

A Comparative Study on the Activity and Antigenicity of Truncated and Full-Length forms of Streptokinase

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Abstract

Application of streptokinase (SK) as a common and cost-effective thrombolytic drug is limited by its antigenicity and undesired hemorrhagic effects. Prior structural/functional and epitope-mapping studies on SK suggested that removal of 59 N-terminal residues led to its fibrin dependency and identified SK antigenic regions, respectively. Following *in silico* analyses two truncated SK proteins were designed and compared for their fibrin specificity and antigenicity with the full-length SK. Computer-based modeling was used to predict the effect of vector (pET41a)-born protein tags on the conformation of SK fragments. SK60-386, SK143-386 and full-length SK (1–414) were separately cloned, expressed in BL21 *E. coli* cells and confirmed by Western-blotting. Functional activity of the purified proteins was evaluated with chromogenic and clot lysis assays and their antigenicity was tested by ELISA assay using rabbit anti-streptokinase antibody. As expected, chromogenic bioassay showed a major activity decline for SK60-386 and SK143-386 (83 and 91 percent, respectively), compared to SK1-414. However, in clot lysis assay, which is a fibrin-dependent pharmacopoeia-approved test, SK60-386 and SK143-386 were respectively 35 and 31 percent more active though lysed the clots slower than full-length SK. Antigenic analysis also indicated significant decrease in ELISA signals obtained for truncated proteins compared to SK1-414 (45 and 28 percent less reactivity for SK143-386 and SK60-386, respectively, $p < 0.0001$). The results of this study for the first time pointed to SK143-386 and SK60-386, as improved SK derivatives with increased fibrin-selectivity and decreased antigenicity as well as acceptable bioactivity profiles in a pharmacopoeia-based analysis, which deserve more detailed pharmacological studies.

Key words: antigenicity, fibrin specificity, protein modeling, truncated streptokinase

Introduction

Several thrombolytic drugs (plasminogen activators; PAs) with different pharmacokinetic and pharmacodynamic properties have been developed for treating diseases such as stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction, among which streptokinase (SK) and tissue plasminogen (Plg) activator (tPA) are the most commonly used agents (Banerjee *et al.*, 2004; Baruah *et al.*, 2004). Although no predominant thrombolytic drug has been introduced to be used for every indication so far (Banerjee *et al.*, 2004), however, several clinical trials comparing the efficacy of SK and tPA generally suggested that streptokinase is the drug of choice for thrombolytic therapy especially in resource limited countries (Banerjee *et al.*, 2004; Baruah *et al.*, 2004).

Streptokinase is produced by different strains of β -hemolytic streptococci and due to its non human origin, is immunogenic and can evoke the immune

system; hence, frequent administrations of SK can result in production of neutralizing antibodies, which in turn reduces the efficacy of therapy, and eventually may lead to extensive allergic reactions (Banerjee *et al.*, 2004; Baruah *et al.*, 2004; Parhami-Seren *et al.*, 1997; Parhami-Seren *et al.*, 1995; Reed *et al.*, 1993; Torrèns *et al.*, 1999). Besides, SK function is not fibrin-specific; therefore its infusion to patients' blood may result in rapid activation of plasminogen and subsequent ectopic emergence of plasmin in circulatory system. This unspecific action of SK has undesirable consequences such as general depletion of plasminogen (Plg) concentration in circulatory system, rapid production of bradykinin resulting in hypotension (Reed *et al.*, 1999) and elevating the risk of hemorrhage (Banerjee *et al.*, 2004; Baruah *et al.*, 2004; Mundada *et al.*, 2003; Reed *et al.*, 1999; Sazonova *et al.*, 2004). Therefore, there is high interest in providing modified or truncated SK molecules with improved fibrin-dependency and less immunogenicity.

