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Role of Lys59 and Lys386 of Streptokinase from Type 12 Group A *Streptococcus* on *In vitro* Stability against Plasmin Cleavage

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Streptokinase (SK), is a plasminogen (PG) activator by forming a complex with PG that activates other PG molecule to form plasmin (Pm). In SK from group C streptococci (SKC), the generated Pm immediately inactivates it by cleaving at two major sites, Lys59 and Lys386. The present work is aimed to study whether both residues that are conserved in streptokinase of group A streptococci (SKA), were primary sites for Pm cleavage. Site-directed mutagenesis was performed to change both Lys residues to Gln to obtain a mutant SKA (SKA2). The stability of both SKAs was investigated by Western blot analysis in the presence of PG. The radial caseinolysis and chromogenic assays were performed to evaluate whether the substitutions affected the activity and the kinetic parameters. Our results demonstrated for the first time that the SKA2 was more stable than SKA to Pm cleavage at both Lys residues. The substitutions did not affect the binding affinity to PG, but increased the activity, the catalytic rate, and the catalytic efficiency for PG activation to some extent. This study shows that the processing of Lys59 and Lys386 by Pm is an important event in SKA instability against Pm.

Key words : Streptokinase, Group A streptoccci, Lys59, Lys386, Pm cleavage.

Streptokinase (SK) is a extracellular protein secreted by group A, C, and G streptococci[1]. This protein is a single-chain polypeptide of 414 amino acids with a molecular weight of 47 kDa[2]. SK from group A streptococci (SKA) is a virulence factor that has an important role in systemic infection of group A streptococci (GAS). SKA facilitates the spread of GAS cells in human body by converting plasminogen (PG) to plasmin (Pm) that degrades fibrin matrix, a vital human component[3].

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The activated complex of equimolar of SK and PG binds substrate PG which is then proteolytically converted to Pm[4]. The full-length of PG (Glu-PG) forms a complex with SK in a lysine binding site (LBS)-dependent manner[5]. However, as soon as Pm is produced, Pm rapidly inactivates SK by cleaving it to several smaller fragments[6]. Two intermediate products with 42-44 kDa in size first appeared and were generated by C-terminal processing of intact SKC[7]. The 37 kDa degradation product was detected later, and this product was relatively stable. The presence of Ser and Lys residues at both ends of the 37 kDa fragment demonstrated that this product was generated by processing both amino- and carboxyl-terminals of SKC. Although this 37 kDa fragment retained it PG binding activity, it possessed only 16% of full activity of SKC in PG activation and was later proteolytically cleaved into smaller fragments[8].

Earlier work in SKC showed that two major processing sites were Lys59 and Lys386 residues. Lys59 and Lys386 subsitutions by Gln generated SKC variant that was more resistant to Pm cleavage in vitro without affecting its activity for PG activation. Processing SKC by Pm at the two sites significantly inactivated SKC activity for PG activation. Lys59 and Lys386 subsitutions by Gln generated a SKC variant that was more resistant to Pm cleavage in vitro. By performing the substitutions, Pm-resistant form of SKC could be engineered without affecting its activity for PG activation[6].

Group A streptococci (GAS) also produces streptokinase (SKA) which functions as an important factor in disease dissemination. There is 85% sequence identity at amino acid level between SKA and SKC. Non-identical residues are found in two major regions, spanning residues 174 to 244 and 270 to 290 which are located in domain of SKA[9]. By accessing NCBI and EMBL data bases, it was revealed that Lys59 and Lys386 which earlier demonstrated to be major Pm cleavage sites in SKC are conserved in many SKA and SKC molecules. This study was aimed to determine the role of Lys59 and Lys386 on Pm cleavage in SKA. Site-directed mutagenesis was performed to substitute Lys59 and Lys386 with Gln residues in SKA. The degradation profile and kinetic parameters of mutant SKA was studied in the presence of PG and compared with those of wild type SKA. The result of this study provides the first evidence of in vitro instability of SKA cleavage by Pm.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture media

Streptococcus pyogenes type M12 strain CS24 was obtained from Prof. Dr. Patrick Cleary of the Department of Microbiology, University of Minnesota, USA. *E. coli* strains JM109 (Promega, Wisconsin, USA) for cloning, Top10 (Invitrogen, California, USA) for transformation of site directed mutagenesis products, BL21(DE3) (Invitrogen) for protein expression are maintained at the laboratory of Pharmaceutical Biotechnology, the School of Pharmacy, Institute of Technology Bandung. The plasmids employed for cloning and overexpression were pGEM-T (Promega, Wisconsin, USA) and pET32b (Novagen, Madison, USA), respectively. The *S. pyogenes* was grown in Todd Hewitt Yeast broth or agar and incubated at 37°C. The *E. coli* strains were grown at 37 C in Luria Bertani (LB) medium containing 100 µg ml^{-1} of ampicillin and the protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-aldrich, Stenheim, Germany) at a final concentration of 0.5 mM10.

