African Journal of Biotechnology Vol. 11(22), pp. 5995-6003, 15 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1690
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Cloning, high-level expression, purification and characterization of a staphylokinase variant, Sakϕ C, from *Staphylococcus aureus* QT08 in *Escherichia coli* BL21

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Accepted 28 February, 2012

The staphylokinase (Sak) is emerging as an important thrombolytic agent for the treatment of patients suffering from cardiovascular disease. Hence in this study, we reported the cloning, high-level expression, purification and characterization of the Sak variant Sakϕ C from *Staphylococcus aureus* QT08 in *Escherichia coli* Bl21. The *sak* gene of 489 bp encoding a protein (163 amino acids) with a predicted molecular mass of 18.5 kDa and pI 7.28 showed 99.8 to 99.6% identity with corresponding sequences from *S. aureus* strains deposited in GenBank (AF332619, X00127, EF122253 and M57455). The DNA sequence (411 bp) encoding the mature Sak (15.5 kDa) truncated 27 N-terminal amino acids was expressed in *E. coli* BL21/pESak under the control of the strong promoter *tac* in the presence of isopropyl-β-D-1-thiogalactopynoside (IPTG) as inducer. The expression level of rSak was estimated at about 42% of the total cellular proteins by densitometry scanning, which is the highest expression level of rSak expressed in any *E. coli* system. The recombinant staphylokinase was purified by Ni^{2+}-ProBond™ column to a single homogeneous 16-kDa band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a specific activity of 15175 U/mg protein, a recovery yield of 58% and a purification factor of 2.56. The optimal pH and temperature for the rSak activity was 9 and 37°C, respectively. rSak was stable over a temperature range of 25 to 50°C and at pH range of 7 to 9. Metal ions and detergents also showed an inhibitory effect on rSak, especially Zn^{2+} and Cu^{2+} which completely inhibited the enzymatic activity.

Key words: *Staphylococcus aureus* QT08, staphylokinase, cloning, high-level expression, purification, characterization.

INTRODUCTION

The obstruction of blood supply to heart muscles by the presence of a pathologic clot can lead to cardiovascular disease, a leading cause of death in human. Thrombolysis is one of the well established treatments for patients with acute myocardial infarction (Hennekens et al., 1995; Guzman and Lincoff, 1997; Sinnaeve and Van de Werf, 2001). The possibility of using blood-clot dissolving agents to dissolve thrombi offers an attractive means to treat this type of disease. Blood clot-dissolving agents currently approved for thrombolytic therapy include tissue plasminogen activator, urokinase, streptokinase, staphylokinase and their derivatives (Collen and Lijnen, 1994; Sinnaeve and Van de Werf, 2001).

Staphylokinase (Sak), an extracellular protein produced by *Staphylococcus aureus* strains, is a promising blood-clot dissolving agent (Collen and Lijnen, 1994). Sak is

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Abbreviations: Sak, Staphylokinase; **IPTG,** isopropyl-β-D-1-thiogalactopynoside.

both potent and safe because it lyses clots effectively in a fibrin-specific manner without causing any significant depletion of plasma proteins, and can also lyse platelet-rich clots in animal model systems (Collen et al., 1993). Sak is a profibrinolytic agent that forms a 1:1 stoichiometric complex with plasminogen that, after conversion to plasmin, activates other plasminogen molecules to plasmin (Lijnen et al., 1991). The plasmin*Sak complex, unlike the plasmin*streptokinase complex, is rapidly inhibited by α_2-antiplasmin. In a plasma milieu, Sak is able to dissolve fibrin clots without associated fibrinogen degradation (Schlott et al., 1994a). In experimental animal models, Sak appears to be equipotent to streptokinase for the dissolution of whole blood or plasma clots, but significantly more potent for the dissolution of platelet-rich or retracted thrombi (Schlott et al., 1994a).

In addition, Sak specifically induces fibrinolysis without fibrinogen depletion and has higher fibrinolytic activity compared with other plasminogen activators like streptokinase, urokinase and tissue plasminogen activator (Vanderschueren et al., 1995). Sak is at least as potent as tissue plasminogen activator (tPA) in the treatment of coronary artery recanalization (Armstrong et al., 2003; Moreadith and Collen, 2003). Furthermore, Sak has been shown to be more efficient than streptokinase for the dissolution of platelet-enriched and retracted blood clots (Lijnen et al., 1995; Abdelouahed et al., 1997). Hence, Sak has become a promising drug and stimulated much structural and protein engineering research. To date, *sak* gene has been cloned and expressed in different expression systems including *Escherichia coli* (Ren et al., 2008; Mandi et al., 2009), *Bacillus subtilis* (Ye et al., 1999), *Streptomyces lividans* (Cheng et al., 1998) and *Pichia pastoris* (Miele et al., 1999; Apte-Deshpnade et al., 2009), and under various *E. coli* promoters *viz*., *T7*, *lambda PR*, *tac* and *ptac* (Lee et al., 1998), *B. subtilis* promoters of *P43*, *Pamy* and *PsacB*, and *P. pastoris AOX1* promoter.

This study aimed at cloning, high-level expression and characterization of a staphylokinase from *S. aureus* strain QT08 isolated from a Vietnamese patient, using pET21a+ vector under the control of the *T7* promoter induced by isopropyl-β-D-1-thiogalactopynoside (IPTG).

MATERIALS AND METHODS

Bacterial strains and vectors

The bacterial strain, *S. aureus* QT08 (GQ247719), isolated from a patient at the Army Hospital No. 103 (Hanoi, Vietnam) was used as the source of Sak gene. *E. coli* DH5α (F$^-$, ø80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1, *endA*1, *hsdR*17(r_k^-, m_k^+), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1) and pJET1.2/blunt vector (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) were used for DNA manipulations and amplification. *E. coli* BL21 (DE3) cells (*F$^-$ ompT gal dcm lon hsdS$_B$*(r_B^- m_B^-) *λ*(*DE3* [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]) (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) and pET21a+ vector (Novagen, Merck Chemicals, Darmstadt, Germany) were used for the expression of Sak. Luria-Bertani (LB) medium containing 1% (w/v) bacto tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; pH 7 to 7.5 was used for cultivation of *E. coli* and *S. aureus* QT08. The LB agar used contained an additional 2% (w/v) agar and 100 µg ampicillin/ml.