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Streptokinase with a molecular mass of 47 kDa, is a 414 amino acid single strand peptide which lacks cystine, cysteine, phosphorous, conjugated carbohydrates and lipids (Banerjee *et al.*, 2004). SK forms an equimolar complex with plasminogen or plasmin and this complex converts plasminogen substrates to plasmin by hydrolyzing the amide bond between Arg₅₆₁ and Val₅₆₂. Eventually the resulted plasmin can degrade and solubilize the fibrin clots (Wang *et al.*, 1998). Crystallography analysis of streptokinase structure (Wang *et al.*, 1998) has shown that it is composed of 3 domains known as α , β and γ (residues 12–150, 151–287 and 287–372 respectively) domains (Reed *et al.*, 1999). All three domains of the protein are involved directly or indirectly in the SK interaction with Plg (Conjero-Lara *et al.*, 1998). γ domain which has a close contact with Plg active site (Wang *et al.*, 1998) plays a central role in amidolytic activity of the activator complex (Conjero-Lara *et al.*, 1998) while β domain is known as the region responsible for high affinity interaction between SK and Plg. Although the exact role of α domain is not clearly defined, but the first 59 residues of this domain are important for streptokinase accurate conformation and full activity (Shi *et al.*, 1994). In fact, deletion of these first 59 amino acids of the N-terminal segment (SK Δ 59 or SK60-414) resulted in major reduction of SK activity in the absence of fibrin, but interestingly in the presence of fibrin the activity was shown to be restored albeit in a longer period of time. In other words, SK Δ 59 showed a fibrin-dependent full-activity characteristic when SK-Plg reaction were awaited for longer times (Mundada *et al.*, 2003; Sazonova *et al.*, 2004). Further studies indicated that presence of plasmin is an essential element for efficient activation of SK Δ 59 (Mundada *et al.*, 2003; Sazonova *et al.*, 2004). In fact, addition of trace amounts of plasmin markedly increased plasminogen activity of SK Δ 59 and declined the previously reported lag time of the reaction. Moreover, in contrast to the wild type SK which protected plasmin from inhibition by α_2 -antiplasmin, SK Δ 59 is more prone to such haemostatic regulations to prevent the risk of hemorrhage. Therefore, since in the real physiological condition, only plasminogen substrates on the surface of fibrin can be fully activated by SK60-414*plasmin complex (Mundada *et al.*, 2003; Reed *et al.*, 1999; Sazonova *et al.*, 2004) and just fibrin-bound plasmin is protected from inhibitory action of α_2 -antiplasmin (Mundada *et al.*, 2003), thus this truncated form of streptokinase was considered as a fibrin targeted plasminogen activator and a potentially improved SK for thrombolytic therapy (Mundada *et al.*, 2003; Sazoana *et al.*, 2009; Sazonova *et al.*, 2004). However, to our knowledge there is no prior study addressing a standard pharmacopoeia comparative analysis based on clot lyses assay between full length SK and SK Δ 59 in allowed reaction period of times to approve the efficiency of this truncated SK as a thrombolytic drug.

It is also well shown that during *in vivo* and *in vitro* Plg activation, SK is cleaved into three fragments spanning amino acid residues of 1–59, 60–386 and 387–414 (Reed *et al.*, 1999). While the N-terminal fragment remains tightly attached to the main segment, the C-terminal part, *i.e.* amino acids 387–414 is released during Plg activation, implying that this part may not be important for SK activity (Reed *et al.*, 1999). Although the SK60-386 may be even a potentially more improved fibrin-dependent PA drug because of losing some immunogenic segments of SK located in its C-terminal (compared to SK60-414) but no prior study has addressed evaluation of its activity by fibrin based methods either.

It was previously demonstrated that 143–386 fragment of streptokinase has only 60 percent of maximal activity over even a longer period of time compared to both full-length streptokinase and SK60-414 (Rodriguez *et al.*, 1995). This fragment lacks the so-called non-essential C terminal part similar to SK60-386 and moreover has an extra 83 residue deletion in the N terminal region. Although SK143-386 has a dramatic reduction of activity in the absence of fibrin, but fibrin degradation potential of this fragment (which lacks more immunodominant regions of SK) or changes of its activity in the presence of fibrin was not previously studied either.

Different regions of streptokinase have been identified as antigenic epitopes by using murine and human monoclonal antibodies as well as sera of patients treated with SK. Many of these regions, including residues 4 to 8 and 96–99 (Coffey *et al.*, 2001), 120–140 (Parhami-Serena *et al.*, 2003), 353–414 (Reed *et al.*, 1993) and 373–414 (Torr ens *et al.*, 1999), are located in regions which are marked out for SK truncations in our study (that is 1–59, 1–142 and 387–414) with the expectation that removal of these parts may result in the reduction of antigenicity in truncated SK molecules.

In the present study, with the final aim of improving the potential therapeutic efficacy of streptokinase by development of more fibrin-specific SKs with less antigenicity, we sought to produce two recombinant truncated streptokinase derivatives spanning the residues of either 143–386 or 60–386 and analyzed their clot lyses activity by a standard pharmacopoeia fibrin-based method with that of full length SK. We also provide comparative data for improved and less antigenic profiles of these truncated molecules.

Experimental

Materials and Methods

Computer-based modeling. To evaluate the impact of the protein tags derived from pET41a (used for expression of SKs and its derivatives in this study) on the conformation of full length and truncated forms of

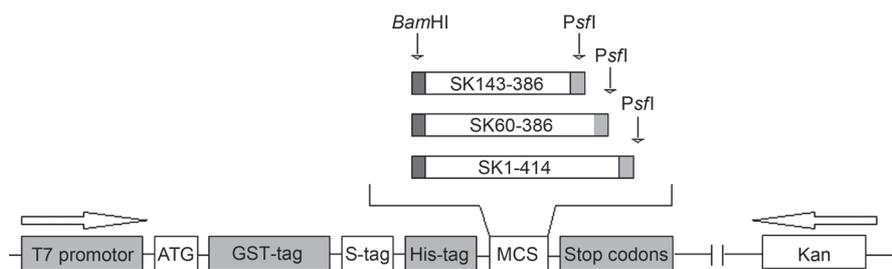


Fig. 1. Schematic illustration for insertion site of 3 different forms of *skc* in pET41a vector.

Truncated (143–386 and 60–386) and full-length(1–414) forms of *skc* were inserted into *Bam*HI and *Pst*I sites of MCS of pET41a; ATG and Kan stands for start translation codon and kanamycin resistance respectively; MCS stands for for multiple cloning sites; GST-tag, S-tag and His-tag are the tags derived from the vector.

