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# Cloning and expression of hybrid streptokinase towards clot-specific activity

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ABSTRACT

Streptokinase (SK) is a thrombolytic agent that is widely used to treat myocardial infarction and pulmonary embolism. The lack of fibrin specificity of SK for the clot lysis is one of the limitations of SK. In this study, we have incorporated the finger and Kringle 2 domains from the human tissue type plasminogen activator gene (t-PA) at the 5′ end of the SK gene. These domains are responsible for specific binding to fibrin. We have used the pRSETB vector in an attempt to express the hybrid streptokinase possessing specificity for fibrin. On this regard, three hybrid streptokinase were constructed and expressed in *Escherichia coli* BL21 (DE3): the finger domain with SK (FSK), the Kringle 2 domain with SK (KSK) and the finger domain + Kringle 2 with SK (FKSK). The activities of the hybrid SKs were assessed by caseinolytic assay and clot lysis assay. All hybrid SKs were found to activate plasminogen in the caseinolytic plate assay. In the clot lysis assay, KSK and FSK were able to dissolute human blood and artificial clots in a fibrin-dependent manner unlike the SK and FKSK proteins.


## 1. Introduction

Thrombolytic therapy with plasminogen activators, such as streptokinase (SK), staphylokinase (STA), tissue plasminogen activator (t-PA) and urokinase (UK), has revolutionized the clinical management of diverse circulatory diseases, e.g., deep-vein thrombosis, pulmonary embolism and myocardial infarction. These agents exert their fibrinolytic effects through activation of plasminogen to plasmin in the circulation. Plasmin is a serine protease that is involved in the dissolution of blood clots in a fibrin-dependent manner (Castellino, 1981; Bachman, 1994).

Streptokinase (SK) is a non-enzymatic protein that is produced by the Lancefield group C strain of β-hemolytic streptococci (Banerjee et al., 2004). SK is a single-chain polypeptide containing 414 amino acids with a molecular mass of 47,000. It consists of 3 domains: α, β and γ (Malke and Ferretti, 1984). SK by itself is not a plasminogen activator; it forms a 1:1 stoichiometric complex with plasminogen known as the streptokinase–plasminogen activator complex. This complex activates other plasminogens to form plasmin (Castellino, 1981; Bajaj and Castellino, 1977). SK has been one of the most frequently used thrombolytic agents in the treatment of acute myocardial infarction (Collen and Lijnen, 1991; Lijnen and Collen, 2000). Unlike streptokinase, t-PA is a trypsin like serine protease that activates plasminogen directly. The t-PA protein has a molecular mass of 70,000 and consists of 527 amino acids with 5 functional domains namely, finger, epidermal growth factor, Kringle 1, Kringle 2, and serine protease. It is produced naturally by vascular endothelial cells (Camiolo et al., 1971) and can be produced using recombinant technology in different cell types including mammalian cell line, yeast and *Escherichia coli* (Gulba et al., 1998). Because of its high affinity for the fibrin, t-PA activates clot bound plasminogen 100 fold more effectively than the circulating plasminogen. Consequently, t-PA is a poor activator for circulating plasminogen or other blood clotting factors (Rouf et al., 1996). The binding of t-PA to fibrin and the modulation of proteolytic activity are primarily mediated by the finger domain and the Kringle 2 domain (Rijken and Collen, 1981; Qiu et al., 1998; Collen and Lijnen, 2004).

The aim of this study was to incorporate the clot-specific regions of t-PA (the finger and Kringle 2 domains) into streptokinase, thereby transforming into a clot-specific hybrid protein. The hybrid constructs were expressed in *E. coli* (BL21) DE3 strain, and then the activities of the recombinant proteins were assessed.

