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Cloning of streptokinase gene in replicase based vector

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Abstract: Streptokinase gene from Streptococcus pyogenes was cloned in replicase based pSin at StuI site of the vector using blunt end ligation. The gene in right orientation was confirmed by RE analysis, PCR using gene specific forward primer and BGH reverse primer, and Sequencing of the insert using BGH sequencing primer.
Key words: Cloning, streptokinase, pSin vector, pTargeT vector.

Introduction

Streptokinase is a single peptide, multi-domain secretory protein of 414 or 415 amino acid residues (47 kDa) produced by various strains of beta hemolytic Streptococcus (1,2,3,4) Many group A treptococci bind plasminogen (5). It was shown that plasminogen, once bound to the streptococcal surface, can be converted to plasmins by tissue-type activator (6). It was also demonstrated plasminogen, once bound to the streptococcal surface, can be readily activated by streptokinase, suggesting a functional link between the two streptococcal molecules (7). Wong et al (8) showed that streptokinase is one of the major blood clot dissolving agents used in many medical treatments.

In the present work, sk gene was cloned in replicase based vector for use in therapeutics.

Materials and Methods

RE digestion of r-plasmid for release of SK gene insert

After checking the integrity and presence of plasmid in eluted buffer, the pTargeT.sk10 plasmid was digested with EcoRI enzyme as per manufacturer's instructions. The reaction mixture contained pTargeT.sk10 DNA 15µl, enzyme buffer (10x) 5µl, EcoRI enzyme 3µl, nuclease free water 27 µl totalling to 50 µl.The reaction mixture was incubated at 37°C in a water bath for 6 hour. A 10µl sample was run to check the digestion and then the digestion product was run on 1% agarose gel and the released insert was extracted from the gel.

DNA extraction from agarose gel

The gel extraction of DNA fragment was done using MinElute gel extraction kit (Qiagen, Germany) following manufacturers instruction. Briefly, the agarose gel containing DNA fragment was excised with a scalpel and weighted in a colourless microfuge tube. Three volumes of buffer QG was added to 1 volume of gel and incubated at 50°C until gel slice had completely dissolved, one gel volume of isopropanol was added to the melted agarose and mixed by inverting several times. The sample was applied to the MinElute column fitted in a collection tube. The column was centrifuged at 13000g for 1 min at room temperature and the flow through was discarded. 500µl of buffer QG was applied to the spin column, centrifuged and the flow through was discarded again. The DNA bound to the column was washed twice 750µl of buffer PE at

1300g and the flow through was discarded. The DNA was eluted from the column in 10µl nuclease free water.

Blunting of EcoRI generated SK gene staggered ends

Because of limited choice of restriction sites in replicase based mammalian expression vector, blunt end cloning was thought to be desirable approach. Although comparatively difficult, it had the dual advantage of getting the clone insert in right orientation with a probability of 50%. So for blunting of staggered ends generated by EcoRI enzyme, T4 DNA polymerase (Promega) was used as per the given instructions. Reaction mixture contained Insert DNA 16 µl, T4 DNA polymerase buffer 10x 2.5µl, T4 DNA polymerase 1µl, dNTP mix (10mM each) 1µl, nuclease free water 4.5µl totalling 25 µl. The reaction mixture was incubated at 37°C for 10 min and then the reaction was stopped by heating at 70°C for 10 min.

Purification of blunted insert

It was done using MinElute reaction clean up kit (QIAGEN, Germany) as per manufacturer's protocol. Added 300µl of buffer ERC to the enzymatic reaction and mixed and checked that the colour of the mixture is yellow similar to buffer ERC without the enzymatic reaction, if the colour of the mixture is orange or violet, added 10µl of 3M sodium acetate, pH 5.0 and mixed. Placed a MinElute column in a 2ml collection tube in a suitable rack and applied the sample to the column to bind the DNA and centrifuged at 13000rpm for 1min, discarded the flow through and placed the column back into the same tube. Centrifuged the column for an additional 1 min at maximum speed. Placed the column in a clear 1.5ml microcentrifuge tube. To elute DNA, added 10µl buffer EB (10mM TrisCl, pH 8.5) or H_2O to the centre of the membrane, let the column stand for 1min and then centrifuged for 1 min.

