Original Article

Construction of tenecteplase coding sequence by a rapid megaprimer PCR method and its cloning in a mammary gland tissue specific expression vector

K. Dormiani[1,2,*], Y. Khazaie[1,*], K. Ghaedi[1,3†], H. Mir Mohammad Sadeghi[2], M. Forouzanfar[1] and M.H. Nasr Esfahani[1†]

[1]*Department of Molecular Biotechnology, Royan Institute for Animal Biotechnology, ACECR, Isfahan, I.R.Iran.*
[2]*Department of Pharmaceutical Biotechnology and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R.Iran.*
[3]*Department of Biology, School of Science, University of Isfahan, Isfahan, I.R.Iran.*

Abstract

Tenecteplase is a variant of tissue plasminogen activator (t-PA) which has better pharmacokinetic properties and more selective thrombolytic activity. In the present study, we describe a rapid method to introduce three sets of mutation into defined positions in t-PA cDNA by a site-directed mutagenesis based on a megaprimer PCR approach to produce tenecteplase coding sequence where amino acids at positions 103, 117 and 296-299 in polypeptide sequence were modified. This sequence was cloned in pTZ57R by T/A cloning method to introduce suitable restriction sites at both ends of the amplified fragment. Vectors containing tenecteplase cDNA were propagated in One Shot TOP10 chemically competent *E. coli* and positive clones were selected by blue/white screening method. After being isolated and purified, the recombinant plasmids were digested by suitable restriction enzymes. Acquired tenecteplase coding sequence was subcloned into a tissue specific expression vector. Upon expression, it is expected that tenecteplase will be expressed exclusively in lactating mammary glands during milk production in transgenic animal. Finally, the recombinant vector was isolated and verified by restriction digestion and sequence analysis to confirm that the tenecteplase coding sequence has been inserted in the proper orientation.

Keywords: Tenecteplase; Tissue plasminogen activator; Cloning; Megaprimer

INTRODUCTION

Tissue plasminogen activator (t-PA) is a multi-domain serine protease whose physiological role is to convert plasminogen into its active form plasmin (1). This enzyme is necessary to initiate or accelerate the fibrinolysis process. t-PA is a relatively poor activator of plasminogen in the absence of fibrin because of its low affinity for the substrate binding, while presence of fibrin enhances markedly its ability to activate plasminogen (2,3). This fibrin specificity decreases systemic activation of plasminogen and the resulting degradation of circulating fibrinogen as compared to a molecule lacking this property. Based on its biochemical proper-

ties, it was discovered that t-PA is much more active in the conversion of plasminogen to plasmin than non-fibrin specific thrombolytics (such as streptokinase or urokinase) (4). Thus, it could be used as a therapeutic agent in the treatment of acute thrombotic disorders such as myocardial infarction (4-7). Due to its rapid clearance from the circulation, t-PA must be infused to achieve thrombolysis effect (3). When t-PA is administered as a bolus (or as a high dose infusion) the plasma level of the enzyme will be increased rapidly and plasminogen can be activated systemically especially on the surface of the clot. This systemic plasmin generation causes decreased levels of circulating plasminogen. An undesirable consequence of systemic activa-

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* These two authors contributed equally to this work: K. Dormiani and Y. Khazaie
† Co-Corresponding athours: K. Ghaedi and M.H. Nasr Esfahani
Tel. 0098 311 2612900, Fax. 0098 311 21605525
Email: kamranghaedi@royaninstitute.org, mh.nasr-esfahani@royaninstitute.org

tion of plasmin is peripheral and intracranial bleeding (8). Since the specific activity of t-PA depends on fibrin binding, systemic activation of plasmin does not occur in the absence of clotted plasma (9).

Tissue-type plasminogen activator is a typical example of a glycoprotein whose biological properties depend on the cooperative interaction of individual protein domains and the oligosaccharides at specific sites within the protein. Therefore many systematic site directed mutations have been applied to modify t-PA with the hope of increasing its fibrin specificity and better pharmacokinetic properties. Mutations in the protease domain to substitute a tetra alanine stretch at positions 296-299 were found to exert this property (9). Moreover, substitution of threonine 103 by asparagine results in a protein with better clearance rate (9). However, these mutations still do not yield full *in vitro* or *in vivo* fibrinolytic activity when compared with the wild-type t-PA. A mutation at position 117 replacing asparagine with glutamine residue removes a glycosylation site and would recover the loss of activity due to previous mutations. This final product was named tenecteplase (9).