Chemicals and reagents

The polymerase chain reaction (PCR) reagents, restriction endonucleases, T4 DNA ligase and *Taq* polymerase, and PCR primers were purchased from Fermentas (Thermo Fisher Scientific Inc. Waltham, USA). Ni^{2+}-ProBond™ resin was from Invitrogen Corp. (Carlsbad, USA), while 4-chloro-1-naphthol (horseradish peroxidase color development reagent) from Bio-Rad Laboratories, Inc. (Berkeley, USA). Human plasminogen was from MP Biomedicals (Santa Ana, USA), N-(p-tosyl)-gly-pro-lys 4-nitroanilide acetate salt (AAS) and anti-rabbit IgG (whole molecule)-peroxidase antibody from Sigma-Aldrich Co. (St. Louis, USA) were employed. All other reagents were of analytical grade unless otherwise stated.

DNA manipulations

Genomic and plasmid DNA isolation was carried out by methods previously described (Quyen et al., 2007). DNA fragments and PCR products were excised from a 0.8% agarose gel and purified by gel extraction kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instruction. DNA sequencing was performed on ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). *E. coli* DH5α and BL21 (DE3) cells were transformed using heat shock method that has been previously described (Quyen et al., 2007).

DNA amplification and analysis

The putative *sak*-coding DNA fragment was amplified from *S. aureus* QT08 genomic DNA by PCR with *Taq* DNA polymerase. Based on the DNA sequence of the *sak* gene from *S. aureus* phage 42D (GenBank: X06603), two oligonucleotides, SakF1 (5'-GCG AAT TCG CTC AAA AGA AGT TTA TTA T-3') and SakR1 (5'-GCC TCG AGT TTC TTT TCT ATA ARA ACC T-3'), were designed as primers for introduction of the underlined *Eco*RI and *Xho*I restriction sites, respectively. The PCR mixture contained 2.5 µL 10X PCR buffer; 2 µL of 2 mM dNTP; 2.5 µL of 25 mM $MgCl_2$; 1 µL genomic DNA (50 to 100 ng); 0.25 µL 5 U *Taq* polymerase and 1 µL each primer (10 pmol), supplemented with 14.75 µL distilled water to a final volume of 25 µL. The thermocycler conditions were as follows: 95°C/4′; 30 cycles of (95°C/45″, 55°C/30″, 72°C/45″); 72°C/10′. The PCR products amplified from the genomic DNA with both primer SakF1 and SakR1 were inserted into the cloning vector pJET1.2/blunt, resulting in pJSak, and then sequenced. DNA sequencing was performed on ABI PRISM 3100 Avant Genetic Analyzer. Sequence alignments were constructed and analyzed using the program MegAlign DNAStar.

Plasmid construction

The DNA fragment (411 bp) encoding the mature staphylokinase (without the signal peptide of 27 N-terminal amino acids) from *S. aureus* QT08 was amplified using pJSak as template and two specific primers SakF2 (5'-GCG AAT TCC ATA TGT CAA GTT CAT TCG ACA AA-3') and SakR2 (5'-GCG AAT TCA AGC TTT TTC

TTT TCT ATA A-3') for introduction of the underlined *Nde*I and *Hin*dIII restriction sites, respectively. The PCR mixture contained 2.5 µL 10X PCR buffer; 2 µL of 2 mM dNTP; 2.5 µL of 25 mM $MgCl_2$; 1 µL pJSak (50 to 100 ng); 0.25 µL 5 U *Taq* polymerase and 1 µL each primer (10 pmol), supplemented with 14.75 µL distilled water to a final volume of 25 µL. The thermocycler conditions were as follows: 95°C/5′; 30x (95°C/30″, 55°C/30″, 72°C/30″); 72°C/7′. PCR products and pET21a+ were digested with NdeI and *Hin*dIII and purified using gel extraction kit (Qiagen) in accordance with the manufacture's instructions. It was followed by ligation of the NdeI-*Hin*dIII digested *sak* products with pET21a+ linearized by the same enzymes, resulting in pESak under the control of the T7-promoter induced by IPTG (isopropyl-β-D-thiogalactopyranoside) and possessing the ampicillin marker. The staphylokinase Sak-his encoded by the plasmid pESak contains the mature staphylokinase fused with the 6x histidine-tag and no leader sequence.

Staphylokinase expression

The transformant *E. coli* BL21/pESak was cultivated in 5 ml of LB medium containing 5 µl of 100 mg ampicillin/ml at 37°C with agitation at 220 rpm. 500 µL of the overnight culture were transferred into 50 ml of LB medium containing 50 µL of 100 mg ampicillin/ml. The culture was cultivated at 37°C with agitation at 220 rpm until an optical density (OD) at 600 nm of 0.8 was reached (for approximately 4 h), then 50 µL of 100 mM IPTG was added. The culture was continuously incubated at 28°C with agitation of 220 rpm for 6 h of induction. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C. Wet cells was used immediately for protein purification or stored at -20°C for later purification.

rSak purification

The fusion form rSak carrying a C-terminal 6xHis tag was expressed in *E. coli* BL21 and purified using affinity chromatography with Ni^{2+}-ProBond™ resin (Invitrogen Corp., Carlsbad, USA) under hybrid conditions. To purify rSak, 350 mg wet cells from a 50-ml culture in LB medium were harvested by centrifugation at 8000 rpm and 4°C for 5 min, and suspended in 10 ml of guanidinium lysis buffer containing 6 M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM NaCl and pH 7.8 for 1 h at 4°C. The cell lysate was then centrifuged at 13000 rpm and 4°C for 5 min. A volume of 8 ml cell lysate was applied to a column containing 2 ml resin, which was equilibrated with denaturing binding buffer and incubated for 45 min at room temperature with gentle hand shaking for several times. The column was washed twice with 8 ml of denaturing wash buffer and twice with 8 ml of native wash buffer. The bound protein was eluted with 8 ml of native elution buffer. The rSak solution was used for characterization.