SKs, three-dimensional structure of the SK proteins fused to the vector derived-tags were modeled using MODELLER program (version 9v5, 2008) which is a non-graphical, command-line based software. To this end, 1bmlC.pdb and 1m9A.pdb atom files (as templates for streptokinase and GST proteins respectively) were downloaded from Protein Data Bank (<http://www.rcsb.org>) and multiple template script files of MODELLER program were used for modeling. Visualization of the models was performed by Deep View Spdb-viewer (version 4.0.1, 2009) and WebLab viewer Lite (version 4, 2000) softwares. The resulted models were evaluated based on three methods: 1. Energy level comparison: in which energy calculation of residues of model and template was performed by applying DOPE potential command line of MODELLER program and energy level of the residues were compared in a graphing program. 2. Root Mean Square Deviation (RMSD) calculation: in which the 3D structures of templates and models were superimposed in Deep View spdb-viewer program (version 4.0.1, 2009) and the quality of the fits were evaluated by calculating Root Mean Square Deviation (RMSD) between carbon α of the residues. 3. Ramachandran plot assessment: which was used to visualize dihedral angles ψ against ϕ of amino acid residues in protein structure by using RAMPAGE server (Lovell *et al.*, 2002).

Bacterial strains and culture media. Plasmid propagation and preparation was performed in *E. coli* DH5 α (Novagen, USA) and *E. coli* BL21 (DE3) (Novagen, USA) was used for protein expression of cloned

genes by IPTG induction. Bacteria were cultivated in LB (Luria-Bertani) broth or TY2X for plasmid extraction and expression induction, respectively. All strains were stored in 20% (v/v) glycerol and stab culture of LB Agar.

Gene amplification, cloning and expression.

Streptokinase gene (*skc*), from a standard streptococcus strain (*S. equisimilis*, ATCC 9542) which is also known as *skc2* (Estrada *et al.*, 1992), was PCR amplified by *Pfu* DNA polymerase. Various *Bam*HI-tailed forward and *Pst*I-tailed reverse primers (Table I) were used to amplify the nucleotide sequences corresponding to full length (SK1-414) and truncated SKs (SK60-386, SK143-386). In this regard *Pf*1 and *Pf*414 were used as primers for amplification of *skc1-414* and pairs of *Pf*60, *Pf*386 and *Pf*143, *Pf*386 were applied for amplification of *skc60-386* and *skc143-386*, respectively. Thermal program was set as 30 cycles of 95°C for 60 sec, 58°C for 45 sec and 72°C for 90 sec, which was followed by a final extension at 72°C for 5 min.

The amplicons, after digestion with *Bam*HI/*Pst*I restriction enzymes, were separately cloned into the same sites of pET41a vector (Novagen, USA) downstream and in frame of the vector-derived tags (GST, S and His-tag), under the control of a T7 promoter (Fig. 1) to construct three recombinant plasmids of pETSK1, pETSK60 and pETSK143. The constructs which respectively encoded SK1-414, SK60-386 and SK143-386 were confirmed by sequencing (SeqLab, Germany) and subsequently were transformed into the *E. coli* BL21 cells. Single colony transformants were

Table I
Nucleotide sequence of primers designed for amplification of truncated and full-length streptokinase molecules.

Pf60 (forward)	5'-CGAGGATCCAGTCCAAAAATCAAAACC-3'
Pf143 (forward)	5'-ATAGGATCCCATGTGCGCGTTAGAC-3'
Pr386 (reverse)	5'-CCGCTGCAGTTACTAGGCTAAATGATAGCTAG-3'
Pf1 (forward)	5'-GAAGGATCCATTGCTGGACCTGAGTG-3'
Pr414 (reverse)	5'-ATCTGCAGTTATTTGTCGTTAGGGTTATCAGG-3'

Underlined sequences denote the restriction sites.

inoculated in TY2X medium ($OD_{600} = 0.6$) to express the recombinant protein for 3–5 hrs following induction by 0.1 mM IPTG (Isopropyl-thiogalactoside).

Protein analysis and purification. Pellets of induced bacteria (5 ml) were lysed in 50 μ l of lysis buffer (100 mM NaH_2PO_4 , 10 mM Tris.Cl and 8 M Urea), resuspended in 50 μ l Laemmli buffer (0.09 M Tris-HCl, 20% (v/v) Glycerol, 2% (v/v) SDS, 0.02% (v/v) bromophenol blue and 2% (v/v) β -ME) and heated at 80°C for 5 min. The supernatants were electrophoresed in 12% SDS-PAGE gel and separated proteins were either stained by coomassie Brilliant Blue or transferred to nitrocellulose membrane. After blocking the membrane in 3% (w/v) bovine serum albumin (BSA) and application of proper dilution of mouse anti-His antibody (Qiagen, Germany) and washing steps, HRP-conjugated goat anti-mouse IgG (Sigma, USA) was utilized as tracking antibody. The bands related to the recombinant SK proteins were finally visualized by application of DAB (3,3' Diaminobenzidine) substrate.