The aim of this study was to use simple megaprimer method to modify three sets of mutation, including amino acid residues at positions 103 and 117 and 296 to 299 by using suitable primers for each mutation site. Since the goal of such design is to express the tenecteplase in animal cell culture or transgenic animals, the most used codons in mammalian systems were substituted. Finally,

the amplified tenecteplase cDNA was inserted into a tissue specific expression vector. This recombinant expression vector will be employed for production of recombinant protein in a transgenic animal.

MATERIALS AND METHODS

Mutagenesis system for the production of tenecteplase cDNA

All PCR experiments were performed in an Eppendorf Mastercycler gradient thermal cycler (Germany) as described below. Primers used during this study were ordered from Bioneer Company (Korea) and are presented in Table 1. All mutagenesis reactions were performed in three stages of PCRs and each stage consisted of two steps (Fig. 1; Tables 2 and 3). pET15b-tPA cDNA was a gift from School of Pharmacy and Pharmaceutical Sciences of Isfahan University of Medical Sciences.

This experiment was carried out as follows: The aim of the first stage was production of one mutation at position 103 in coding sequence of t-PA, introducing threonine in place of asparagine residue by alteration of three nucleotides (Tables 2 and 3).

Step 1: 50 µl of mutagenesis reaction containing 200 ng template DNA (pET15b-tPA), 10 pmol (100 nM) each of TPAF forward primer and mutagenic TNK103R reverse primer, 1.25 U *Pfu* DNA polymerase (Fermentas, Lithuania), 1 µl dNTPs at 10 mM (Cinnagen, Iran) and 5 µl 10X buffer for *Pfu* (200 mM Tris-HCl with pH 8.8 at 25 °C, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton

Table 1. List of primers used in different stages

| No.	Name	Sequence	characteristics
1	TPAF	5´ggcctcgagatggatgcaatgaagagagggct 3´	Upstream of t-PA coding strand as forward primer
2	TPAR	5´ggcctcgagtcacggtcgcatgttgtcacg 3´	Downstream of t-PA coding strand as reverse primer and reverse primer for colony PCR
3	TNK103R	5´tctccgctgtgctcca**gtt**gcccctgtagctgatg 3´	Mutagenic primer for 103rd amino acid as reverse primer
4	TNK117R	5´caacgcgctgct**ctg**ccagttggtgca 3´	Mutagenic primer for 117th amino acid as reverse primer
5	TNK296-9R	5´gctctccgggcga**ggcggcggcggc**ggcaaagatggca 3´	Mutagenic primer for amino acids 296 to 299 as reverse primer
6	CSF	5´ggaatcgcggatcctcgagatggatgca 3´	Forward primer for colony PCR