Electrophoresis analysis and protein concentration

The homogeneity and molecular mass of the rSak were determined by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) with Biometra equipment (Göttingen, Germany). Proteins were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250. Protein concentrations were estimated by the method of Bradford with the bovine serum albumin as standard (Bradford, 1976).

Home-made production of anti-Sak serum

Two New Zealand rabbits (Central Company of Veterinary Vaccine, Vetvaco, Hanoi, Vietnam) were used for anti-Sak antibody production. Pre-immunized blood samples were taken from the ear veins of each rabbit. For the initial immunization, rabbits were immunized subcutaneously with purified Sak (200 µg per rabbit) emulsified in Freund's complete adjuvant. Subsequent booster injections were given every week with 200 µg purified Sak in Freund's incomplete adjuvant. Ten days after the fourth immunization, the rabbits were sacrificed. The sera were collected from blood, portioned into Eppendorf tubes (1 ml per tube) and stored in deep freezer at -80°C.

Western blot analysis

Western blot analysis was carried out according to the protocol described in "Protein Methods" (Bollag et al., 1996). The polyvinyl difluoride (PVDF) membrane was embedded in the primary antibody (anti-Sak) serum solution diluted to 1:1000 in 1% (w/v) nonfat milk and incubated in the secondary anti-rabbit IgG (whole molecule)-peroxidase antibody solution diluted to 1:10000 in 1% (w/v) nonfat milk. Finally, the membrane was colored with 4-chloro-1-naphthol (HRP color development reagent).

rSak assay

The rSak activity was determined by using plasminogen coupled chromogenic substrate according to the method of Hernandez et al. (1990). The Sak activity was assayed by mixing 2 µL plasminogen (0.025 U/µL) in 10 µL of 20 mM Tris-HCl buffer pH 7.5 and 10 µL of appropriate dilution of rSak (0.1 µg) in 50 mM potassium phosphate buffer pH 7.0, and incubated at 37°C for 30 min. The plasminogen*Sak complexes were mixed with 40 µL of 1 mM chromogenic substrate AAS in 50 mM phosphate buffer, pH 7.0 and incubated at 37°C for 15 min. The reaction was stopped by adding 10 µL of 0.4 M acetic acid and the absorbance was measured at 405 nm. One unit (U) of Sak is defined as one unit of standard streptokinase from Sigma. One unit of standard streptokinase liberates a standard clot of fibrinogen, plasminogen and thrombin at pH 7.5 and 37°C for 10 min. The units of Sak were calculated by using a standard curve of pure standard streptokinase (Sigma).

Temperature and pH optimum

The pH and temperature optimum of rSak were determined by measuring the activity as described above using 100 mM potassium acetate buffer (pH 4 to 6), 100 mM potassium phosphate buffer (pH 5.5 to 7.5) and 100 mM Tris-HCl buffer (pH 7.5 to 10) at 37°C for 30 min, and in the temperature range of 20 to 45°C at pH 7 for 30 min, respectively.

Temperature and pH stability

For the determination of temperature and pH stability, purified rSak (0.1 µg for each reaction) was pre-incubated in 50 mM potassium phosphate buffer pH 7 at different temperatures 25 to 70°C for 0 to 8 h, and pH range (100 mM potassium phosphate pH 6 to 7 and 100 mM Tris-HCl pH 8 to 9) at 37°C for 0 to 8 h, respectively. The residual activity was then determined as above mentioned.

Effect of metal ions and EDTA

Purified rSak, 0.1 µg for each reaction, was incubated with 5 mM of various metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+} and K^{+}) and ethylenediaminetetraacetic acid (EDTA) at 37°C for 60 min, and in 2% (w/v) of different detergents (Tween 80, Tween 20, SDS

and Triton X-100) at 37°C for 30 min. The residual activity was then determined. All measurements were carried out in triplicate with the resulting values being the mean of the cumulative data obtained.

DNA and amino acid sequence analysis

Homologies of the DNA and amino acid sequences were determined with the program MegAlign DNAStar. Signal peptide was predicted using signal peptides prediction program SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al., 1997).

RESULTS AND DISCUSSION

Gene cloning and analysis

The recombinant plasmid pJSak was sequenced and the putative staphylokinase gene was aligned with sequences from GenBank using DNAStar. Nucleotide sequence analysis of the *sak* gene from *S. aureus* QT08 (Figure 1A) showed a 489-bp open reading frame that encoded a protein of 163 amino-acid residues, with a predicted molecular mass of 18.5 kDa and pI 7.28 (Figure 1A). Using the signal peptides prediction program, SignalP 3.0 (Nielsen et al., 1997), it was revealed that the most likely cleavage site between position 27 and 28 (Figure 1A) was the predicted signal peptide site in the Sak sequence. The NH_2-terminal amino acid sequence of the mature staphylokinase from *S. aureus* QT08 was identified as SSSFDKGKYKKGDDA of Sak reported (Schlott et al., 1994b) and Sak from *S. aureus* QT08 belonged to the variant SakϕC among three natural variants SakϕC, Sak42D and SakSTAR which differ in only three amino acids 34, 36 and 43 in the mature protein: GGH, GRR, and SGH, respectively (Schlott et al., 1997).

The coding sequence of *sak* from *S. aureus* QT08 showed 99.8 to 99.6% identity with the corresponding sequences from *S. aureus* strains (AF332619, X00127, EF122253 and M57455), and 98.8 to 97.8% with sequences from *S. aureus* strains (U77328, EU146839 and A17529) (Figure 1B). The deduced amino acid sequence of Sak had homology of 100, 99.4 to 97.8 and 90.8% with the corresponding amino acid sequences from three *Staphylococcus* strain groups (X00127; AF332619, M57455, U77328, EU146839, and A17529; and EF122253, respectively) (Figure 1C). The nucleotide sequence encoding Sak from *S. aureus* QT08 was deposited in the GenBank with an accession number FJ868207.

