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Engineered Variants of Streptokinase: Molecular Analysis of Their Plasminogen Activation and Proteolytic Processing

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ABSTRACT

Streptokinase (SK) is a major blood-dissolving agent that used in the treatment of various circulatory disorders. Since the original host of Streptokinase is pathogenic and secretes several toxins which complicate the purification, (SK) gene was cloned in a strain of *Bacillus subtilis* as a nonpathogenic expression system. The role of the promoter and signal sequence of *B. subtilis* in the production of streptokinase was assessed by two different constructs, (vSK_a) and (vSK_b). Secreted streptokinase from both constructs were analyzed and specific activity of sucrose induced streptokinase from the construct of (vSK_b) was about 1.2 to 1.4 fold higher than that from (vSK_a). Streptokinase produced from (vSK_a) and vSK_b has an N-terminal sequence identical to the native streptokinase of *S. equismilis* H46A. Upon incubation of the immobilized plasmin (hPlm) with SK, several peptide fragments were observed by PAGE electrophoresis. After purification, molecular masses and the N-terminal sequences of these fragments were determined. Data obtained from these sequences indicated that there are different processing sites. To prevent proteolytic processing of SK at the C-terminus, two variants (SK1 and SK2) were engineered via site directed mutagenesis. Characterization of streptokinase derived from SK1 and SK2 by Western blot analysis demonstrated that both variants were resistant to the proteolytic processing but a low percentage (10-20%) of the secreted streptokinase from SK1 appeared in a degraded form. However, variant of SK2, showed a biological activity 2.5 times higher than that of the wild-type streptokinase. To prevent processing of SK at the N-terminus, Lys⁵⁹ in SK2 was changed by site directed mutagenesis either to glutamine yielding the mutant variant SK2^{K59Q} or glutamic acid yielding SK2^{K59E}. Purified SK protein from SK2^{K59Q} was resistance to proteolysis by (hPlm) and showed better plasminogen activation in the radial caseinolysis assay. This study indicates that blocking of the N-terminal cleavage at K⁵⁹ site is critical to generate a proteolytic-resistant streptokinase variant.

Key words: Streptokinase, variants, plasminogen, activation, proteolytic processing, *B. subtilis*

INTRODUCTION

Streptokinase (SK) is a 47-kDa protein secreted by several species of β -hemolytic streptococci. SK combines in equimolar manner with the human plasminogen (Hpg) molecules forming the complex SK-HPG which activates HPG to plasmin (Hplm). Plasmin can degrade the fibrin matrix of blood clots through the conversion of the HPG molecules by the hydrolysis of specific activating peptide bond of HPG, such as Arg⁵⁶⁰-Val⁵⁶¹ (Castellino, 1981; Shi and Wu, 1988; Shi *et al.*, 1993). Thus, during the treatment of various circulatory disorders, e.g., myocardial infarction, deep vein thrombosis, pulmonary embolism, HPN generated by the activation of HPG helps restore blood flow to the afflicted part by proteolytic dissolution of the fibrin in the pathological clot. SK is a single

polypeptide chain of 414 amino acid residues which organized into three structurally similar and independently folding domains designated α , β and γ . These domains are separated by two coils and small regions at both of the N- and C-termini of the protein (Conejero-Lara *et al.*, 1996; Parrado *et al.*, 1996; Wang *et al.*, 1998; Bean *et al.*, 2005; Joshi *et al.*, 2012). All three domains play a key role for functioning of SK (Malke *et al.*, 1987; Nihalani and Sahni, 1995; Reed *et al.*, 1995; Rodriguez *et al.*, 1995; Conejero-Lara *et al.*, 1998; Nihalani *et al.*, 1998; Fay and Bokka, 1998).

Production of streptokinase through a nonpathogenic expression system would be ideal since the original host is pathogenic and secretes several other toxins that complicate the purification (Ferretti *et al.*, 1987). Structural gene of SK from *Streptococcus equisimilis* H46A has been cloned (Malke and Ferretti, 1984) and sequenced (Malke *et al.*, 1985). SK was expressed by using the intact streptokinase gene including its promoter region in various gram-negative and gram positive hosts including *Escherichia coli* (Malke and Ferretti, 1984), *Streptococcus sanguis* (Jackson *et al.*, 1986), *Proteus mirabilis* (Laplace *et al.*, 1989), *Bacillus subtilis* (Klessen and Malke, 1986), *Streptococcus lactis* (Laplace *et al.*, 1989) and from *Streptococcus pyogenes* (Cook *et al.*, 2012). *S. equisimilis* H46A, however and all of these hosts produce at least two major forms of streptokinase. One of them is the intact mature streptokinase with a molecular weight of 47 kDa while the other is a 44-kDa degradation product.