Recombinant proteins harboring the N-terminally tagged 6xHis amino acids were purified in denaturing condition by the application of Nickel-TitriloTriacetic Acid (Ni-NTA) agarose columns (Qiagen, Germany), according to manufacturer protocol. Briefly, the pellets of 200 ml of induced bacterial culture were lysed in 5 ml of lysis buffer (100 mM NaH_2PO_4 , 10 mM Tris.Cl and 8 M Urea) for 15 minutes. After centrifugation for 20 minutes at 10000 rpm, the upper lysate was mixed with 2 ml of Ni-NTA resin for 30 minutes in a Falcon tube. The mixture was subsequently loaded into appropriate column (Qiagen, Germany), washed with 4 ml of washing buffer (containing the same lysis buffer, pH 6.3) twice and finally the SK proteins were eluted by elution buffer (containing the same lysis buffer, pH 4.5) in four fractions (0.5 ml each one). Purity and concentration of protein fractions were analyzed by SDS-PAGE and Bradford methods, respectively.

Bioassay of recombinant SK proteins. Streptokinase activity was determined by three methods:

Caseinolysis – In this semiquantitative standard method (Saksella, 1981) a plate containing 5% (w/v) skim-milk, 1% (w/v) agarose, 10 mM NaCl and 50 mM Tris base was prepared and different dilutions of standard SK (B. Braun, Germany) and the same amounts of eluted proteins were applied in the 5-mm wells previously prepared in the plate. All the standard and test wells were filled with 1 mg ml^{-1} concentration of plasminogen solution (Fluka, Sweden) to have the plasminogen in an excess molarity. Two other wells containing standard SK alone or plasminogen alone were also prepared as negative controls. After 24 h incubation at room temperature the caseinolysis diameter surrounding the wells, reflecting the functional activity of SK, was measured.

Chromogenic assay – This method that monitors the amount of formed plasmin by an endpoint assay of a synthetic substrate (British Pharmacopoeia, 1998) (Couto *et al.*, 2004) was conducted with slight modifications. In brief, recombinant SK molecules (0.4 nM) and Plg (1 nM) were mixed together in 96-well plates and incubated at 37°C for 10 min to construct activator complexes. 0.75 mM S2251 (H-D-valyl-L-leucyl-L-lysine- ρ -nitroanalide) was added to the enzymatic complex as substrate and the mixture was incubated at 37°C for 20 min. The reaction was stopped with 20% (v/v) acetic acid and absorbance was read in 405 nm. Biological activity (IU ml^{-1}) and specific activity (IU mg^{-1} and IU nM^{-1}) of the samples were calculated according to OD_{405} of reference streptokinase with known biological activity (B. Braun, Germany).

Clot lysis assay – This method measures the SK activity in the presence of fibrin (British Pharmacopoeia, 1998). Briefly, 0.2 ml of SK (0.8 nM) (either reference or purified recombinant proteins) was mixed with 0.2 ml of citric-phosphate buffer, 0.1 ml of thrombin (20 IU ml^{-1}) and 0.5 ml of euglobulin (10 mg ml^{-1}), which was purified according to the method used by Couto *et al.* (Couto *et al.*, 2004), in a test tube. After the formation of clots, the required times for complete dissolution of clots was noted. Biological activity of the unknown purified samples was determined based on the plotted standard curve showing the clot lysis time against the IU ml^{-1} of different concentrations of reference SK. Biological activity (IU ml^{-1}) and specific activity (IU mg^{-1} and IU nM^{-1}) of samples were calculated according to reference streptokinase plot with known biological activity (B. Braun, Germany).

Antibody preparation and Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was used to evaluate antigenic discrepancy between full-length and truncated SK using polyclonal anti-SK antibodies generated in rabbits. Full length purified SK (SK1-414) emulsified in Complete Fraunds' Adjuvant (CFA, Sigma) was intramuscularly injected to six female New Zealand rabbits (0.25 mg/rabbit). Booster administration was performed with the same amount of SK mixed with Incomplete Fraunds' Adjuvant (IFA, Sigma) 4 wks later. The anti-sera were prepared at 4 wk post-booster injection and used in an indirect ELISA to detect the truncated SK molecules. ELISA plate was coated with 0.4 nM of the 3 proteins (SK143-386, SK60-386 and SK1-414). After blocking with 3% (w/v) BSA, serial dilutions of anti-sera from individual injected rabbits (1/800, 1/1600, 1/3200, 1/6400, 1/12800) were added to streptokinase coated wells. Following the washing steps, horse-radish peroxidase (HRP)-conjugated anti-rabbit antibody was applied to the wells. Finally and after further washes, addition of the chromogenic substrate; TMB (Tetramethyl-benzidine) led to the color

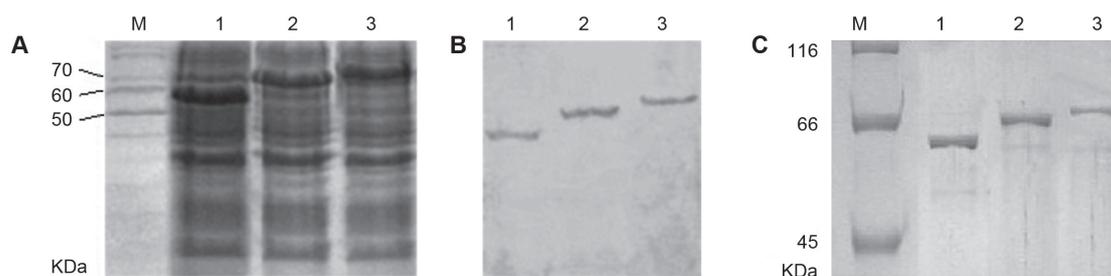


Fig. 2. Analysis of the expressed SK proteins.