## 2. Materials and methods

### 2.1. Bacterial strains, vectors and reagents

The T7 expression vector pRSETB (Invitrogen, USA) were used for cloning the constructs. The different SK constructs were expressed in the *E. coli* BL21 (DE3) strain (Invitrogen, USA). The restriction enzymes (*Eco*RI, *Nde*I and *Kpn*I), *Taq* DNA polymerase and T4 DNA ligase were procured from NEB (USA). PCR purification kit (cat. no. 28704) was obtained from Qiagen (USA). The oligos for PCR were synthesized by Eurofins Genomics India Pvt, Ltd (India).

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### 2.2. Construction of the native streptokinase

The 1.3-kb SK gene was PCR amplified using gene-specific primers SK F1 and SK R1 (Table 1), with an annealing temperature of 53 °C. The forward primer contained an *Nde*I site (in italics), and the reverse primer contained an *Eco*RI site (in italics). The PCR amplicons were digested with the restriction enzymes *Nde*I and *Eco*RI and then ligated into the pRSETB expression vector, which was also digested with the same enzymes.

### 2.3. Construction of FSK and KSK

The DNA fragment encoding the finger and Kringle 2 domains of the t-PA protein was amplified using the specific primers, namely, FD F1 and FD R1, K2 F1 and K2 R1, respectively (Table 1). The optimal annealing temperature for PCR amplification of the finger and Kringle 2 domain with the above reported set of primers were 50 °C and 52 °C, respectively. Both the 5′ and the 3′ ends of the finger and Krigle 2 domain amplicons were flanked with *Nde*I restriction sites by inclusion of the restriction site in the primers (Table 1, in italics). The amplicons were subjected to restriction enzyme digestion with the *Nde*I so as to enable the insertion of the individual domains at the 5′ end of the SK gene in the SK-pRSETB construct. The ligation products were transformed into competent DH5α cells, and the recombinants were selected against ampicillin (100 μg/ml) background on LB agar. The sequences of the hybrid constructs were confirmed by DNA sequencing (MWGAG Biotech, India).

### 2.4. Construction of the finger domain + Kringle 2- SK

The finger domain was prepared using the forward primer FD F1 (*Nde*I) and the reverse primer FD R2 (Table 1), which has a *Kpn*I restriction site (in italics). The Kringle 2 domain was amplified using the forward primer K2 F2, which contains a *Kpn*I restriction site (in italics) and the reverse primer was K2 R1 (*Nde*I).

The amplified fragments were digested with *Kpn*I restriction enzyme and ligated together to obtain FK (Finger–Kringle 2) chimera. The chimeric fragment (FK) was amplified using the finger domain forward (FD F1) and the Kringle 2 reverse (K2 R1) primers with an annealing temperature of 52 °C. The amplicons were digested with the *Nde*I enzyme, and the purified fragments were ligated at the 5′ end of the SK gene in the SK-pRESTB construct.

### 2.5. Expression analysis of the constructs

For recombinant protein expression, the constructs were transformed into *E. coli* BL21 (DE3) expression host, using the Mandel and Higa protocol (Mandel and Higa, 1970). The transformants were grown in the Luria–Bertani medium (LB: 10% tryptone, 5% yeast extract, 10% NaCl and 0.8% glucose). The media were supplemented with 100 μg/ml ampicillin. The cells were grown by shaking with 200 rpm at 37 °C until the culture reached an $O.D_{600}$ of 0.6. The target protein expression was induced by adding 1 mM IPTG. After 3 h of induction period, and all the uninduced and induced cultures reached to $O.D_{600}$ 1.5, the cultures were harvested by centrifugation at 4000*g* for 10 min. The bacterial pellets were re-suspended in phosphate-buffered saline (1×). The expression profiles of the constructs were analyzed by 10% SDS–PAGE as per Laemmli protocol (Laemmli, 1970), and the gels were stained with Coomassie brilliant blue G-250.