Preparation of pSin vector (9)

2µl of pSin vector DNA was added to 200µl of *E.coli* DH5α competent cells. It was kept on ice for 1 hour. The mixture was heat shocked at 45°C for 2min and immediately chilled on ice till further use. 600µl of freshly prepared SOC media was added to heat shocked mixture of plasmid and competent cell and then incubated at 37°C for 1 hour in shaking incubator at 110 rpm. Cell were plated on LB agar medium in petri dish containing 100µg/ml ampicillin and grown overnight. A few colonies were picked up and streaked plates to store for use in future. The same loop was used to inoculate fresh LB broth (5ml). Plasmid (pSin vector) was isolated from the freshly inoculated overnight grown culture and checked on 1% agarose gel electrophoresis against 1kb DNA ladder marker for the presence and its size.

Creation of cloning site in pSin vector

Among the available few restriction site at MCS, StuI was chosen as preferred site to create blunt end. A reaction mixture was prepared with StuI enzyme (Biolabs 1000U/ml) (cutting site in vector 7775 bp) The reaction mix contained StuI enzyme (Biolab 10,000U/ml) 5µl, pSin vector (100 ug/µl) 60µl, buffer 10x 10µl, nuclease free water 25µl totalling 100µl. The reaction mixture was incubated at 37°C overnight, the linearised plasmid was checked and quantitated on 1% agarose gel electrophoresis, linerarized plasmid was then gel extracted and quantified as per method described earlier.

Dephosphorlyation of 5' ends of pSin vector

The (calf intestinal alkaline phosphate) CIAP was used to remove 5'phosphate group from both the ends of the linearized plasmid. The reaction mixture contained linearized vector pSin 50µl, CIAP enzyme 2µl, buffer (10x) 6µl , nuclease free water 2µl, totali9ng 60µl. The protocol provided with Fermentas CIAP was followed with slight modification. Briefly, the reaction mixture was incubated at 34°C for 30 min. But to be on safer side 1µl of CIAP was added again and reincubated at 37°C for 30min. Enzyme was then inactivated by heating the reaction mixture at 70°C for 10 min.

Purification of dephosphorylated linearized vector

It was done using MinElute reaction cleanup kit as described earlier.

Blunt end ligation of pSin vector and SK gene

A 10µl reaction mixture was standardized with 30% PEG 8000 for blunt end ligation. It contained T4 DNA ligase (Fermentas) 1µl, pSin vector 2µl, SK gene insert 5µl, ligation buffer (10x) 1µl, 30% PEG 8000 (Amresco) 1µl.The time temperature relationship was maintained as described in data sheet of T4 DNA ligase provided by Fermentas. The reaction mixture was incubated at 16°C overnight

Transformation of *E.coli* (DH5α) cells with ligation product pSin vector and SK gene.
It was done as per method described earlier (9).

Screening of recombinant clones

A large number of colonies 25 were picked from the overnight grown transformants. The individual colonies were inoculated in ampicillin (50µg/ml) containing LB broth and allowed to grow for 18 to 24 hour. Plasmid DNA was isolated from these colonies by alkali lysis method as well using Promega SV miniprep Kit and checked on 1% agarose gel electrophoresis.

Isolated plasmids were checked for the presence of insert by digestion with XcmI enzyme and then electrophoresed on 1% agarose. The **. r**eaction mix contained plasmid pSin.sk 4µl, XcmI enzyme 1.5µl, NE buffer 2 (10x) 1.5µl, nuclease free water 8µl t0otalling 15µl.

Sequencing of cloned SK gene in pSin

Once the recombinant plasmids were identified as containing SK gene in correct orientation, a part of the same plasmid DNA was sent for sequencing to Chromous Biotech Bangalore. The sample was sequenced with BGH reverse primer. The obtained sequence was analysed by blast at NCBI in the light of it's similarity with existing sequence of SK gene and the sequence from which primer was designed.

Results and Discussion

The SK gene insert was released from pTargeT.sk10 by digestion with EcoRI (Fig.1) and blunted. The pSin vector was digested with StuI to obtain blunt ended vector which was ligated with SK gene and *E.coliDH5α* was transformed and plated on ampicillin containing agar plates. Large numbers of colonies were observed after overnight growth and 12 colonies were processed for plasmid DNA isolation which yielded good amount of DNA. The plasmid DNA from 12 clones were digested with enzyme XcmI to check the presence of insert as well as orientation of the gene (Table1).