(A) SDS-PAGE and (B) western blot analysis of crude lysis of *E. coli* BL21 cells expressing truncated and intact forms of streptokinase; lane M: molecular weight marker, lane 1–3: SK143-386, SK60-386 and SK1-414 respectively; (C) SDS-PAGE analysis of purified proteins; lane M: molecular weight marker, lane 1–3: SK143-386, SK60-386 and SK1-414 respectively.

appearance that was stopped by 10% (w/v) acetic acid. While the background absorbance resulting from the pre-immune sera was subtracted from the tests, the means of OD_{450} for different groups were statistically compared using student *t*-tests.

Results

Modeling of truncated and full-length proteins.

Expression of N-terminally truncated proteins is shown to be usually less efficient (Nihalani *et al.*, 1998; Reed *et al.*, 1999). We hypothesized that expression of N-terminally truncated SK proteins fused with N-terminal tags (derived from vectors such as pET41a) that are efficiently expressed may be facilitated. Although Reed *et al.* (Reed *et al.*, 1999) previously reported that maltose binding protein (MBP) fused to streptokinase did not affect its activity, however for us an existing concern with this strategy was the risk of unwanted changes in the configuration of these proteins. To gain insights into this possibility we took advantage of computer-based modeling using the MODELLER software.

To avoid complexity in modeling, the small 6His-tag (6 residues) and S-tag (15 residues) were ignored but GST tag, a protein with 220 amino acids was included in the modeling procedure. Several models were created by using 'multiple-template' scripts of MODELLER software and subsequently models with the lowest energy were selected for assessments (data not shown).

DOPE scores of models resulted from model evaluation scripts of MODELLER software were compared with DOPE scores of templates by depicting the data as plots (Fig. 3). There are gaps in the DOPE plots due to deletion of some residues in 1bmlC (template of SK), which is obtained from Protein Data Bank, however analogous points related to these gaps in the models show good level of energy (less than $-1E^{-2}$). Fortunately residues related to γ domain, which is in close contact with catalytic site of plasminogen in the complex (Wang *et al.*, 1998), in all 3 models of streptokinase had very near level of energy to their template (Fig. 3).

Assessment of selected models by Ramachandran plot showed that residues of outlier region for full-length SK (SK1-414), SK60-386 and SK143-386 were 4.9%, 2.9% and 3.5% respectively. These scores compared to the scores of their templates (6.1% for 1bmlC and 1% for 1m9A) seemed very reasonable.

RMSD of selected models for the 3 proteins after superimposing to 1bmlC as template for streptokinase and from 1m9aA as template GST-tag, were calculated separately. These results indicated that SK143-386 model has the least RMSD in comparison to the other models which means it was the best model (RMSD less than 2 Å is considered as excellent, between 2 and 6 Å as reasonable and more than 6 Å as improper). Altogether the results of all three kinds of evaluations showed that the fusion tags may not have considerable impact on the conformation of our proteins.

Cloning, expression and purification of recombinant full length and truncated streptokinase proteins.

Two truncated SK genes encoding 143–386 and 60–386 amino acid residues (*skc143-386* and *skc60-386*, respectively) in addition to the full-length SK gene (*skc1-414*) were PCR-amplified from the previously isolated *skc2* template (Estrada *et al.*, 1992). The amplicons were cloned into the *Bam*H1/*Pst*I sites of pET41a plasmid, in the same open reading frame (ORF) of vector-born N-terminal fusion-tag (Fig. 1). Transformation and subsequent expression by IPTG induction of plasmids in *E. coli* BL21 (DE3) resulted in the appearance of protein bands with expected molecular weights for vector derived tags fused to the proteins (57, 66 and 78 kDa for SK143-386, SK60-386 and SK1-414, respectively) in SDS-PAGE (Fig. 2A) and Western-blot analyses (Fig. 2B).

Induction of protein expressions in large scale cultures (200 ml) and purification of His-tagged SK proteins using Ni-NTA affinity chromatography finally provided us with approximately 150 mg of full length and truncated proteins with a purity of more than 90 percent for each protein that was shown by SDS-PAGE (Fig. 2C). These proteins were further evaluated for bioactivity and antigenicity.