Several intermediate products with different molecular weights are accumulated upon activation of plasminogen with recombinant streptokinase (Shi *et al.*, 1994; Caballero *et al.*, 1999; Laha *et al.*, 2011; Joshi *et al.*, 2012). The 44-kDa intermediate product appears first and developed by processing at the C-terminus, where they have identical N-terminal residues to those observed in the intact streptokinase (McClintock *et al.*, 1974). The 37-kDa intermediate appears later and is relatively stable (Brockway and Castellino, 1974; McClintock *et al.*, 1974; Shi *et al.*, 1994; Caballero *et al.*, 1999). Sequencing analysis of the N-terminal of the 37-kDa intermediate product suggests that this fragment has the sequence corresponding to Ser⁶⁰ to Lys³⁸⁶ from the native streptokinase. Therefore, this intermediate generated by processing at both of N- and C-terminals. Further-processing of the 37-kDa product at a series of cleavage sites results in the complete degradation of streptokinase into small fragments (Shi *et al.*, 1994). Until now we are not sure whether the processing of streptokinase by plasmin is essential for generating the active form this enzyme or it simply a consequence of positioning lysine and arginine residues at the surface exposed regions.

As Streptokinase is processed by plasmin, a trypsin-like serine protease which process lysine or arginine (Weinstein and Doolittle, 1972) it would be interesting to investigate whether mutating lysine and arginine residues at the processing sites of SK would generate mutant SK-variants that can resist the processing by plasmin. In this work we modified sequences encoding the protease cleavage sites by site directed mutagenesis. Streptokinase derivatives resistant to C and N terminals cleavage were produced in protease deficient *B. subtilis* AZ₂₂₆ strain as an expression system.

MATERIALS AND METHODS

Materials: Human plasminogen Hpg was purified from human plasma using affinity chromatography (Deutsch and Mertz, 1970). DEAE-Sepharose and Chelating Sepharose were from Amersham Biosciences and phenyl-agarose was from Affinity Chromatography Ltd. Restriction enzymes used in this study were from New England Biolabs Canada, Ltd. (Mississauga, Ontario, Canada), Pharmacia Biotech Inc. (Baie d'Urfe', Quebec, Canada) and GIBCO BRL Canada (Burlington, Ontario, Canada).

Bacterial strains and growth conditions: Strains of *S. equisimilis* H46A (ATCC 12449), *B. subtilis* AZ₂₂₆ (*trpC2 nprA apr epr bfp mpr::ble nprB::ery*) and *E. coli* [(XL1-Blue) (*recA1 endA1 gyrA46 thi-1 hsdR17 supE44 relA1 Lac [F' proAB⁺ lacI^q Z M15 Tn10 (tet^r)*)] were used for routine experiments of expression and transformation. Bacteria were grown at 37°C for 8 h in brain heart infusion broth (Difco) as standing cultures at 37°C. *E. coli* (XL1-Blue) strain was grown in LB medium with added selective agent [ampicillin (Ap), 50 mg L⁻¹]. Transformed cells were grown on tryptose blood agar (TBAB; Difco, Detroit, Mich.) plates amended by 10 mg of kanamycin mL⁻¹. Cell density in the culture was adjusted to approximately 10⁶ cells mL⁻¹. Sucrose was added at a final concentration of 2% to induce the expression. After 5 h of induction, cells were collected by centrifugation at 5000 rpm.

Construction of vSK₃: The 2.5-kb PstI fragment carrying the structural gene of streptokinase (SK) from the parent strain of *S. equisimilis* H46A (ATCC 12446) was cloned into pBR322 as described in (Malke and Ferretti, 1984). This plasmid is named vSK. To construct plasmid vSKa, the 2.5-kb PstI fragment from vSK was ligated to the PstI digested pUB18. One of the resulting plasmids was named as vSKa where the insert carries the promoter of Sk. the expression of SK is presumed to be under the control of this sequence element.

Construction of vSK_b: It was constructed firstly by inactivating the HindIII site in the polylinker region and the insertion of the *sacB* promoter and signal peptide cassette (a 570-bp EcoRI-PstI fragment). Secondly, a 923-bp HindIII -PstI fragment encoding the 3' end region of *sk* was inserted into the HindIII-PstI double-digested pUB-SP to generate pUB-SK. The following pair of oligonucleotides (5'AGCTTTCGCGATCGCTG3' and 3'AAGCGCTAGCGACCTG 5' were used to generate the mature form of streptokinase with an identical N-terminal sequence. The resulting product had a HindIII site at one end and an Ava II site at the other end. Two restriction enzyme sites, NruI and PvuI, were also introduced within this synthetic sequence. These annealed oligonucleotides were ligated to a 1.37-kb Ava II fragment isolated from SK. The resulting ligated product was then digested by HindIII. A 760-bp HindIII fragment was gel purified and ligated to the HindIII-cut pUB-SK.

Site-directed mutagenesis for the production of streptokinase variants: Using the inverse PCR method, site-directed mutagenesis protocol was used to change Lys⁵⁹ to either glutamine or glutamic acid as described by Hemsley *et al.* (1989). vSK_b plasmid as the template DNA with two sets of oligonucleotide primers were used for PCR. Set -I [5' AGC T(A,C)T CAT A(C,A)T TCT CA(T,A) GAT CAA 3' and 3' GATCG A(T,G)A GTA T(G,T)A AGA GT(A,T) CTA GTT 5'] and Set-II [5' AGC TCT CAT A(G,A)T (A,G)AT CAA GAT (C,A)AA 3' and 3' GATCG AGA GTA T(C,T) A (T,C)TA GTT CTA (G,T)TT 5']. The amplified fragment was then recircularized by ligation. Produced plasmid DNA was transformed to AZ₂₂₆.