Table 1. pSin.sk digested with XcmI.

Right orientation	Wrong orientation
9.741kb, 2.418 kb	8.634kb, 3.515kb

The clone 11 was found to contain SK gene in right orientation and was designated as pSin.sk11 (Fig.2& 3). This recombinant plasmid was sequenced using BGH reverse primer and result showed that the gene was in right orientation.

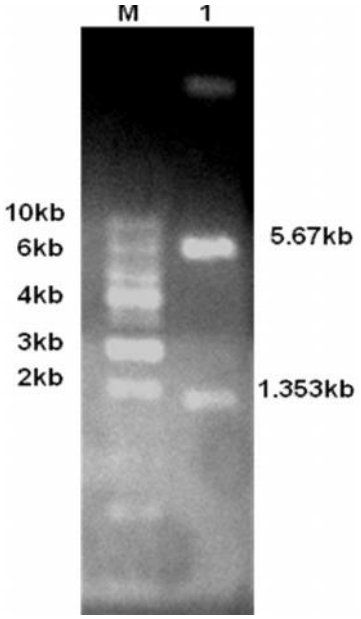

Fig. 1. pTargeT.sk10 digested with EcoRI releasing sk gene insert. Lane M,1 kb DNA ladder (B Genei); 1, pTargeT and sk gene released after EcoRI digestion.

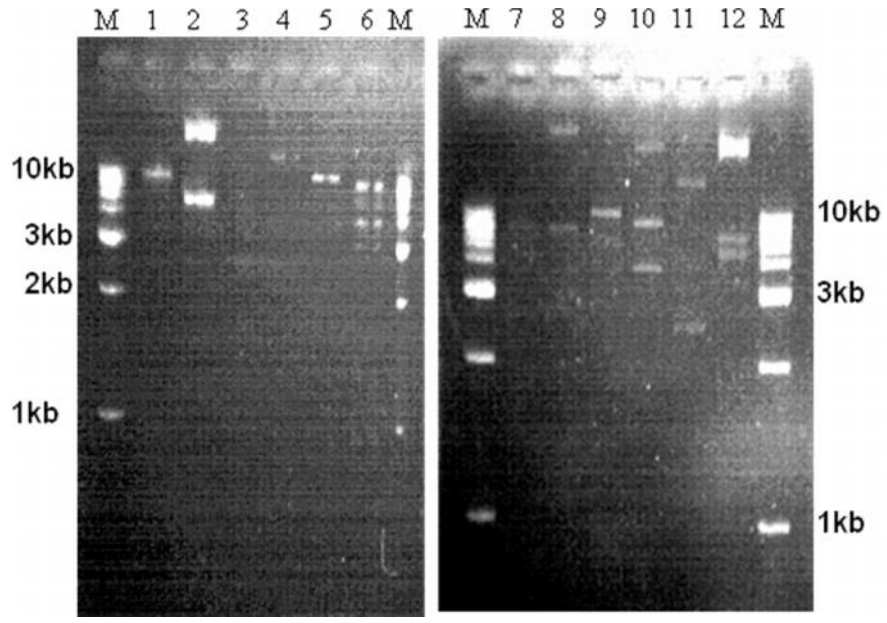

Fig.2. Plasmid DNA pSinCMV.sk cut with XcmI to check right orientation gene containing clone. Lane M, 1kb DNA ladder (B Genei); 1-12, clones 1-12 digested with XcmI. Clone 11 yielding fragments of 9.741 kb, and 2.418 kb is in right orientation**.**

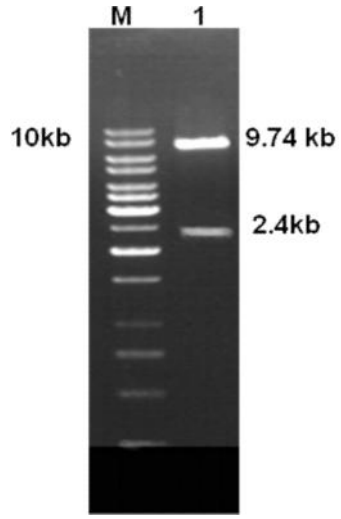

Fig.3. pSinCMV.sk 11 cut with XcmI. Lane M-1 kb DNA ladder ; 1- XcmI cut rplasmid producing 9.74 and 2.4 kb fragments confirming right orientation of the gene.