Modified codons are underlined
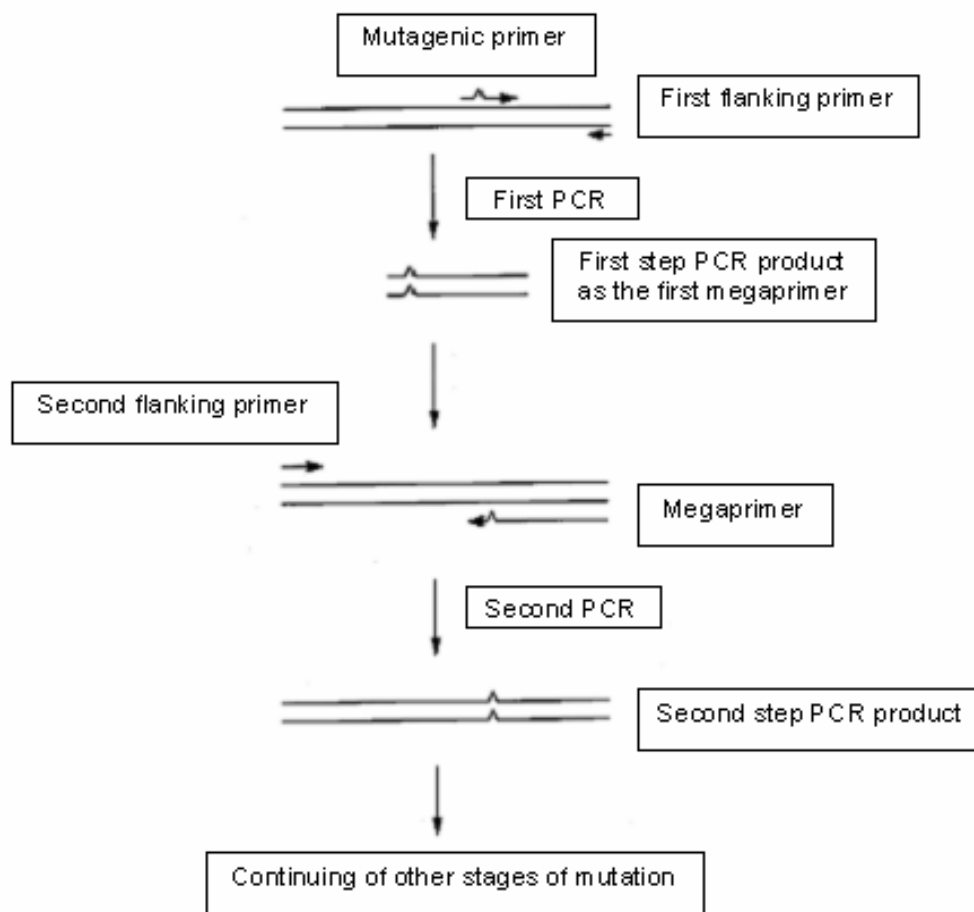
Fig. 1. Schematic outline of the mutagenesis protocol used in each stage to introduce one set of mutation into t-PA coding sequence.

Table 2. Brief description of different steps and stages employed to produce tenecteplase coding sequence

Stages	steps	Primers		Template	Product size (bp)	Product
		Forward	**Reverse**			
Stage 1	Step1	TPAF	TNK103R	t-PA coding strand	439	First megaprimer
	Step2	First megaprimer	TPAR	t-PA coding strand	1707	t-PA coding strand with one mutation
Stage 2	Step1	TPAF	TNK117R	t-PA coding strand with one mutation	477	Second megaprimer
	Step2	Second megaprimer	TPAR	t-PA coding strand	1707	t-PA coding strand with two mutations
Stage 3	Step1	TPAF	TNK296-299R	t-PA coding strand with two mutations	1024	Third megaprimer
	Step2	Third megaprimer	TPAR	t-PA coding strand	1707	t-PA coding strand with three mutations

Table 3. PCR reaction conditions

Stages	Reaction condition					
	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
1	94 °C, 2 min	94 °C, 1 min	55 °C, 1 min	72 °C, 2 min	30	72 °C, 10 min
2	94 °C, 2 min	94 °C, 1 min	55 °C, 1 min	72 °C, 2 min	30	72 °C, 10 min
3	94 °C, 2 min	94 °C, 1 min	65 °C, 1 min	72 °C, 5 min	30	72 °C, 20 min

X-100, 1 mg/ml BSA and 20 mM $MgSO_4$) (Fermentas, Lithuania) were used for PCR using the following conditions: 5 min of denaturation at 94 °C followed by 35 cycles of amplification (94 °C 1 min, 55 °C 2 min and 72 °C 3 min) and ended with one cycle of 10 min at 72 °C. The amplified product of this step used as a megaprimer with the length of 439 bp that started from the first amino acid codon of t-PA protein with two modified nucleotides at the 413 and 414 positions of t-PA coding strand. The product was purified by QIAprep Spin Miniprep kit (Qiagen, Germany) and its concentration was determined by CECIL, CE7250 spectrophotometer (UK). The amplified product was used for step 2.

Step 2: 50μl PCR reaction containing 200 ng template DNA (coding strand of t-PA), 10 pmol (100 nM) each of the 439 bp megaprimer (produced in the step1) as forward and TPAR downstream primer as reverse, 1.25 U *Pfu* DNA polymerase, 1 μl dNTPs at 10 mM and 5 μl 10X buffer of *Pfu* were utilized. The PCR cycles were the same as above. The product of this step was the entire t-PA coding strand with a mutation at amino acid 103.