Expression and purification of rSak

The DNA fragment (411 bp) encoding the mature staphylokinase (rSak) truncated 27 N-terminal amino acids from *S. aureus* QT8 was inserted into pET21a+ vector at the *Nde*I and *Hin*dIII sites resulting in the recombinant plasmid pESak. The transformant *E. coli* BL21/pESak was grown in LB medium for the rSak production. After IPTG induction, the cells were collected and used for purification and characterization. The expression level of rSak by *E. coli* BL21/pESak was determined as high as 42% of the total proteins (Figure 2A, lanes 3 to 5) using Dolphin 1D software, which is the highest level of rSak expressed in any *E. coli* system till date. The expression level of rSak in *E. coli* system was previously reported to be 10 to 15% (Schlott et al., 1994a), 22% (Schlott et al., 1997) and 35% (Mandi et al., 2009).

The yield of rSak was estimated as high as 300 mg/L culture in this study, whereas it was reported to be 200 mg/L fermentation broth (Schlott et al., 1994a), 70 to 500 mg/L culture (Schlott et al., 1997), 20 mg/L (Lee et al., 1998) and 2.8 g/L of fermentation broth (Mandi et al., 2009), which was reported to be highest yield of rSAK expressed in any bacterial system till date. However, the yield of rSak was dependent on the cell mass productivity per liter fermentation broth. If any high cell-density culture (HCDC) technique has been developed for growing recombinant *E. coli* strain in fed-batch cultures at concentrations greater than 100 g (dry cell weight) per liter (Lee, 1996), then the yield of rSak could be enhanced to 3 or 4 g/L fermentation broth. The yield of rSak expressed in *Bacillus* system was also different, as low as 25 mg/L of culture supernatant (Behnke and Gerlach, 1987) and 50 mg/L (Gerlach et al., 1988), and as high as 337 mg/L (Ye et al., 1999) and 255 mg/L (Kim et al., 2001). The eukaryote system *P. pastoris* produced the prokaryote protein Sak with a lower yield of 33 to 50 mg/L fermentation broth (Miele et al., 1999) or with a high yield of 1 g/L (Apte-Deshpnade et al., 2009) but with negligible plasminogen activation activity. In another study, we have expressed this rSak from *S. aureus* QT08 in *P. pastoris* in the fully active form with a specific activity of 20658 U/mg and a level of 19 mg/L culture broth.

rSak from *S. aureus* QT08 over-expressed by *E. coli* BL21/pESak cells was purified through affinity chromatography column of Ni^{2+}-ProBond™ resin to the homogeneity on SDS-PAGE with a molecular weight of approximately 15.5 kDa (Figure 2B, lanes 7 to 9). The purified rSak gained a specific activity of 15175 U/mg protein with a purification factor of 2.56 and a yield of 58% (Table 1). Also, the Western blot analysis using rabbit anti-Sak antibody showed two forms of rSak, monomeric (~15.5 kDa) and dimeric protein (~31 kDa) (Figure 2C, lane 10).

Temperature and pH optimum

rSak showed an optimum temperature of 30 to 37°C and was active at a broad temperature range from 20 to 45°C with the activity of over 93% in comparison to the optimal activity (Figure 3A). rSak activity showed the maximum

(A)