The pSin vector is a replicase gene based mammalian expression vector which produces 100 to 1000 times more copies of mRNA of the gene insert and hence its requirement for therapeutic use will be very less. The cloning of SK gene was done in this vector also successfully and efficiently since out of 12 colonies processed, one clone (clone 11) yielded the plasmid with gene insert in right orientation which was confirmed by digestion with enzyme XcmI as well as sequencing with BGH reverse primer. The PCR amplification and cloning of SK gene has seen reported by different workers (8,7,10,11) using *E.coli* while some workers have used *Pichia pastoris* for cloning the SK gene and purified the SK protein for therapeutic use (12). Sazonova et al (13) showed that reprogrammed streptokinases develop fibrin targeting and dissolve blood clots with more potency than tissue plasminogens activator. They observed that reprogramming SK's mechanism of action markedly enhances fibrin-targeting and creates, in comparison with TPA, activators with greater fibrinolytic potency. Hardig et al (14) studied the changes in clot analysis levels of reteplase and streptokinase following continuous wave ultrasound exposures at ultrasound intensities following attenuation from the skull bone. The rplasmid constructed in the present work can be evaluated for use in human as thrombolytic agent.

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References:

1. Malke H, Roc B, Ferretti JJ (1985). Nucleotide sequence of the streptokinase gene from *Streptococcus equisimilis* H 46A. Gene(Amst)., 34: 357-362.
2. Jakson KW, Tang J (1982). Complete amino acid sequence of streptokinase and its homology with serine proteases. Biochemistry., 21: 6620-6625.
3. Tillet WS, Garner RL (1933). The fibrinolytic activity of haemolytic streptococci. J Exp Med., 58: 485-502.
4. Nihalani D, Kumar R, Rajagopal K, Sahni G (1998). Role of the amino-terminal region of Streptokinase in the generation of a fully function plasminogen activator complex probed with synthetic peptides. Protein Sci., 7: 637-648.
5. Ullberg M, Kronvall G, Wiman B (1989). New receptor for human plasminogen on Gram positive cocci. APMIS., 58: 996-1002.
6. Kuusela P, Ullberg M, Sakula O, Kronvall G (1992). Tissue-type plasminogen activator-mediated activation of plasminogen on the surface of group A, C and G streptococci. Infect Immun., 60: 196-201.
7. Berge A, Ulf S (1993). PAM, a Noval Plasminogen-binding Protein from Streptococcus pyogenes. J Biol Chem., 268: 25417-25424.
8. Wong SL, Ruiqiong Y, Shyroze N (1994). Engineering and Production of Streptokinase in a *Bacillus subtilis* Expression-Secretion System. Applied and Environmental Microbiology., 60: 517-523.
9. Sambrook J, Russell D (2001). Molecular Cloning: A laboratory manual 3rd ed. GSHL Press., New York.
10. Wakeham N, Terzyan S, Zhai P, Loy JA, Tang J, Zhang XC (2002). Effect of deletion of Streptokinase residues 48-59 on Plasminogen activation. Protein Engineering., 15: 753-761.
11. Goyal D, Sahni G, Sahoo DK (2009). Enhanced production of recombinant Streptokinase in Escherichia coli using fed-batch culture. Bioresour Technol., 100: 4468-4474.
12. Vellanki RN, Potumarthi R, Mangamoori LN (2009). Constitutive expression and optimization of nutrients for Streptokinase production by Pichia pastoris using statistical methods. Appl Biochem Biotechnol., 158: 25-40.
13. Sazonova IY, McNamee RA, Houng AK, King SM, Hedstrom L, Reed GL (2009). Reprogrammed streptokinases develop fibrin-targeting and dissolve blood clots with more potency than tissue plasminogen activator. J Thromb Haemost., 7: 1321-1328.
14. Hardig BM, Carlson J, Roijer A (2008). Chance in clot lysis level of reteplase and Streptokinase following continuous wave ultrasound exposure, at ultrasound intensities following attenuation from the skull bone. BMC Cardiovasc Disord., 26: 8-19.

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