At the second stage, for creating second mutation located at amino acid residue 117 (nucleotide 454 to 456 of t-PA coding strand), the same process was accomplished as described above (Tables 2 and 3).

Step1: All conditions were the same as step1 of the previous stage except for the template which was the final product of stage 1 with the size 1707 bp instead of pET15b-tPA and TNK117R instead of TNK103R as the reverse primer. The amplified product of this step with the length of 477 bp was used as a megaprimer for the next reaction encompassing three modified nucleotides at positions of 454 to 456 of t-PA coding strand.

Step 2: 50 μl PCR reaction containing 200 ng template DNA (pET15b-tPA), 10 pmol (100 nM) each of the 477 bp megaprimer as

forward and TPAR downstream primer as reverse, 1.25 U *Pfu* DNA polymerase, 1μl dNTPs at 10 mM and 5 μl 10X buffer of *Pfu* were used. The conditions of PCR reaction were similar to step 1. The final product of this stage was t-PA coding strand with two mutations at amino acid residues 103 and 117.

The last stage was again performed in two steps in order to construct the third mutation site resulting in a four amino acids substitutions at residues 296 to 299 (991 to 1002 nucleotides of t-PA coding strand), with a tetra-alanine stretch (Tables 2 and 3).

Step 1: The conditions were exactly the same as step 1 of previous stage except for these modifications: the template was the final product of stage 2 with the size 1707 bp and mutagenic TNK296-299R primer as an antisense primer. The annealing time of PCR reactions was set at 65 °C, which was due to the length, GC reach contents, and the high melting point of the primer TNK296-299R (95.8 °C). The amplified product of this step was collected and used as a megaprimer for the next step. This product had a length of 1024 bp that started from the initiation codon of t-PA sequence and contained two already described mutated sites besides four alanine residues at positions 296 to 299 in native t-PA molecule.

Step 2: 50 μl of reaction containing 200 ng template DNA (pET15b-tPA cDNA), 10 pmol (100 nM) each of the 1024 bp megaprimer as forward and TPAR downstream primer as reverse, 2.5 U *Ex taq* (Takara) DNA polymerase, 1 μl dNTPs at 10 mM and 5 μl 10X buffer of *Ex taq* were utilized. The final product of this stage was the entire length of t-PA coding strand (size of 1707) with three mutation sites at amino acid residues 103, 117 and 296 to 299. In this final step, *taq* polymerase instead of *Pfu* DNA polymerase was used in order to clone the final product in a t-vector (Fermentas).

Cloning of tenecteplase cDNA in a t-vector

This amplified fragment was directly inserted into pTZ57R vector (Fermentas) by T/A cloning method according to the instruction of manufacturer. The ligation reaction mixture was subjected to transform in One Shot TOP10 chemically competent *E. coli* cells (Invitrogen, USA). The positive clones were selected by blue/white screening method on LB agar and recombinant plasmids (pTZ57R-tenecteplase) were extracted and purified by QIAquick Gel Extraction Kit (Qiagen, Germany). To confirm that tenecteplase coding sequence was cloned in pTZ57R, the purified plasmid was examined by restriction digestion and sequence analysis. As primer pairs, TPAR and TPAF, were designed to have *Xho*I recognition site in their 5´ ends cloned tenecteplase coding sequence had *Xho*I recognition sites at the both of its ends.

Cloning of tenecteplase cDNA into a tissue specific expression vector

Recombinant vector (pTZ57R-tenecteplase cDNA) was digested by *Xho*I (Fermentas) and the resultant tenecteplase sequence was purified using QIAprep Spin Miniprep Kit (Qiagen) and subcloned into pBC1 tissue specific expression vector (Invitrogen) in *Xho*I site for expression of recombinant proteins into the milk by a ligation reaction according to Sambrook *et al.* (10). This tissue specific expression vector is a specific milk expression vector which contains the goat β-casein promoter and other proprietary DNA sequences that has a size about 22 kb. The ligation mixture was used to transform One Shot TOP10 chemically competent *E. coli* according to the instructions of manufacturer. PCR method using specific PCR primers to amplify tenecteplase cDNA was used for selecting positive colonies as well as determining the orientation of the insert. Two Recombinant vector containing tenecteplase sequences with correct orientation were chosen and were send for sequencing to the Bioneer Copmany (Korea). Finally one of them was propagated in One Shot TOP10 chemically competent *E. coli* and purified by

Endofree Plasmid Purification Kit (Qiagen) for further use.