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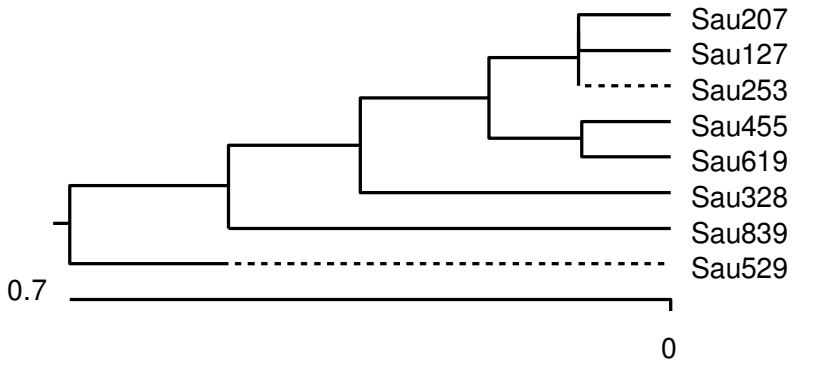
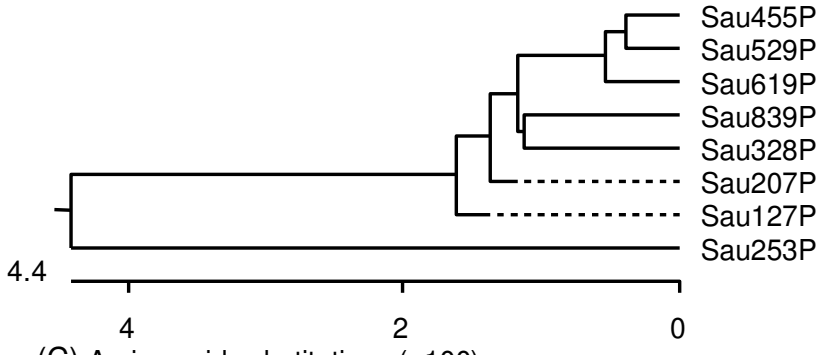
atgctcaaaagaagtttattattttaactgttttattgttattattctcattttcttca   60
 M  L  K  R  S  L  L  F  L  T  V  L  L  L  L  F  S  F  S  S    20
attactaatgaggtaagtgcatcaagttcattcgacaaaggaaaatataaaaaaggcgat  120
 I  T  N  E  V  S  A  S  S  S  F  D  K  G  K  Y  K  K  G  D    40
gacgcgagttattttgaaccaacaggcccgtatttgatggtaaatgtgactggagttgat  180
 D  A  S  Y  F  E  P  T  G  P  Y  L  M  V  N  V  T  G  V  D    60
Ggtaaaggaaatgaattgctatcccctcattatgtcgagtttcctattaaacctgggact  240
 G  K  G  N  E  L  L  S  P  H  Y  V  E  F  P  I  K  P  G  T    80
Acacttacaaaagaaaaaattgaatactatgtcgaatgggcattagatgcgacagcatat  300
 T  L  T  K  E  K  I  E  Y  Y  V  E  W  A  L  D  A  T  A  Y   100
Aaagagtttagagtagttgaattagatccaagcgcaaagatcgaagtcacttattatgat  360
 K  E  F  R  V  V  E  L  D  P  S  A  K  I  E  V  T  Y  Y  D   120
Aagaataagaaaaaagaagaaacgaagtctttccctataacagaaaaaggttttgttgtc  420
 K  N  K  K  K  E  E  T  K  S  F  P  I  T  E  K  G  F  V  V   140
Ccagatttatcagagcatattaaaaaccctggattcaacttaattacaaaggttgttata  480
P  D  L  S  E  H  I  K  N  P  G  F  N  L  I  T  K  V  V  I    160
gaaaagaaa                                                     489
 E  K  K                                                      163
```



(B) Nucleotide substitutions (×100)



(C) Amino acid substitutions (×100)

Figure 1. (A) The nucleotide and deduced amino acid sequences of Sak from *S. aureus* QT08. The putative Sak signal sequence is underlined beneath the amino acid sequence. The bold amino acids are Sakϕ C variant (GGH). (B) Phylogenetic analysis of the staphylokinases, based on comparison of DNA sequence and (C) deduced amino acid sequences of the mature proteins. The phylogenetic tree was constructed using the DNAStar program. The following sequences were obtained from GenBank: Sau207 (*S. aureus* strain QT08, accession no. FJ868207); Sau127 (*S. aureus* strain, accession no. X00127); Sau253 (*S. aureus* strain, accession no. EF122253); Sau455 (*S. aureus* strain, accession no. M57455); Sau619 (*S. aureus* strain, accession no. AF332619); Sau328 (*S. aureus* strain, accession no. U77328); Sau839 (*S. aureus* strain, accession no. EU146839); Sau529 (*S. aureus* strain, accession no. A17529).

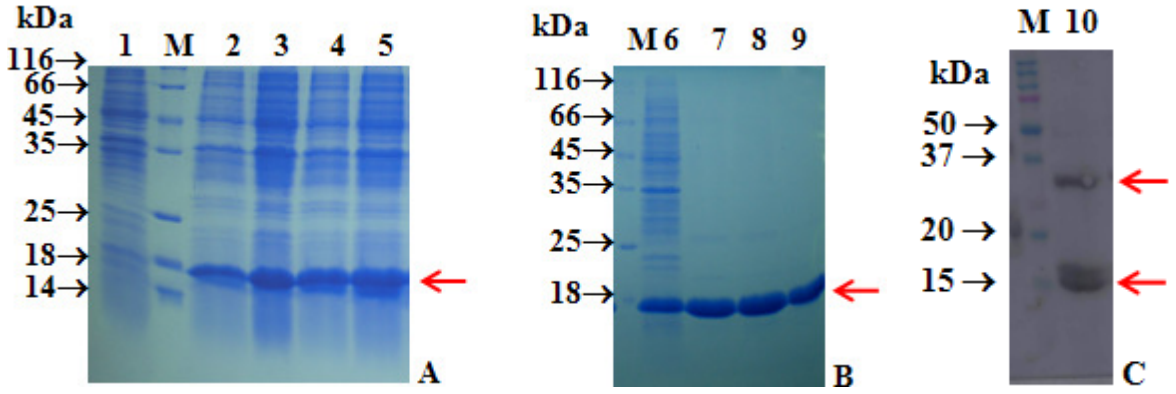


Figure 2. SDS-PAGE of the overexpressed (A) and Ni-NTA purified (B) rSak in *E. coli* BL21. Proteins were stained with Coomassie Brilliant Blue. Lane M, Molecular standards (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) indicated in kDa; A, *E. coli* pESak cell lysate before (lane 1) and after IPTG induction for 2, 4, 6 and 8 h (lanes 2, 3, 4 and 5, respectively); B, *E. coli* pESak cell lysate after IPTG induction for 6 h (lane 6) and Ni-NTA purified rSak (from *E. coli* pESak lysate) (lanes 7, 8 and 9). Western blot (C) of rSak expressed in *E. coli* (lane 10) and standard proteins (Bio-Rad Laboratories, Inc., Berkeley, USA) (lane M).

Table 1. Purification procedure of rSak from the cell lysate of *E. coli* BL21/pESak.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Cell lysate	141714	23.93	5923	100	1
Ni^{2+}-ProBond™ resin column	81609	5.38	15175	58	2.56

(100%) at pH 9 and 83 and 97% at pH 8.5 and 9, respectively (Figure 3B) in 100 mM Tris-HCl buffer. Moreover, in 100 mM phosphate buffer, rSak showed optimum activity at pH 7 (94%) and 83 and 64% at pH 6.5 and 7.5, respectively. Our results are consistent with the report of the wild type Sak (Vesterberg and Vesterberg, 1972); the optimal pH value for the Sak activity was 8.5 for Tris-HCl buffer (pH 7.6 to 9.2) and 7.5 for phosphate buffer (pH 6.8 to 7.7).

Temperature and pH stability