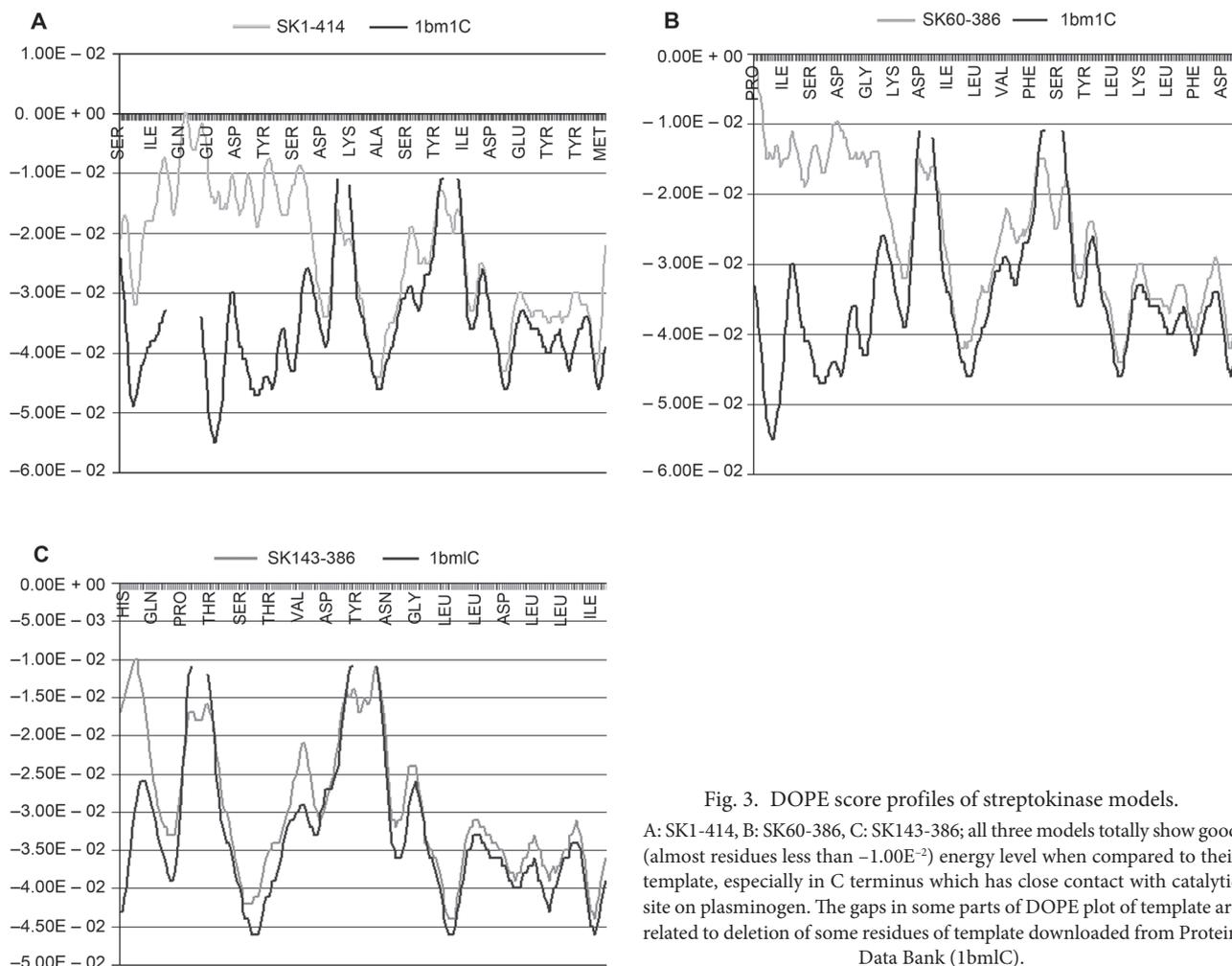


Fig. 3. DOPE score profiles of streptokinase models.

A: SK1-414, B: SK60-386, C: SK143-386; all three models totally show good (almost residues less than $-1.00E^{-2}$) energy level when compared to their template, especially in C terminus which has close contact with catalytic site on plasminogen. The gaps in some parts of DOPE plot of template are related to deletion of some residues of template downloaded from Protein Data Bank (1bm1C).

Comparison of biological activity for truncated and full length purified SK proteins. The caseinolysis method (Saksella, 1981) was used for preliminary activity assessment of the recombinant SK proteins. Two other methods used in this study were chromogenic plasminogen activation assay and a fibrin clot lysis assay which are recommended assays for biological activity assessment of streptokinase in British Pharmacopoeia (BP, 1998). Caseinolysis zones, surrounding the wells of samples together with different amounts of standard streptokinase, indicated that full length and truncated forms of SK proteins were biologically active (data not shown). Following this primary screening, specific activities of proteins were measured in terms of International Unit per milligram and per nanomole. The quantitative chromogenic assay indicated that, compared to SK1-414, specific activities of SK60-386 and SK143-386 had respectively reduced by 81 and 88 percent in terms of $IU\ mg^{-1}$ 83 and 91 percent in terms of $IU\ nM^{-1}$ (Table II). Similarly, the clot lysis assay (lysis of one milliliter of clot by one microgram or nanomole streptokinase) showed a reduction of 68 and 80% of $IU\ \mu g^{-1}$ and 72 and 85% of $IU\ nM^{-1}$, respectively, compared to SK1-414 (Table III). This method that mea-

sures the activity of SK in the presence of fibrin may be considered as a criterion for the fibrin dependency of SK molecule in physiologic condition (Reed *et al.*, 1999). Of note, all SK proteins (both full length and truncated SKs) were able to completely lyse the clots during 20 minutes which was acceptable and allowed time for a thrombolytic drug with potential therapeutic

Table II

Biologic and specific activity of truncated and full-length streptokinase molecules measured by chromogenic method.