RESULTS

To achieve the entire length of tenecteplase cDNA starting from t-PA coding sequences, PCR products of each stage contained different amino acid residues in one of above mentioned positions. At the first stage, step one was performed to replace the amino acid threonine by asparagine at position 103. The product of this stage was the first megaprimer with size of 439 bp containing two modified nucleotides 413 and 414. Considering the size of the obtained product using forward primer (TPAF) and the first mutagenic reverse primer (TNK103R), a size of 439 bp was expected (Fig. 2a). This product was used as a mega-primer in the step 2 which was performed for the production of complete fragment of t-PA with the desired mutation.

The PCR product of the second step with this megaprimer and reverse flanking primer (TPAR) was about 1700 bp that was compatible with the size of t-PA coding strand. The second stage of PCR was performed with using the product of the stage one as the template and TPAF and TNK117R as primers. The size of the megaprimer of this stage (the product of TPAF and TNK117R primers) was expected to be 477 bp and was confirmed by the agarose gel (Fig. 2b). Second step PCR reaction of the second stage was carried out successfully by the 477 bp megaprimer (second megaprimer) as forward and TPAR as reverse primer using the t-PA coding strand as the template to produce a strand with two mutation sites.

Finally, for the first step PCR reaction of stage 3, TPAF and TNK266-299R were used to produce the final megaprimer with a size of 1024 bp (Fig. 2c). Consequently, t-PA with three mutations at nucleotides 413 and 414, 454 to 456 and 991 to 1002 was obtained by a PCR mutational insertion approach with the third megaprimer and TPAR and the original t-PA coding strand as template (Fig. 2d). The sequencing of the final product confirmed all of these mutations. Using *taq* DNA polymerase to construct the final step of
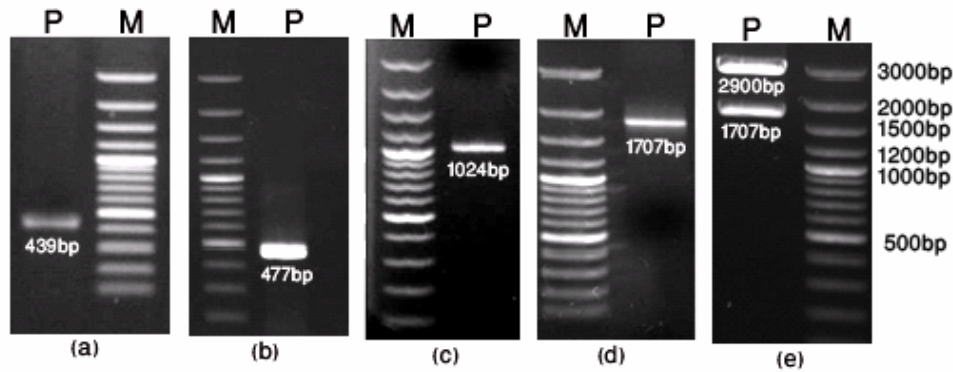
Fig. 2. Ethidium bromide stained 0.7% agarose gel. Lane M: DNA molecular size marker (100 bp DNA ladder plus, Fermentas) was the same for all the gels. (a) Lane P: the amplified product of stage 1 with the size 439 bp. (b) Lane P: the amplified product of stage 2 with the size 477 bp as the second megaprimer. (c) Lane P: the amplified product of stage 3 with complete three sets mutation of interest. (d) Lane P: The final product, tenecteplase coding sequence that contains full length of t-PA cDNA (1707 bp) and complete three sets of mutations in amino acid positions 103, 117 and 296 to 299. (e) Lane P: tenecteplase with size 1707 bp and linear pTZ57R with size about 2900 bp after digestion of recombinant vector (pTZ57R-tenecteplase cDNA) by *Xho*I.