Induction of streptokinase by sucrose: Strain of *B. subtilis* AZ₂₂₆ was cultivated in super-rich medium. Sucrose at a final concentration of 2% was only added to one set of the AZ₂₂₆ (transformed with the construct vSK_b) to induce the production of streptokinase under of the *sacB* promoter and signal peptide. Both AZ₂₂₆ cultures (induced and non-induced) shared an identical growth profile under the specified culture conditions. Streptokinase secreted to the culture medium was determined by monitoring the plasmin activity with Chromozym PL as the substrate.

Purification of plasminogen and streptokinase: Human plasminogen was purified according to Deutsch and Mertz (1970) from fresh frozen plasma by lysine-Sepharose chromatography. Activated Sepharose 4B with CnBr was coupled to L-Lysine to according to Kohn and Wilchek (1982). 0.2 M α -aminocaproic acid in 0.1 M phosphate buffer (pH 7.2) containing 5 mg of aprotinin mL⁻¹ was added to bound plasminogen for elution. Fractions-containing-plasminogen were concentrated by ultrafiltration, washed with 50 mM Tris-Cl buffer (pH 7.5) and stored at -80°C. Streptokinase was precipitated from the culture supernatant according to the method of Karimi *et al.* (2012).

Plasminogen activation with recombinant streptokinase: Immobilized Plasminogen activation was performed by a modification of the procedure of Lizano and Johnston (1995). The 100 mg of streptokinase in 10 mM Tris-Cl (pH 8.0) and 100 mM NaCl were added to metalchelating (100 mL) IMAC affinity matrix. Tubes were incubated at 22°C for 5 min; the slurry was applied to a Spin-X micro-centrifuge filter fitted with a 0.45-mm cellulose acetate filter. The matrix was centrifuged for 3 min at pelleted by centrifugation at 4000 rpm. Matrix was washed with 20 mM Tris-Cl, pH 7.4 for several times and then removed from the Spin-X unit, placed in a microcentrifuge tube and resuspended in 200 mL of 50 mM Tris-Cl buffer, pH 7.4. An equimolar amount of plasminogen (in 50 mM Tris-Cl buffer, pH 7.4) was added to a series of micro-centrifuge tubes containing 25 mL aliquots of the matrix. Samples were placed on a rotating platform to keep the matrix in suspension and incubated at 22°C and. A sample was selected at different intervals (0 to 90 min) and the reaction was terminated by addition of 0.1 volumes of 10X stop buffer. The sample was transferred to a Spin-X microcentrifuge tube and pelleted by centrifugation at 4000 rpm for 3 min. Immobilized reactants of plasminogen and streptokinase were eluted by addition of 25 mL of 100 mM EDTA, followed by centrifugation at 10000 rpm for 10 min. Finally the samples were subjected for 10% SDS-PAGE analysis.

Protein blotting and N-terminal amino acid analysis: SDS-PAGE was performed according to Laemmli (1970). The upper buffer reservoir of the electrophoretic chamber contained 1 mM reduced glutathione to act as a scavenger of potential acrylamide polymerization by-products for sequencing of electro-transferred proteins. SDS-PAGE gels were equilibrated in carbonate buffer (10 mM NaHCO₃-3 mM Na₂CO₃ [pH 9.9]-20% [vol/vol] methanol) for 10 min and electrophoretically transferred to 0.45 mm nitrocellulose membranes (Bio-Rad, Hercules, Calif.) at 900 mA and 4°C for 30 min, according to Dunn (1986), in a Hoefer mini-blotting transfer chamber (Hoefer Scientific, San Francisco, Calif.) or to Immobilon-CD-PVDF transfer membranes (Millipore, Bedford, Mass.) for subsequent analysis of the N-terminal amino acid sequence on an Applied BioSystems model 4767A protein sequencer by the LSUMC Core Laboratories staff.

Miscellaneous: Isolation of chromosomal DNA, restriction analysis, electro-elution of DNA fragments, ligation and DNA sequence determination were performed as described in Sambrook *et al.* (1989). Protein concentrations were determined by the method Bradford (1976). To identify colonies that show streptokinase activity, cells were plated on TBAB agar plates overlaid with a thin layer of agarose (0.5% [wt/vol] agarose in physiological buffered saline with 0.5 mg of plasminogen and 0.1 g of skim milk in a final volume of 10 mL). The activity of streptokinase was determined by two methods: The colorimetric method (Castellino *et al.*, 1976) with tosyl-glycyl-prolyl-lysine-4-nitroanilide acetate (Chromozym PL; Boehringer Mannheim

Canada, Laval, Quebec, Canada) as the substrate and the radial caseinolysis method (Saksela, 1981) with agarose containing both plasminogen and skim milk. To determine the kinetic parameters for the activation of plasminogen by streptokinase variant forms, the conditions described by Shi *et al.* (1994) were used except that Chromozym PL was used as the substrate.