	SK1-414	SK60-386	SK143-386
Biological activity ($IU\ ml^{-1}$)	3758	634	346
Specific activity ($IU\ mg^{-1}$)	11743	2186	1443
Specific activity ($IU\ nMol^{-1}$)	952	158	88

Table III

Biologic and specific activity of truncated and full-length streptokinase molecules measured by clot lysis method.

	SK1-414	SK60-386	SK143-386
Biological activity ($IU\ ml^{-1}$)	3211	1464	1011
Specific activity ($IU\ mg^{-1}$)	10703	3405	2106
Specific activity ($IU\ nMol^{-1}$)	868	244	128

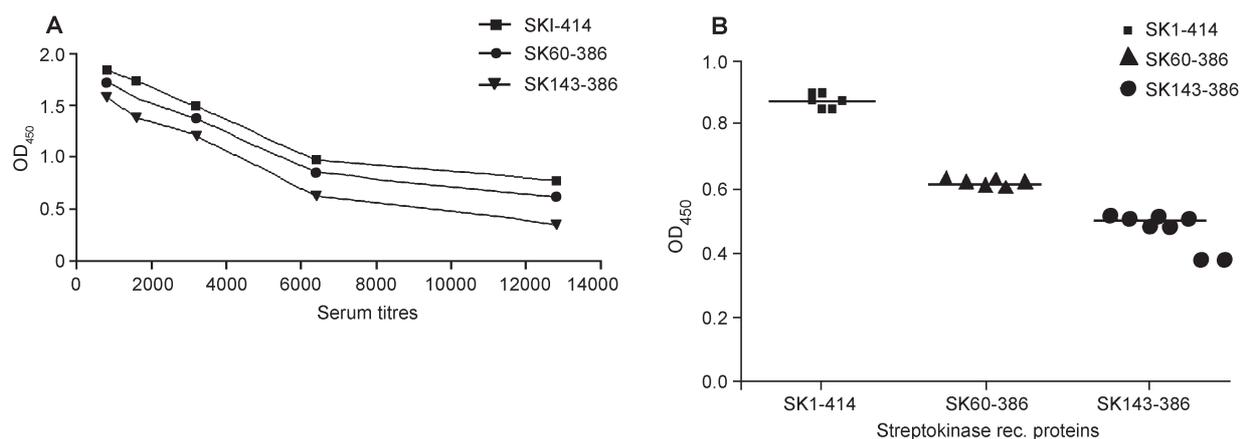


Fig. 4. Antigenicity analysis of truncated forms of streptokinase in comparison to full length SK.

(A) Reactivity of five serial dilutions of anti-SK polyclonal antisera on SK143-386, SK60-386 and SK1-414 by indirect ELISA. (B) Comparison of ELISA signals obtained with 1/12800 dilution of antisera in pairs of SK1-414 & SK143-386, SK1-414 & SK60-386 and SK60-386 & SK143-386. Deletion of residues 1-59, 1-142 and 387-414 in streptokinase resulted in significantly less antigenic proteins ($p < 0.0001$ for all comparisons).

efficacy in the British pharmacopoeia. Comparison of the results obtained from clot lysis and chromogenic assays (in terms of IU nM⁻¹) also indicated that while the specific activity of full length SK in the presence of fibrin was reduced approximately by 9%, but both truncated molecules showed an increased activity of 35% and 31% for SK60-386 and SK143-386 respectively.

Although there was significant difference in the specific activity between truncated and full-length streptokinase it was observed that by equal amount (0.8 nM), SK60-386 and SK143-386 completely lysed the clot approximately in 12 and 16 minutes, respectively, whereas SK1-414 solubilized the clot in about 3 minutes; *i.e.* the truncated molecules had full activity in longer times but still in the range of allowed time for a thrombolytic drug with potential therapeutic efficacy in the British pharmacopoeia (20 minutes).

Antigenicity analysis of full length and truncated SK molecules. Immunological characteristics of streptokinase can be evaluated by developing anti-SK antisera in different animals, including rabbits (Houba and Hana, 1966) and monkeys (Torrrens *et al.*, 1999). In this study for comparison of antigenicity of the proteins, rabbit polyclonal antisera raised against purified SK1-414, were used to analyze antigenicity of truncated streptokinase proteins. Reactivity of five serial dilutions (1/800, 1/1600, 1/3200, 1/6400 and 1/12800) of the antisera with SK143-386, SK60-386 and SK1-414 which was measured by indirect ELISA pointed to lower signals (OD₄₅₀) in the case of truncated molecules (Fig. 4A). Dilution of 1/12800 was selected to statistically compare the observed differences of antigenicity between the 3 proteins (Fig. 4B). Interestingly, this analysis showed a significant decrease of reactivity for both truncated molecules in comparison with full length SK. SK143-386 showed to be 45% less reactive to anti-SK than SK1-414 ($P < 0.0001$) and SK60-386 had 28%

lower reactivity in comparison to SK1-414 ($P < 0.0001$). Also reactivity of SK60-386 to anti-SK was 23% higher than SK143-386 ($P < 0.0001$). These data, in accordance with previous study (Torrrens *et al.*, 1999) on evaluation of C terminally mutated streptokinase, indicated that removal of the selected fragments (59 or 142 residues of N terminal and 28 residues of C terminal) from full length SK leads to the production of considerably less immunogenic SK derivatives.