rSak from *S. aureus* QT08 was stable at a temperature range from 25 to 50°C. Meanwhile, after incubation for 8 h, the rSak activity retained above 50% in comparison to the original activity (Figure 4A), but less than 20% at temperature range of 60 to 70°C. rSak from *S. aureus* QT08 showed a similar profile in the temperature stability to native Sak (Fujimura et al., 1974; Schlott et al., 1994b). The activity of the native Sak from *S. aureus* after heating at 60°C for 2 h decreased to 11% of that of the original sample (Fujimura et al., 1974). Both wild type Sak42D and SakSAR showed a different thermal stability; Sak42D was inactivated more rapidly while SakSAR gradually inactivated for several hours at high temperature (70°C) (Schlott et al., 1994b). rSak from *S. aureus* QT08 showed pH stability at a pH range of 7 to 9. The residual rSak activity was above 40% in comparison to the original activity after treatment for 8 h (Figure 4B).

Effect of metal ions

In this study, the effect of various additives on the purified on the rSak activity was investigated. K^+ and Ca^{2+} at the concentration of 5 mM showed a slightly inhibitory effect on rSak activity, the residual activity was accounted for 93% and 90% of the original activity, respectively (Table 2). Moreover, the rSak activity retained over 50% by addition of 5 mM of Mg^{2+} and EDTA and less than 20% by addition of 5 mM of Co^{2+}, Fe^{2+}, Ni^{2+} and Ag^+. However, the addition of 5 mM of Zn^{2+} and Cu^{2+} completely inhibited the activity of the purified rSak (Table 2). These similar phenomena were also observed in the catalytic activity of the wild type Sak treated with 20 mM of the metal ions (Vesterberg and Vesterberg, 1972). The addition of 20 mM of Cu^{2+} and Ag^+ completely inhibited the Sak activity. Other metal ions showed a Sak inhibitory activity of 98 to 70% in order of Zn^{2+}> Co^{2+}> Mn^{2+} > Ca^{2+} > Pb^{2+}, while Na^+ and Mg^{2+} did not show any significantly inhibitory activity (Vesterberg and Vesterberg, 1972). In contrast, Ca^{2+} and Mg^{2+} showed an opposite effect on the rSak activity in this study and the native Sak activity studied by Vesterberg and Vesterberg (1972). The same results is also found in the report that Cl^-, Na^+, K^+ and

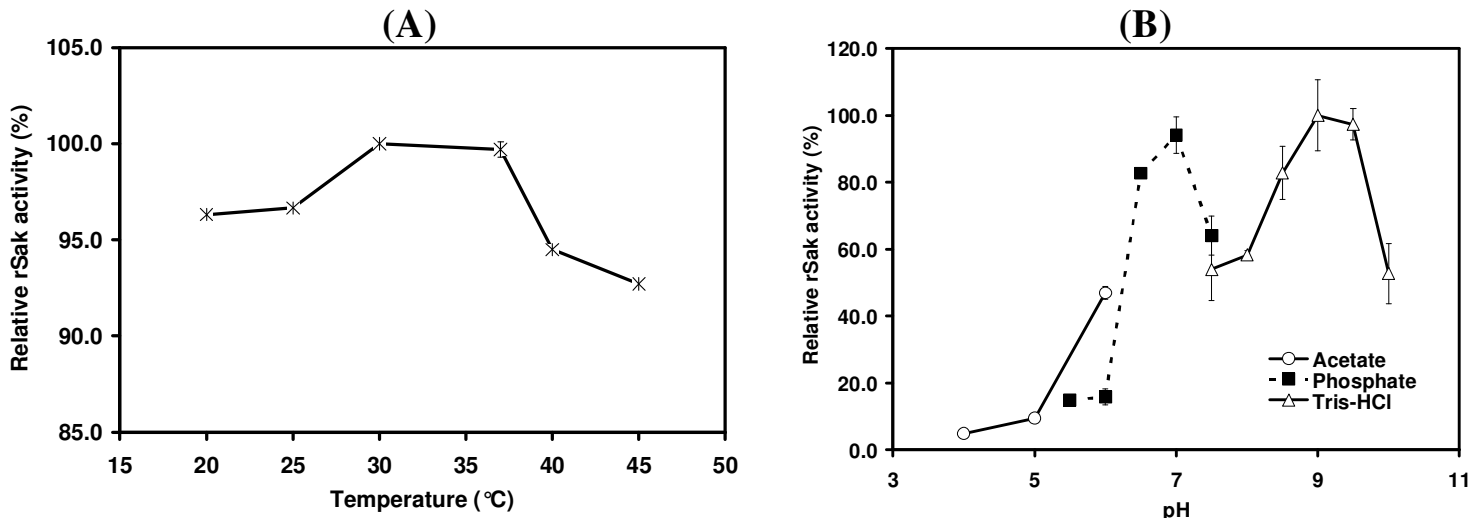


Figure 3. Temperature (A) and pH (B) optimum of rSak.

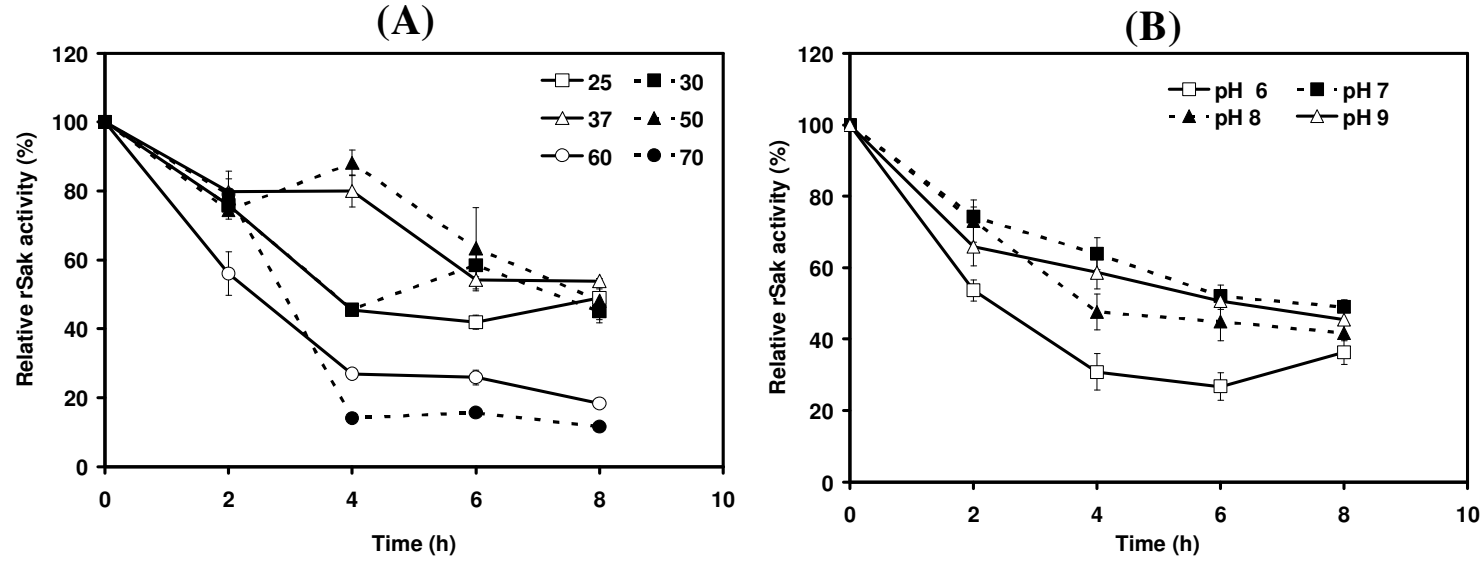


Figure 4. Temperature (A) and pH stability (B) of rSak.

Ca^{2+} affected the rate at which plasminogen was activated by rSak (Yarzábal et al., 1999).

In addition, the sodium salt of EDTA at concentrations higher than 10 mM showed an inhibitory effect on the Sak activity (Vesterberg and Vesterberg, 1972), but at lower concentrations there was no inhibitory effect on the enzyme activity. In this study, at the concentration of 5 mM, EDTA inhibited 32% enzyme activity.

Effect of detergents on rSak activity

All tested detergents inhibited enzyme activity (Table 3). The addition of Triton X-100 and Tween 20 decreased the rSak activity slightly by 2 to 5%, but Tween 80 by 22%. An addition of SDS caused the most loss of enzyme activity; it remained only 7.3% of the original activity. There have been no reports so far regarding this issue yet.

The study on the influence of metal ions, detergents, temperature and pH on the Sak activity was rather complicated since, in fact, two reactions were studied in the assay: first, the activation of plasminogen to plasmin by Sak, and secondly, the digestion of substrate by plasmin. By adding metal salts simultaneously with the substrate, it was possible to observe the influence of this on the fibrinolytic activity, but it was not possible to isolate the influence of additives on the activation of plasminogen

Table 2. Effect of metal ions on the rSak activity.

Additive[a]	Residual activity (%)[b]
Control (no additive)	100 ± 0.87
$AgNO_3$	8.54 ± 1.86
$CaCl_2$	90.02 ± 0.12
$Co(NO_3)_2$	20.06 ± 3.06
$CuSO_4$	0
$FeSO_4$	13.34 ± 6.67
KCl	93.38 ± 5.57
$MgCl_2$	49.53 ± 3.44
$NiCl_2$	19.78 ± 0.39
$ZnSO_4$	0
EDTA	67.63 ± 0.53

[a]The final concentration of additive (EDTA or inorganic salt) in the reaction mixture was 5 mM. [b]Relative activity was expressed as a percentage of control (100% rSak activity was 15675 U/mg).