RESULTS

Engineering of streptokinase gene in *Bacillus subtilis* as an expression system:

Although mature streptokinase can be produced intracellularly in *E. coli* it has to be purified from many other *E. coli* intracellular proteins. *B. subtilis* is an attractive host for streptokinase production since it is capable of secreting extracellular proteins directly to the culture medium and nonpathogenic (Park *et al.*, 1991; Behnke, 1992; Fabian, *et al.*, 1992; Harwood, 1992). In a trial to assess the role of the promoter and signal sequence of *B. subtilis* promoter and signal sequence in directing the production and secretion of streptokinase, two plasmids were constructed. The first one (vSK_a) carries the intact streptokinase gene (Sk) including its promoter region while the second plasmid (vSK_b) carries the sequence encoding the mature part of streptokinase fused to the *B. subtilis* levansucrase (sacB) promoter and signal sequence. Transformed strain *B. subtilis* AZ₂₂₆ with (vSK_a) and (vSK_b) were cultivated in superrich medium and their secreted streptokinase was monitored by western blot analysis as shown in Fig. 1.

Sucrose at a final concentration of 2% was added to one set of *B. subtilis* AZ₂₂₆ culture which transformed by the construct (vSK_b) to induce the production of streptokinase. Streptokinase secreted to the culture medium was determined by monitoring the plasmin activity with Chromozym plasminogen as substrate. Transformed *B. subtilis* AZ₂₂₆ by the construct (vSK_a) produces streptokinase constitutively. Induced production of streptokinase was observed by sucrose in case of AZ226 (vSK_b) which has the promoter and signal sequence derived from SacB. By

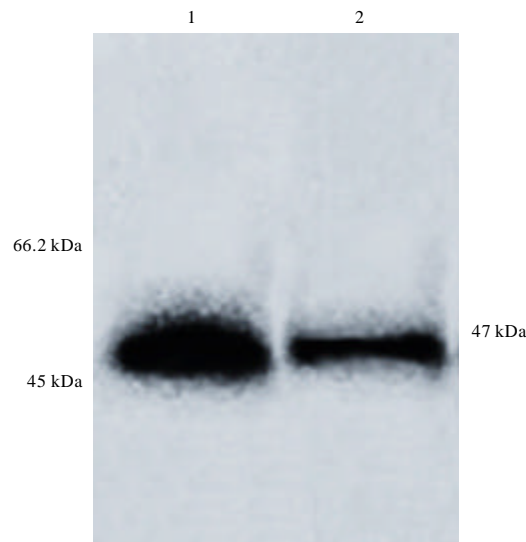


Fig. 1: Western blot analysis of streptokinase gene. Total proteins were extracted from transformed strain *B. subtilis* AZ226 with (vSK_a) and (vSK_b), analyzed by 12% SDS and immunoblotted using streptokinase-specific polyclonal antibodies. Lane 1: SK from the construct (vSK_b) clone 9 and Lane 2: SK from the construct (vSK_a) clone 11

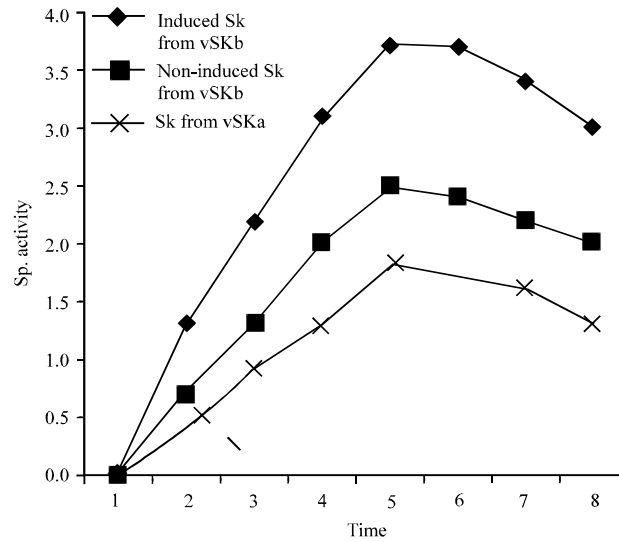


Fig. 2: Comparison of streptokinase production by transformed *B. subtilis* AZ226 either by vSKa or vSKb. Streptokinase specific activity from *B. subtilis* AZ226 (vSK_a) and AZ226 (vSK_b) in the presence or absence of sucrose (2%) was determined by using chromozym plasminogen as a substrate

comparing specific activity of the produced streptokinase it was noticed that the streptokinase specific activity of AZ226 (vSK_b) was about 1.2 to 1.4 fold higher than that from AZ226 (vSK_a) and the secreted streptokinase activity reached the highest level 4.30 h after inoculation in both cases (Fig. 2). N-terminal of the streptokinase produced from both constructs was analyzed to determine whether it has the N-terminal sequence identical to the native streptokinase. The first five amino acid residues were found to match exactly that of the native streptokinase produced from *S. equisimilis*, strain H46A (i.e., Ser-Lys-Pro-Phe-Ala).