tenecteplase construction, a single adenine nucleotide was added to each 3′ end of the sequence; hence it could be inserted into pTZ57R as a t-vector. In all steps except the last one, *Pfu* DNA polymerase was employed as it had been reported that its fidelity is 12 times higher than *taq* polymerase due to the presence of a 3' to 5' exonuclease activity (11). Thus, the choice of *Pfu* polymerase was critical for getting high overall fidelity and efficiency of the method. However, *Pfu* polymerase lacks terminal transferase activity (12), which is present in *Taq* polymerase that adds an extra A to the 3' ends of amplified double stranded DNAs (13). After transformation, positive clones were selected by blue/white screening method using LB plates containing IPTG and X-gal. After extraction and purification of recombinant pTZ57R (pTZ57R-tenecteplase), the tenec-teplase sequence was isolated simply form the rest of the vector by *Xho*I digestion (Fig. 2e). After purification of tenecteplase with two cohesive ends, it was subcloned into the tissue specific expression vector and propagated in One Shot TOP10 chemically competent *E. coli* which is a suitable strain for transforming by large size plasmids. Using a large vector for cloning requires the screening of large numbers of *E. coli* transformants for the presence of the insert of interest. A simple protocol for screening transformants by colony

PCR was employed to identify those containing tenecteplase coding sequence with correct orientation in the vector. In this protocol two primers were used. One of them was CSF, as forward primer which could anneal to the vector upstream of tenecteplase sequence and the reveres primer was TPAR which would attach to the 3′ end of the inserted DNA. PCR was carried out based on Invitrogen protocol.

Colony PCR and gel electrophoresis of amplified fragments showed a sharp band with the size of about 1700 bp colonies containing tenecteplase sequence having correct orientation, while those colonies containing the insert with reversed orientation showed a band with size about 150 bp (Fig. 3). After purification of recombinant vector, to confirm that inserted fragment was bona fide fragment of tenecteplase, recombinant plasmid was digested by *Xho*I and digested materials were electrophoresed on agarose gel. On the gel a band about 1700 bp and another one with size about 21000 bp showed tenecteplase and linear vector, respectively (Fig. 4). Finally to verify tenecteplase coding sequence, recombinant plasmid was sent for sequencing with appropriate primers and data approved the absence of any undesired mutation in the tenecteplase sequence (Fig. 5).
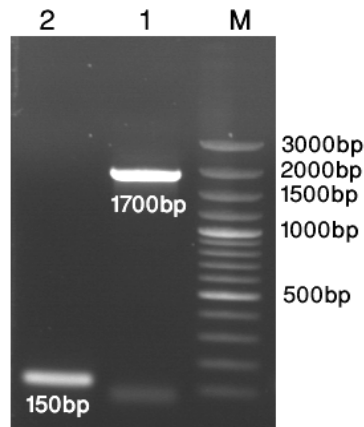
Fig. 3. Colony PCR for identification of correct colonies. Ethidium bromide stained 0.7% agarose gel. Lane M: DNA molecular size marker (100bp DNA ladder plus, Fermentas). Lane 1: the amplified product with correct orientation by specific primers after colony PCR. Lane 2: the amplified product with reversed orientation by the same primers after colony PCR.

Fig. 4. Digestion of recombinant tissue specific vector by *Xho*I. Ethidium bromide stained 0.7% agarose gel. Lane M1: DNA molecular size marker (100 bp DNA ladder plus, Fermentas). Lane P: tenecteplase with size 1707 bp and linear vector with size about 21000 bp after digestion of recombinant vector by *Xho*I. Lane M2: DNA molecular size marker III (0.12-21.2 kpb, Roche).

DISCUSSION

Oligonucleotide-directed site-specific muta-genesis is a method used for introducing desired mutations into target DNA sequences. Different protocols have been established to achieve efficient mutagenesis, including some utilizing PCR (14). Among the PCR-based protocols, the 'megaprimer' approach (15-17) appears to be simple and rapid. This method involves two rounds of PCR that uses two 'flanking' primers and one internal mutagenic primer containing the desired base substitution(s). The first PCR is performed using the mutagenic internal primer and the first flanking primer. The product of this first PCR, the 'megaprimer', is purified and used, along with the second flanking primer, as a primer for a second PCR. The final PCR product contains the desired mutation in a particular DNA sequence (Fig. 1). One advantage of megaprimer PCR mutagenesis is eliminating the agarose gel electrophoresis step (18,19) using a convenient two-step PCR protocol which provides rapid and highly efficient site-directed mutagenesis without any additional manipulation or treatment of PCR products.