Table 3. Effect of detergents on the rSak activity.

Detergent[a]	Residual activity (%)[b]
Control (no additive)	100
SDS	7.3 ± 0.18
Tween 20	95 ± 1.45
Tween 80	78 ± 1.67
Triton X-100	98 ± 2.00

[a]The final concentration of surfactant in the reaction mixture was 2.5%. [b]Relative activity was expressed as a percentage of control (100% rSak activity was 16379 U/mg).

to plasmin by Sak. Therefore, additives had an inhibitory effect on plasmin and on the total activity (Vesterberg and Vesterberg, 1972).

Conclusion

Sak is a promising blood-clot dissolving agent for the treatment of patients suffering from a heart attack. It would be desirable to produce this protein in large quantities for biochemical characterization and clinical trials. In the present study, a *sak* gene from *S. aureus* QT08 was cloned and analyzed. The gene sequence showed only one nucleotide different from that from *S. aureus* (AF332619). The mature polypeptide chain (rSak) consisting of 136 amino acids was overexpressed in *E. coli* Bl21 at a high level of 42% of the total protein. The temperature and pH optimum for rSak were 37°C and 9, respectively. rSak was stable in a temperature range of 37 to 50°C and pH 7 to 9. Additives (metal ions and detergents) also showed an inhibitory or no effect on rSak.

ACKNOWLEDEGMENTS

This study was supported by the National Foundation for Science and Technology Development (Nafosted), Ministry of Science and Technology, 2010-2011 with the project 106.05.169.09 “Cloning, sequence analysis, expression of a gene encoding the thrombolytic agent staphylokinase (Sak) from *Staphylococcus aureus* isolates in microbial expression systems, characterization of thrombolytic properties and application-oriented investigation”. We appreciate Prof. Dr. Dinh Duy Khang for the home-made production of the anti-Sak serum.

REFERENCES

Abdelouahed M, Hatmi M, Helft G, Emadi S, Elalamy I, Samama MM (1997). Comparative effects of recombinant staphylokinase and streptokinase on platelet aggregation. J. Thromb. Haemost. 77: 815-817.

Apte-Deshpnade A, Mandal G, Soorapaneni S, Prasad B, Kumar J, Padmanabhan S (2009). High-level expression of non-glycosylated and active staphylokinase from *Pichia pastoris*. Biotechnol. Lett. 31: 811-817.

Armstrong PW, Burton J, Pakola S, Molhoek PG, Betriu A, Tendera M, Bode C, Adgey AA, Bar F, Vahanian A, Van de Werf F (2003). Collaborative angiographic patency trial of recombinant staphylokinase (CAPTORS II). Am. Heart J. 146: 484-488.

Behnke D, Gerlach D (1987). Cloning and expression in *Escherichia coli*, *Bacillus subtilis*, and *Streptococcus sanguis* of a gene for staphylokinase - a bacterial plasminogen activator. Mol. Gen. Genet. 210: 528-534.

Bollag DM, Rozycki MD, Edelstein SJ (1996). Protein Methods, 2nd edn. Wiley Publishers.

Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Cheng Y, Li Y, Liu B, Guo L (1998). Cloning and secretory expression of staphylokinase in *Streptomyces lividans*. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 20: 428-432.

Collen D, De Cock F, Stassen J-M (1993). Comparative immunogenicit and thrombolytic properties toward arterial and venous thrombi of streptokinase and recombinant staphylokinase in baboons. Circulation, 87: 996-1006.

Collen D, Lijnen HR (1994). Review: Staphylokinase, a fibrin-specific plasminogen activator with therapeutic potential? Blood 84: 680-686.

Fujimura S, Makino T, Hayashi TTA (1974). Occurrence of a complex form of staphylokinase in the course of cultivation of *Staphylococcus aureus*. Appl. Microbiol. 28: 5-10.

Gerlach D, Kraft R, Behnke D (1988). Purification and characterization of the bacterial plasminogen activator staphylokinase secreted by a recombinant *Bacillus subtilis*. Zentralbl. Bakteriol. Mikrobiol. Hyg. A, 269: 314-322.