Characterization of streptokinase proteolytic fragments: Data presented in Fig. 3 depicting the fragmentation of streptokinase upon incubation with the human plasmin (HPI_m). It was observed that SK molecule cleaved into peptide fragments with different molecular masses. These peptide fragments were found in the supernatant after 10 min of incubation with HPI_m (Lane, 2). Also, the 47 kDa peptide of Sk decreased while the other produced peptide fragments increased as the reaction proceeded for more than 30 min (Lane, 5).Molecular weights of the SK-peptide fragments were determined by SDS-PAGE electrophoresis.

Analysis of the N-terminal amino acid sequences showed that many different cleavage sites occurred in the Sk-peptide based on the incubation period with the HPI_m. Peptide fragments arises by proteolysis at both the NH₂ and COOH regions of native streptokinase. The 44-kDa intermediate appears first and are generated by C-terminal processing since it has identical N-terminal residues to those observed in the intact streptokinase. Isolation of a short C-terminal peptide with the N-terminal sequence corresponding to Tyr⁴⁰² indicates that one of the C-terminal cleavage events takes place between Arg⁴⁰¹ and Tyr⁴⁰². Also, another three large SK peptide fragments with molecular masses of 7 kDa, 18 and 30 kDa were produced. They consisted of Ile¹-Lys⁵⁹, for the one of the 7 kDa, Glu¹⁴⁸-Lys³⁸² for the one of the 18 kDa and Ser⁶⁰-Lys³⁸² for the one of the 30 kDa.

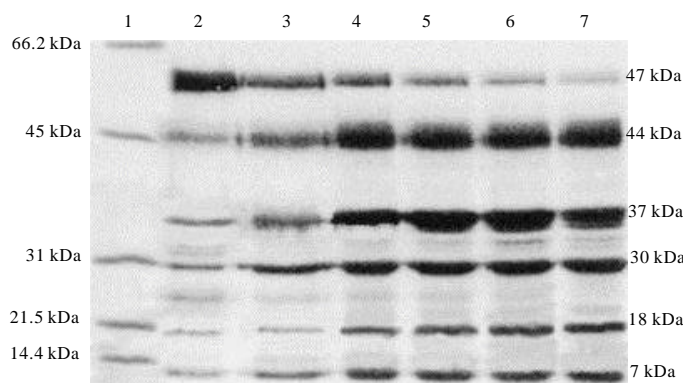


Fig. 3: SDS-PAGE analysis of the interaction of immobilized streptokinase with Immobilized human plasmin. HPlm (5 μ M) and Sk (5 μ M) were incubated in 1 mL of 0.05 M phosphate 0.1 M NaCl 0.02 M Lysine 25% glycerol buffer (pH 6.5) at 25°C. Samples were diluted at a final concentration of 2 nM. and collected at 10 min, (Lane 2); 20 min, (Lane, 3); 30 min, (Lane, 4); 45 min, (Lane, 5); 60 min, (Lane, 6); 90 min, (Lane, 7). Molecular masses compared by protein marker applied in Lane 1

Protection of streptokinase from the proteolytic cleavage: Based on the data in Fig. 1 and 3 it can be indicated that a processing event for the 44 kDa takes place on the C-terminus. Cleavage site of processing event could be determined by isolation of a short C-terminal peptide with the N-terminal sequence corresponding to Tyr⁴⁰². In light of these observations it can be indicated that one of the C-terminal cleavage events takes place between Arg⁴⁰¹ and Tyr⁴⁰². Since plasmin is a trypsin-like serine protease that specifically cleaves the peptide bond after lysine or arginine (Weinstein and Doolittle, 1972) it would be interesting to investigate whether changing nonpolar or hydrophobic amino acid residues in the C-terminal region to either polar or charged ones can generate streptokinase variants that can resist proteolytic processing at the C terminus. To achieve this objective, two streptokinase variants (SK1 and SK2) were constructed using vSk₆ as a template. In these variants the four hydrophobic residues K²⁹, Y³¹, A³², L³³ and Y³⁵ numbered from the C terminus of streptokinase were converted to polar residues and glutamine, respectively, via site directed mutagenesis.

Streptokinase variants (SK1 and SK2) were characterized by Western blot analysis and radial caseinolysis assay. Supernatant from both cultures were normalized to have equivalent amounts of streptokinase. As shown in Fig. 4a, both variants of SK1 and SK2 produce a protease-resistant form streptokinase. However, a low protein percentage of about (10 to 20%) exists in a new processed form marked by asterisk (lanes, 1 and 3) in case of the wild type and SK1. Variant of SK2 showed a biological activity 2.5 times higher than that of the wild-type streptokinase (Fig. 4b).