Discussion

Streptokinase is one of the most important drugs for thrombolytic therapy; however, it has shortcomings such as immunogenicity and fibrin-non specificity (Banerjee *et al.*, 2004; Baruah *et al.*, 2004; Reed *et al.*, 1999; Sazonova *et al.*, 2004). In the present study, based on previous reports on antigenic mapping and functional characteristics of SK regions, two truncated SK-molecules lacking the 59 N-terminal or the 142 N-terminal amino acids plus 28 C-terminal residues (SK60-386 and SK143-386 respectively) were considered for construction and analysis of antigenicity/activity. To the best of our knowledge there was no prior report on evaluation of activity of SK143-386 and SK60-386 as potential thrombolytic drugs for their therapeutic efficacy by a fibrin based and pharmacopoeia approved method. Further, no prior study addressed for comparison of the antigenicity of these truncated SK proteins with full-length SK.

Bioassay of truncated SK proteins by chromogenic method indicated a dramatic decline of activity compared to the full length SK (up to 84 and 91 percents for SK60-386 and SK143-386, respectively (Table II). According previous reports, on structure/function analysis of streptokinase, this lower activity of truncated SK

proteins in chromogenic assay was expectable (Reed *et al.*, 1999; Rodriguez *et al.*, 1995). Analysis by fibrin clot lysis assay also evidenced for significant activity reduction for truncated SK proteins compared to full-length SK (Table III), but comparison of activities by two methods (Chromogenic assay versus fibrin clot assay) indicated 35% and 31% increase of activity for SK60-386 and SK143-386 respectively and reduction of 9% for full-length SK by clot lysis assay compared to chromogenic assay (Tables II, III). These observations are somehow in accordance with prior studies which demonstrated SK lacking N terminal 59 amino acids restores the activity in the presence of fibrin, however in the present study the truncated SK proteins did not restore full activity. Accordingly, while it was already shown that during a long analyzing time (6 hours) SK60-414 is more active than full-length SK in all ranges of the tested concentrations (0–50 nM), (Reed *et al.*, 1999), other studies showed that SK60-414 is superior than full-length SK only in high concentrations (Mundada *et al.*, 2003). The results of our clot lysis test are consistent with the later report in that our truncated proteins had less activity than full-length SK using a lower concentration (0.8 nM).

The lag time in fibrinolysis, which was the major reason for lower activity in our clot lysis assay, may be explained by the fact that both truncated molecules (lacking 59 first residues) were only able to form efficient activator complexes with plasmin and not with plasminogen (Mundada *et al.*, 2003; Sazonova *et al.*, 2004) which were generated in trace amount in the beginning of the interaction. By more plasmin formation, further plasminogen substrates would be subsequently activated in a time manner. Delay in fibrin degradation can be, in fact, a positive feature, since it gives the opportunity to SK to become active only when it reaches to the loci occluded by fibrin clots. This feature can minimize activity of the SK protein in the regions lacking fibrin clots and may potentially reduce the risk of hemorrhage.

The other side of our rationale for the truncation of streptokinase was to reduce its antigenicity. Antigenicity analysis by ELISA which was carried out in our study was based on methods previously developed for evaluation of the immunological characteristics of streptokinase using anti-SK antisera developed in different animals, including rabbits (Houba and Hana, 1966 and Torrèns *et al.*, 1999). These analyses showed a less reactivity of the SK-specific rabbit polyclonal antisera against the truncated SK molecules (Fig. 5), so that a significant decrease in the obtained signals was observed for truncated molecules in comparison with the full length protein, which was still significantly lower for SK143-386 compared to the SK60-386. These findings were somehow expected and compatible with

other studies (Parhami-Serena *et al.*, 2003; Reed *et al.*, 1993; Torrèns *et al.*, 1999) which mapped and analyzed antigenic epitopes of streptokinase. Accordingly, lower reaction of anti-SK1-414 antibodies with truncated molecules confirmed the accumulation of antigenic determinants in the excised regions. Moreover, in a confirmatory experiment we prepared anti-truncated SK antibodies by immunizing the rabbits with SK60-386 and SK143-386 molecules and found that in comparison with anti-full length SK antibodies, antisera raised against the shortened molecules generally had a lower reactivity with their corresponding proteins (data not shown). Overall, these findings verified the less antigenicity/immunogenicity of the truncated SK proteins introduced in this study.

Altogether, based on the available structure/function and antigenic mapping reports of SK molecule, to our knowledge the present study for the first time attempted to engineer truncated recombinant SK molecules with a simultaneous more fibrin specificity and less antigenicity. According to the obtained results, truncation of SK in both N- and C-terminal ends was successful to create fibrin targeted SKs with comparable activities and considerably lower antigenic properties. In a preliminary analysis, both of these proteins (SK143-386 and SK60-386) passed the pharmacopoeia standard for streptokinase activity assessment by a clot lysis assay for evaluation of a thrombolytic drug with potential therapeutic efficacy and hence may be considered for further pharmacological assessments.

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