into the expression vector and could be expressed using T7 RNA polymerase.

Expression analysis: Protein expression was induced by IPTG. The expressed protein was electrophoresed using SDS-PAGE. After staining of proteins by Coomassie brilliant blue (R-250) and destaining, in addition to other bands, a weak band with an estimated size of ~ 39 kDa on lane of the induced sample was observed.

DISCUSSION

K2S is a non-glycosylated variant of t-PA which in this study was cloned and expressed in different strains of *E. coli* using pET expression system. The process of glycosylation is a very complex step in the production of eukaryotic proteins like t-PA and it has been shown that by omitting this step, the pharmacokinetic properties of K2S compared to the native compound, t-PA, would improve. For example duration of the action of this compound and therefore its half life as a drug will increase due to the fact that the hepatic receptor for glycosyl residues cannot bind to the K2S (Lau *et al.*, 1987; Hotchkiss *et al.*, 1988; Johannessen *et al.*, 1990). Furthermore differences in glycosylation pattern of the protein may affect its activity (Wittwer *et al.*, 1989; Spellman *et al.*, 1989).

We used the pET system as cloning and expression vector in *E. coli*. These series of vectors have several

advantages that persuaded us to use one of them, pET15b, for cloning and expression K2S gene. These vectors have a powerful T7 promoter that if fully induced, almost all of the cells resources are converted to target gene expression making the cloned K2S eligible for use in industrial scale. It is also possible to attenuate expression level simply by lowering the concentration of inducer. Another important feature of this system is its ability to maintain target gene transcriptionally silent in uninduced state (Studier *et al.*, 1886; Studier, 1991). Also, this vector adds polyhistidine tag to proteins which makes it easy to purify these fusion proteins by affinity chromatography (Hochuli, 1990; Kweon *et al.*, 2004). On the other hand this tag can be removed by thrombin making it possible to obtain the desired protein.

In conclusion, in the present study a variant of t-PA was cloned and expressed in *E. coli*. Further experiments are needed to optimize the expression conditions and to produce this protein in large scales.

ACKNOWLEDGMENTS

This work was supported by the Isfahan University of Medical Sciences, Isfahan, Iran.

REFERENCES

- Dowdle, E.B.D., 1990. Plasminogen Activator. US Patent 490623.
- Hotchkiss, A., C.J. Refino, C.K. Leonard, J.V. O'Connor and C. Crowley *et al.*, 1988. The influence of carbohydrate structure on the clearance of recombinant tissue-type plasminogen activator. *Thromb. Haemostas.*, 60: 255-261.
- Hochuli, E., 1990. Purification of recombinant proteins with metal chelate adsorbent. *Gene. Eng.*, 12: 87-98.
- Johannessen, M., V. Diness, K. Pingel, L.C. Petersen, D. Rao, P. Lioubin, P. O'Hara and E. Mulvihill, 1990. Fibrin affinity and clearance of t-PA deletion and substitution analogues. *Thromb. Haemostas.*, 63: 54-59.
- Kohnert, U., R. Rudolph and J.H. Verheijen, 1992. Biochemical properties of the kringle 2 and protease domains are maintained in the refolded t-PA deletion variant BM 06.022. *Protein Eng.*, 5: 93-100.
- Kweon, D.H., S.G. Kim and J.H. Seo, 2004. Purification and refolding of cyclodextrin glycosyltransferase expressed in *Escherichia coli*. *J. Inclusion Phenomena and Macrocyclic Chem.*, 50: 37-41.
- Lau, D., G. Kuzman, C.M. Wei, D.J. Livingston and N. Hsiung, 1987. Modified human tissue plasminogen activator with extended half-life *in vivo*. *Bio/Technology*, 5: 953-958.
- Manosroi, J., C. Tayapiwatan and F. Gotz, 2001. Secretion of active recombinant human tissue plasminogen activator derivatives in *Escherichia coli*. *Applied Environ. Microbiol.*, 67: 2657-2664.
- Martin, U., R. Bader and E. Bohem, 1993. BM 06.022: A novel recombinant plasminogen activator. *Cardiovasc. Drug Rev.*, 11: 299-311.
- Martin, U., B. Kaurfmann and G. Neugebauer, 1999. Current clinical use of reteplase for thrombolysis. A pharmacokinetic-pharmacodynamic perspective. *Clin. Pharmacokinet.*, 36: 265-276.
- Rao, D. and M.Y. Insly, 2003. Expression of tissue-type plasminogen activator in bacteria. US Patent H2055H.
- Sambrook, J. and D.W. Russell, 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
- Smalling, R.W., C. Bode and J. Kalbfleish, 1995. More rapid, complete and stable coronary thrombolysis with bolus administration reteplase compared with alteplase infusion in acute myocardial infarction. *Circulation*, 91: 2725-2732.
- Spellman, M.W., L.J. Basa, C.K. Leonard, J.L. Chackel, J.V. O'Connor, S. Wilson and H. van Halbeek, 1989. Carbohydrate structure of human tissue plasminogen activator in Chinese hamster ovary. *J. Biol. Chem.*, 264: 14100-14111.
- Studier, F.W. and B.A. Moffatt, 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.*, 189: 113-130.
- Studier, F.W., 1991. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.*, 219: 37-44.
- Wittwer, A.J., S.C. Howard, L.S. Carr, N.K. Harakas, J. Feder, R.B. Parekh, P.M. Rudd, R.A. Dwek and T.W. Rademacher, 1989. Effects of N-Glycosylation on *in vitro* activity of bowes melanoma and human colon fibroblast derived tissue plasminogen activator. *Biochemistry*, 28: 7662-7669.