N-terminal sequencing of the 37-kDa fragment appears in Fig. 3 suggests that this fragment has the sequence corresponding to Ser⁶⁰ to Lys³⁸⁶ from the native streptokinase. This means that this product is generated through both N- and C-terminal processing events. In a trial to check whether the processing of streptokinase at the N-terminal region is an essential step in the generation of active streptokinase, Lys⁵⁹ in the engineered variant (SK2), was changed by site directed mutagenesis either to glutamine yielding the mutant variant SK2^{K59Q} or glutamic acid yielding

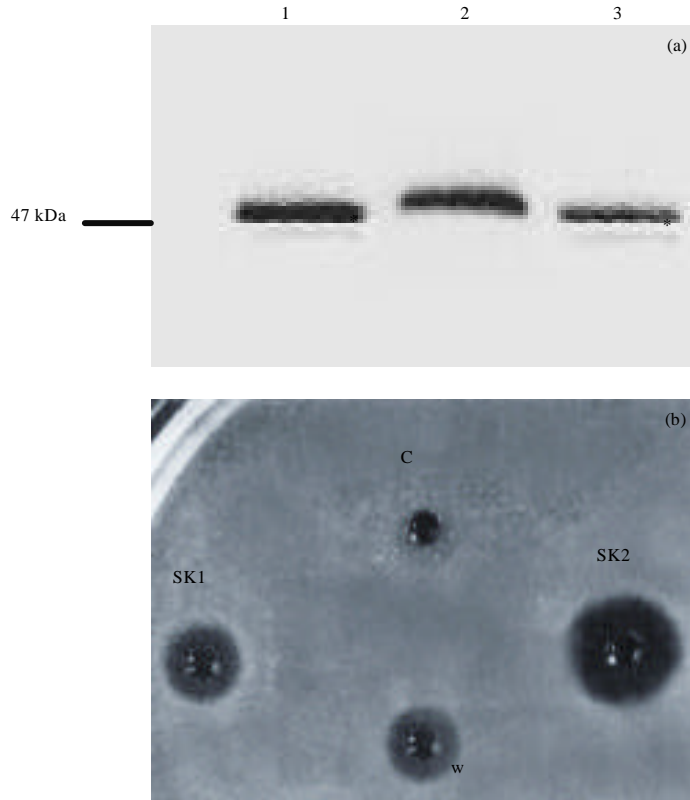


Fig. 4(a-b): (a) Western blot analysis of wild type streptokinase and radial caseinolysis assay of the two variants of SK1 and SK2. Lane, 1: Native streptokinase; Lane, 2: Streptokinase variant of SK2; Lane, 3: Streptokinase variant of SK1 SK was detected by streptokinase-specific polyclonal antibodies. (b) Streptokinase activity by the radial caseinolysis assay. C: Negative control (strain transformed by empty vector); w: Native streptokinase; SK1 and SK2; variants of streptokinase

another mutant variant SK2^{K59E}. Around 30 transformants were selected and spotted on to TBAB agar plates. One group of the transformants (12) exhibited the largest halo zone on TBAB agar plates that had been overlaid with a thin layer of agarose containing both plasminogen and skim milk. The second group (11) had halos smaller than those in group 1 but still larger than positive control strain, which produces the wild-type streptokinase. Seven transformant colonies (third group) did not show any halo surrounding the colony (Fig. 5a). Based on the nucleotide sequence, the first group carried the A-to-C mutation which converts Lys⁵⁹ to glutamine while group carried the A-to-G mutation which converts Lys⁵⁹ to glutamic acid. The expression of these variants compared to the native form of streptokinase is given in Fig. 5b.

Expressed proteins of both native streptokinase and variant SK2^{K59Q} were purified from the culture supernatant and used to study their processing by plasmin. Native streptokinase was rapidly processed and the 37-kDa intermediate form could also be observed 5 min after reaction with plasmin and became stable 20 min after reaction as presented in Fig. 6a. For the streptokinase variant of SK2^{K59Q}, the intermediate processing products were

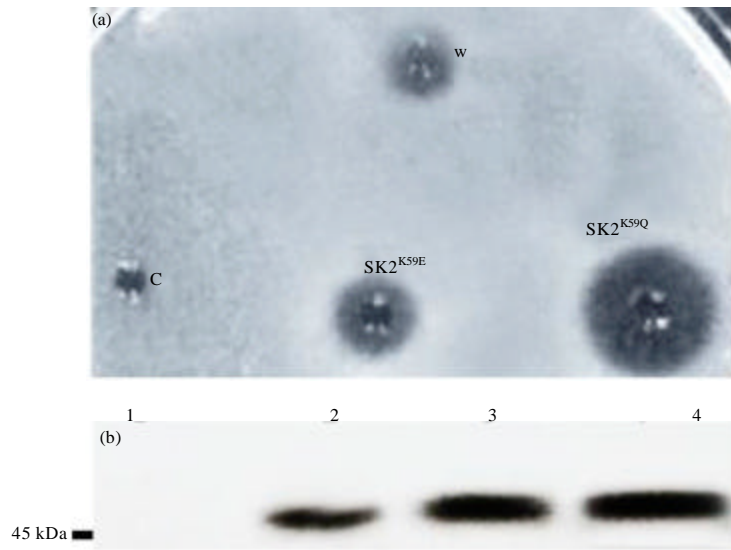


Fig. 5(a-b): (a) Radial caseinolysis activity of engineered variants of streptokinase in comparison to its native form. Equal amount was loaded into each wells and incubated at 37°C for 12 h. W, native form of streptokinase; C: Control strain of *Bacillus subtilis* transformed by empty vector as a negative control and (b) Western blot analysis of native type and mutant variants of streptokinase using streptokinase-specific polyclonal antibodies. Lane 1: Negative control, Lane 2: Native streptokinase, Lane 3: Mutant variant of SK2K59E and Lane 4: Mutant variant SK2K59Q