We have applied three stages of PCR based mutagenesis using megaprimer to get an efficient variant of t-PA, tenecteplase. The structure of tenecteplase is similar to t-PA except for 6 amino acid residues at positions 103, 117 and 296-299. As the aim of this study was the expression of related recombinant protein in mammalian cell models or trans-genic animal and since different organisms employ different codons, the most common used codons in eukaryotes were chosen for substitution. Moreover, to our knowledge this is the first report using the megaprimer approach as a simple and rapid method for production of t-PA variants such as tenecte-plase.

ACKNOWLEDGMENT

(a)

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   1-ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTT-60
  61-TCGCCCAGCCAGGAAATCCATGCCCGATTCAGAAGAGGAGCCAGATCTTACCAAGTGATC-120
 121-TGCAGAGATGAAAAAACGCAGATGATATACCAGCAACATCAGTCATGGCTGCGCCCTGTG-180
 181-CTCAGAAGCAACCGGGTGGAATATTGCTGGTGCAACAGTGGCAGGGCACAGTGCCACTCA-240
 241-GTGCCTGTCAAAAGTTGCAGCGAGCCAAGGTGTTTCAACGGGGGCACCTGCCAGCAGGCC-300
 301-CTGTACTTCTCAGATTTCGTGTGCCAGTGCCCCGAAGGATTTGCTGGGAAGTGCTGTGAA-360
 361-ATAGATACCAGGGCCACGTGCTACGAGGACCAGGGCATCAGCTACAGGGGCA CG TGGAGC-420
                                         Tenecteplase   413- AC -414
 421-ACAGCGGAGAGTGGCGCCGAGTGCACCAACTGGAA CA GCAGCGCGTTGGCCCAGAAGCCC-480
                       Tenecteplase  454-C G -456
 481-TACAGCGGGCGGAGGCCAGACGCCATCAGGCTGGGCCTGGGGAACCACAACTACTGCAGA-540
 541-AACCCAGATCGAGACTCAAAGCCCTGGTGCTACGTCTTTAAGGCGGGGAAGTACAGCTCA-600
 601-GAGTTCTGCAGCACCCCTGCCTGCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGG-660
 661-TCAGCCTACCGTGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAAT-720
 721-TCCATGATCCTGATAGGCAAGGTTTACACAGCACAGAACCCCAGTGCCCAGGCACTGGGC-780
 781-CTGGGCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG-839
 841-CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGC-900
 901-CTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCC-960
 961-TCCCACCCCTGGCAGGCTGCCATCTTTGCC AAGCA C AGGAGG TCGCCCGGAGAGCGGTTC-1020
              Tenecteplase  991- GCCGC   GCCGCC -1002
1021-CTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCGCCCACTGCTTCCAG-1080
1081-GAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACCGGGTGGTCCCT-1140
1141-GGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGGAATTCGATGAT-1200
1201-GACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATTCGTCCCGCTGTGCC-1260
1261-CAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGGACCTGCAGCTGCCGGAC-1320
1321-TGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAGGCCTTGTCTCCTTTCTATTCG-1380
1381-GAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCATCCAGCCGCTGCACATCACAACAT-1440
1441-TTACTTAACAGAACAGTCACCGACAACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGG-1500
1501-CCCCAGGCAAACTTGCACGACGCCTGCCAGGGCGATTCGGGGAGGCCCCCTGGTGTGTCTG-1560
1561-AACGATGGCCGCATGACTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAG-1620
1621-GATGTCCCGGGTGTGTACACCAAGGTTACCAACTACCTAGACTGGATTCGTGACAACATG-1680
1681-CGACCGTGA-1689
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AGCTACAGGGGCAACTGGAGCACAGC          GTGCACCAACTGGCAGAGCAGCGCG          CATCTTTGCCGCCGCCGCCGCCTCGCCCGGAG
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(b)   **(c)**   **(d)**

Fig. 5. cDNA sequence data of t-PA and tenecteplase. a) ORF sequence of t-PA. Boxes indicate bases which are different in t-PA and tenecteplase coding sequences. b), c) and d) indicate partial sequence data of tenecteplase that show modified neucleotides in t-PA sequence.

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