Guzman LA, Lincoff AM (1997). Thrombolytic therapy: The treatment of choice in acute myocardial infarction thrombolytic therapy. J. Thromb. Thrombolys, 4: 337-343.

Hennekens CH, O'Donnell CJ, Ridker PM, Marder VJ (1995). Current issues concerning thrombolytic therapy for acute myocardial infarction. J. Am. Coll. Cardiol. 25: S18-S22.

Hernandez L, Rodriguez P, Castro A, Serrano R, Rodriguez MP, Rubiera R, Estrada MP, Perez A, de la Fuente J, Herrera L (1990). Determination of streptokinase activity by quantitative assay. Biotechnol. Appl. 7: 153-160.

Kim JH, Wong SL, Kim BG (2001). Optimization of staphylokinase production in *Bacillus subtilis* using inducible and constitutive promoters. Biotechnol. Bioprocess Eng. 6: 167-172.

Laemmli UK (1970). Clevage of structure proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lee SJ, Kim C, Kim DM, Bae KH, Byun SM (1998). High level secretion

of recombinant staphylokinase into periplasm of *Escherichia coli*. Biotechnol. Lett. 20: 113-116.
Lee SY (1996). High cell-density culture of *Escherichia coli*. Trends Biotechnol. 14: 98-105.
Lijnen HR, Van Hoef B, Cock FD, Okada K, Ueshima S, Matsuo O, Collen D (1991). On the mechanism of fibrin-specific plasminogen activation by staphylokinase. J. Biol. Chem.2 66: 11826-11832.
Lijnen HR, Van Hoef B, Collen D (1995). Interactions of staphylokinase with human platelets. Thromb. Haemost. 73: 472-477.
Mandi N, Soorapaneni S, Rewanwar S, Kotwal P, Prasad B, Mandal G, Padmanabhan S (2009). High yielding recombinant staphylokinase in bacterial expression system: cloning, expression, purification and activity studies. Protein Exp. Purif. 64: 69-75.
Miele RG, Prorok M, Costa VA, Castellino FJ (1999). Glycosylation of asparagine-28 of recombinant staphylokinase with high-mannose-type oligosaccharides results in a protein with highly attenuated plasminogen activator activity. J. Biol. Chem. 274: 7769-7776.
Moreadith RW, Collen D (2003). Clinical development of PEGylated recombinant staphylokinase (PEG-Sak) for bolus thrombolytic treatment of patients with acute myocardial infarction. Adv. Drug Deliv. Rev. 55: 1337-1345.
Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10: 1-6.
Quyen DT, Nguyen SLT, Dao TT (2007). A novel esterase from *Ralstonia* sp. M1: gene cloning, sequencing, high-level expression and characterization. Protein Exp. Purif. 51: 133-140.
Ren D, Li D, Yang W, Li Y, Gou X, Liang B, Li B, Wu Q (2008). Novel preparation protocol for the expression and purification of recombinant staphylokinase. Biotechnol. Appl. Biochem. 51: 9-13.
Schlott B, Gührs KH, Hartmann M, Röcker A, Collen D (1997). Staphylokinase requires NH2-terminal proteolysis for plasminogen activation. J. Biol. Chem. 272: 6067-6072.
Schlott B, Hartmann M, Gührs K, Birch-Hirschfeid E, Pohl HD, Vanderschueren S, Van de Werf F, Michoel A, Collen D, Behnke D (1994a). High yield production and purification of recombinant staphylokinase for thrombolytic therapy. Biotechnology, (N.Y.), 12: 185-189.
Schlott B, Hartmann M, Gührs KH, Birch-Hirschfeld E, Gase A, Vettermann S, Collen D, Lijnen HR (1994b). Functional properties of recombinant staphylokinase variants obtained by site-specific mutagenesis of methionine-26. Biochim. Biophys. Acta 1204: 235-242.
Sinnaeve P, Van de Werf F (2001). Thrombolytic therapy. State of the art. Thromb. Res. 103 S71-S79.
Vanderschueren S, Stockx L, Wilms G, Lacroix H, Verhaeghe R, Vermyle J, Collen D (1995). Thrombolytic therapy of peripheral arterial occlusion with recombinant staphylokinase. Circulation, 92: 2050-2057.
Vesterberg K, Vesterberg O (1972). Studies on staphylokinase. J. Med. Microbiol. 5: 441-450.
Yarzábal A, Serrano RL, Puig J (1999). Modulation of staphylokinase-dependent plasminogen activation by mono- and divalent ions. Brazil. J. Med. Biol. Res. 32: 39-43.
Ye RQ, Kim JH, Kim BG, Szarka S, Sihota E, Wong SL (1999). High-level secretory production of intact, biologically active staphylokinase from *Bacillus subtilis*. Biotechnol. Bioeng. 62: 87-96.