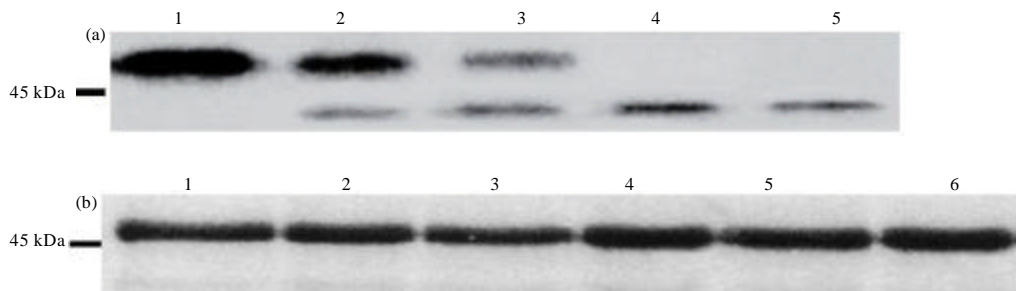


Fig. 6(a-b): (a) Processing of native streptokinase and (b) Streptokinase variant of SK2K59Q. Purified proteins of native streptokinase and its variant SK2K59Q were mixed with plasmin in a 1:1 molar ratio, incubated at 37°C and collected at different time points (in minutes). The reaction was terminated by adding SDS-PAGE buffer. Samples were analyzed by 12% SDS-PAGE and detected by Western blot using streptokinase-specific polyclonal antibodies. Lanes 1-5 in (a): Native streptokinase at 0 min; at 5 min; at 10 min; 20 min and at 60 min, respectively. Lanes 1-6 in (b): Streptokinase variant of SK2K59Q at 0 min; at 5 min; at 10 min; 20 min; at 60 min and at 90 min, respectively

not observed even after 60 min of reaction (Fig. 6b) which means changing of Lys⁵⁹ to glutamine blocked the processing of streptokinase at the N-terminus.

DISCUSSION

Streptokinase, a group of extracellular proteins produced, has been reported to be produced by various bacterial expression systems including the pathogenic *S. equismilis* H46A, the natural streptokinase (Martin, 1982). SK was expressed by using the intact streptokinase gene including its promoter region in various gram-negative and gram positive hosts including *Escherichia coli* (Malke and Ferretti, 1984), *Streptococcus sanguis* (Jackson *et al.*, 1986), *Proteus mirabilis* (Laplace *et al.*, 1989), *Bacillus subtilis* (Klessen and Malke, 1986), *Streptococcus lactis* (Laplace *et al.*, 1989) and from *Streptococcus pyogenes* (Cook *et al.*, 2012). Using a protease-deficient *B. subtilis* strain (AZ₂₂₆), the possibility of applying this nonpathogenic expression-secretion system to produce streptokinase was examined. A slightly higher level of streptokinase production was noticed upon using the *B. subtilis* sacB promoter and signal sequence than the use of *S. equismilis* Sk promoter and signal sequence (Fig. 1 and 2).

All of the expression systems produce a 44-kDa streptokinase degradation product to a different degree. This proteolysis causes a reduction in both the biological activity and the shelf life of this medically important enzyme (Castellino *et al.*, 1976). Another degradation product with a molecular mass of 37-kDa, isolated from the activation of the plasminogen by streptokinase and behaved in a similar way to that of the native SK in the activation of plasminogen (Malke and Ferretti, 1984; Klessen and Malke, 1986). Also, other several degradation peptide fragments of streptokinase were observed by PAGE electrophoresis. These fragments had molecular masses of 30, 18 and 7 kDa. No obvious difference between the degradation pattern of SK fragments in the supernatant mixture with immobilized HPlm between 30 and 90 min. However, the only difference that noticed was the reduction of the 47 kDa fragment while the fragment of the 44 kDa was increased.

Several approaches have been conducted to prolonging the half-life of blood clot-dissolving agents. These include attachment of polyethylene glycol (Brucato and Pizzo, 1990) or maltose binding protein to streptokinase (Houng *et al.*, 1995), chemical coupling of human serum albumin to urokinase (Breton *et al.*, 1995), the preparation of the streptokinase-acylated plasminogen complex known as APSAC (Smith *et al.*, 1981) and site-directed mutagenesis of glycosylation sites and domains in tissue plasminogen activator (Keyt *et al.*, 1994; Madison, 1994; Sharp *et al.*, 2009).