**Table 1**
Oligonucleotides used in this study.

| No. | Name of primer | Primer sequence |
|---|---|---|
| 1 | SK F1 | 5′ -GGGATTC*CATATG*ATTGCTGGACCTGAG-3′ |
| 2 | SK R1 | 5′ - CCG*GAATTC*TTATTTGTCTTTAGG-3′ |
| 3 | FD F1 | 5′-GGGATTC*CATATG*GTGATCTGCAGAGAT-3′ |
| 4 | FD R1 | 5′-GGGATTC*CATATG*ACTTTTGACAGGCACTGA–3′ |
| 5 | K2F1 | 5′-GGGATTC*CATATG*GGAAACAGTGACTGCTAC-3 |
| 6 | K2R1 | 5′-GGGATTC*CATATG*GGAGCAGGAGGGCACATC-3′ |
| 7 | FD R2 | 5′-CGG*GGTACC*ACTTTTGACAGGCACTGA-3′ |
| 8 | K2F2 | 5′-CGG*GGTACC*GGAAACAGTGACTGCTAC-3′ |

### 2.6. Activity assays

#### 2.6.1. Caseinolytic assay

The activities of the different hybrid streptokinase were assessed by caseinolytic plate assay. For this assay, the caseinolytic–agarose base was prepared by melting 90 mg of agarose in 9 ml of the buffer solution (50 mM Tris–HCl, 150 mM NaCl), containing 500 μg plasminogen and 1% skim milk powder. Wells of 5 mm in diameter were created on the caseinolytic–agarose base after gelling of the solution. Fifty microliters of purified hybrid SKs (4 μg/10 μl) were loaded into the wells and incubated for 8 h at 37 °C. The clear zone formed around the wells was measured.

#### 2.6.2. Fibrinolytic assay

This test was performed to determine the clot lysing abilities of the hybrid streptokinases. The *in vitro* clot lysis was preformed with human blood clots and artificial clots. Human blood clots were prepared by incubating 100 μl of fresh blood at 37 °C for 45 min in a microfuge tube (1.5 ml). The clots were then washed 2–3 times with normal saline (Prasad et al., 2006). Artificial clots were prepared by adding 3 μl of bovine thrombin (10 mg/ml) and 2 μl of human plasma to the 20 μl of buffer (50 mM Tris–HCl, 150 mM NaCl) containing 10 μl of fibrinogen (100 mg/ml). The clots were formed when the mixture was incubated at 37 °C for 5 min.

Aliquots of 1 ml of purified hybrid SKs (4 μg/10 μl) were added to the human blood and artificial clots. All the tubes were then incubated at 37 °C. The time taken for the different hybrid proteins (SK) to perform clot lysis were determined and compared with that of the native streptokinase.

## 3. Results

### 3.1. Cloning and expression of native streptokinase (SK)

The 1.3-kb gene encoding the SK protein by was amplified with gene-specific primers. The purified amplicons were subcloned into the pRSETB vector, resulting in the pRSETB-SK construct. The pRSETB-SK construct was then transformed and expressed in *E. coli* BL21 (DE3) strain. The expression profile of the native streptokinase (molecular mass 47,000) in BL21 (DE3) was confirmed on SDS–PAGE (10%) (Fig. 1a).

### 3.2. Cloning and expression of the hybrid streptokinase constructs

#### 3.2.1. Construction and expression of FSK and KSK

The finger domain is the first domain in the protein encoded by the human t-PA gene; the region contains 138 bp and encodes for 46 amino acid residues (aa 4–50). The finger domain that was amplified using specific primers was cloned in the pRSETB-SK construct. This hybrid construct containing the finger domain with streptokinase was named as FSK. The recombinants (pRSETB-FSK) were confirmed by restriction digestion and DNA sequencing (data not shown). The molecular size of the FSK clone was 1.438 kb. The expression levels of FSK in *E. coli* BL21 (DE3) was found to be moderate when compared with the native SK expression (Fig. 1a). The hybrid protein (FSK) contains 460 amino acids residues and has a molecular mass of 52,180.

The Kringle 2 domain is the 4th domain in human t-PA; it contains 261-bp encoding 87 amino acid residues (aa 175–262 in t-PA). The Kringle domain that was amplified using specific primers was cloned in the pRSETB-SK construct. This hybrid streptokinase (the Kringle 2