Some of these approaches have shown promising results, others have lower activity or become heterogeneous nature because of the chemical modification. In this report the degradation peptide fragments of SK from its reaction with immobilized hPlm were purified and the amino acid sequence of their N-terminal was conducted. Sequencing of the N-terminal of these peptides identified different cleavage sites. All of these sites were located in the hydrophilic domains of SK and nine of them were found in the β -domain (Radek and Castellino, 1989). In light of the N-terminal sequencing data of degradation peptides, mutations within the C-terminal region of streptokinase would be an alternative way for the protection of the streptokinase from proteolytic processing and for the engineering protease resistant streptokinase. To achieve this objective, two engineered variants (SK1 and SK2) were constructed. The hydrophobic amino acid residues located between 380 and 384 were converted to amino acids with hydrophilic side chains via site directed mutagenesis. During the secretory production of streptokinase, these mutations would eliminate the proteolytic processing within the region of 382 to 384 by proteases from *B. subtilis* AZ₂₂₆.

About 20 transformants were isolated and they showed a biological activity of about 90% in case of SK₂ comparable to that of the wild-type streptokinase (Fig. 4b). Characterization of streptokinase derived from SK1 and SK2 by Western blot analysis demonstrated that both forms of streptokinase were resistant to the proteolytic processing but a low percentage (10 to 20%) of the secreted

streptokinase from SK1 and the wild type appeared in a degraded form (Fig. 4a). This result suggests that the C-terminal region directly or indirectly plays a key role in streptokinase processing. Comparing the sequence of SK1 and SK2 demonstrates the importance of K³⁸⁶ which is located at the 29th position from the C terminus. SK1 has retained K³⁸⁶ at that position while SK2 has K³⁸⁶ changed to glutamine. Increasing of the biological activity of SK2 (2.5 times higher than that of the wild-type) may be attributed to the direct or indirect role of the C-terminal region in plasminogen activation and the importance of lysine residue K³⁸⁶ which is located at the 29th position from the C terminus.

Logically, lysine residue K⁵⁹ was another goal for site-directed mutagenesis, since streptokinase is processed N-terminally between K⁵⁹ and S⁶⁰ and C-terminally between K³⁸⁶ and D³⁸⁷ to generate the 37-kDa intermediate which retains only 16% of the intact streptokinase activity during the plasminogen activation process (Saksela, 1981). To block the proteolytic processing between K⁵⁹ and S⁶⁰ two new engineered variants of streptokinase were developed. These variants carried a mutation that led to the conversion of K⁵⁹ to glutamine (SK2^{K59Q}) or glutamic acid (SK2^{K59E}). SK2 variant was selected as a template for this conversion step because it showed a biological activity 2.5 times higher than that of the wild-type streptokinase and no degradation activity developed with it. To avoid perturb of the three-dimensional structure of streptokinase, glutamine was selected to replace K⁵⁹ because the length of its side chain is comparable to that of lysine. Also it does not introduce a positive charge to streptokinase. Therefore, the engineered streptokinase at these sites should not be cut by plasmin with the trypsin-like substrate specificity. This prediction was supported by the observed increase in biological activity of on radial caseinolysis assays (Fig. 5a) and the processing results (Fig. 5b). Streptokinase produced from SK2^{K59Q} was more stable than that of the wild type even after 90 min of reaction (Fig. 6b). This could be explained by the abolition of the N-terminal processing at k⁵⁹. Also, this result showed that only the conversion of K⁵⁹ to glutamine was important in extending the functional half-life of the intact streptokinase. This is consistent with the idea that processing K³⁸⁶ does not affect the function of plasminogen activation.

Although, replacement of K⁵⁹ and K386⁵⁹ with glutamine does not affect either the binding or the catalytic activity of streptokinase, some other residues of lysine in streptokinase are critical for its function. Lysine residues at positions 256 and 257 of streptokinase are important for affinity binding to plasminogen and lysine residues at positions 332 and 334 are required for the catalytic activity (Lin *et al.*, 1996). Many other evidences indicate that the first 59 amino acids have multiple functional roles for streptokinase. Mutation of Val19 (Lee *et al.*, 1997) and G²⁴ (Lee *et al.*, 1989) by Site-directed mutagenesis inactivates streptokinase. A plasminogen binding site is suggested to exist between residues between F⁶⁷ and K⁵¹ (Nihalani and Sahni, 1995). The conformation stability of streptokinase needs the presence of the first 59 residues (Shi *et al.*, 1994; Young *et al.*, 1995). The streptokinase fragment (residues 60 to 414) exhibited much lower activity and shows a disordered secondary structure without these N-terminal amino acids (Young *et al.*, 1995). This work, also illustrates that proteolytic processing of streptokinase by plasmin leads only to the inactivation of streptokinase as the plasminogen activator and these cleavages are not required to convert streptokinase to the active form. The design of SK2^{K59Q} allows the generation of plasmin-resistant streptokinase and its production in intact form using *B. subtilis* as secretory production system.

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