Gene cloning

To obtain ska open reading frame (ORF), *S. pyogenes* cells were grown from glycerol stock on Todd Hewitt Yeast agar and incubated at 37°C for 18 h. For isolation of chromosomal DNA, the *S. pyogenes* was grown in Todd Hewitt Yeast broth at 37°C for 18 h. The chromosomal DNA isolation was performed as described in QIAamp DNA Mini Qiacube kit protocol (Qiagen, Hilden, Germany). The ska ORF was amplified from chromosomal DNA as a template using Polymerase Chain Reaction (PCR). A pair of primer was designed based on the nucleotide sequences of ska gene from a type of M12 *S. pyogenes* strain MGAS2096 (EMBL accession number ABF36759), using DNASTAR program (DNASTAR, Inc., Wisconsin, USA). The nucleotide sequence of primers is presented in Table 1. The PCR was done as follows: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, elongation by Taq DNA polymerase at 72°C for 1 min, and final elongation at 72°C for 10 min. The PCR product was analyzed by 1% agarose gel electrophoresis and cloned into pGEM-T. Several recombinant pGEM-Ts isolated from ampicillin resistant white *E. coli* JM109 transformants were characterized by migration, enzyme digestion, and nucleotide sequencing analyses. After confirmation, the DNA insert was subcloned into pET32b and was transformed into *E. coli* Top10. The recombinant pET32b isolated from E. coli Top10 was subjected to migration, double digestion and nucleotide sequencing analyses. All sequencing was performed using the T7 promoter and T7 terminator, and ska specific primers (Table 1) to confirm the presence of intended mutations and the absence of any other unwanted mutations. One recombinant plasmid was chosen for further study, designated as (pETska) and transformed into *E. coli* BL21(DE3).

Site directed mutagenesis

Previous work showed that two major targets of Pm cleavage in SKC molecule were Lys59 and Lys3866. To study whether the two lysines are also the major sites of Pm cleavage in SKA molecule, we altered Lys59 (K59) and Lys386 (K386) with Gln (Q59 and Q386) using site directed mutagenesis. Two pairs of primer were designed for the mutagenesis (Table 1). FskaK59Q and RskaK59Q were used in the first mutagenesis (AAA59 CAA) and FskaK386Q and RskaK386Q were used in the second mutagenesis (AAA386 CAA) to obtain pETskaK59Q-K386Q (pETska2). The composition of mutagenesis reaction was 210 ng of DNA template, 2.5 µl of 40 µM of each primer, 2 µL of 10 mM dNTP, 3.75 U of Pfu DNA polymerase (Stratagene, California, USA), and 5 µL of 10 x buffer *Pfu* DNA polymerase in a final volume of 50 µL. The condition of mutagenesis reaction was predenaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extention step at 72°C for 2.5 min and additional extention step was done at 72°C for 10 min. The site-directed mutagenesis product was digested with the *Dpn*I enzyme and the digested product was transformed into *E. coli* Top10. Plasmids were isolated from ampicillin resistant transformants and DNA inserts was sequenced as mentioned above. The plasmid harboring the intended mutation and containing no other mutations was isolated and transformed to *E. coli* BL21(DE3). After an extensive plasmid characterization, one transformant was chosen for protein overproduction and designated as *E. coli* (pETska2).

Protein overproduction and purification

Two recombinant cells, *E. coli* (pETska) to obtain wild type SKA and *E. coli* (pETska2) to obtain mutant SKA (SKA2) were used in the present study. Both proteins were overproduced in *E. coli* as fusion proteins with thioredoxin 6xHis-tag at its amino terminus. Both *E. coli* cultures were grown in an Erlenmeyer flask containing selective LB broth medium at 37°C, on a rotary shaker at an agitation speed of 200 rpm. The protein overproduction was induced by an addition of IPTG to a final concentration of 0.5 mM at mid-log phase. The cells were harvested after three h of incubation at 37°C by cold centrifugation at 3400 x g for 20 min and resuspended in lysis buffer of pH 8.0 (50 mM NaH2PO4, 300 mM NaCl) containing 1 mM of phenylmethyl sulphonyl fluoride. Cell disruption was done by sonication on ice at 25% amplitude for 2 min with 5 seconds burst with 5 seconds interval. Intracellular total proteins were obtained by cold centrifugation at 3400 x g for 2 x 15 min. The target protein was subjected to purification on a Protino Ni-TED packed column according to the manufacturer's protocol (Protino, Macherey-Nagel, Duren, Germany). The SKA proteins were recovered from the column with an elution using 3 mL of 250 mM imidazole (pH 8). The overproduction and protein purification were monitored by a 10% Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) at 125 V, 400 mA for 1 h. The quantification of protein was done using the program ImageJ v.1.43u, using a known amount of bovine serum albumin (BSA) (Sigma-aldrich, Steinheim, Germany) as a reference13.

Protein confirmation

To confirm the protein identity, a coomassie blue stained protein band predicted to be SKA was submitted for peptide sequencing by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF/TOF) mass spectrometer using a 4800 Proteomics Analyzer (Applied Biosystems) by vendor (Proteomics International Pty Ltd, East Perth, Australia). Protein samples were trypsin digested and peptides were extracted according to standard techniques[11]. Spectra were analysed to identify protein of interest using Mascot sequence matching software (Matrix Science).

Protein stability assay

To determine the stability of SKA and SKA2 to Pm cleavage, 50 µmol SKA or SKA2 was each mixed with 50 µmol Glu-PG (Sigma-aldrich, St. Louis, USA) and incubated at 28°C. The samples were withdrawn at 0, 10, 20, and 30 min, and added to tubes containing 5x sample buffer for 10% SDS PAGE analysis. Western blot analysis was performed as previously described[12]. SKA-specific polyclonal antibody used in the Western blot analysis was previously generated in our laboratory by injecting the purified SKA into a rabbit strain Japanese white. The rabbit polyclonal antibody specific to SKA (1 : 2500) was used as the primary antibody and alkaline phosphatase conjugated goat anti-rabbit IgG as the secondary

(Sigma-aldrich, Steinheim, Germany) (1 : 10.000) was used as the secondary antibody. The intensity of intact form of SKA and SKA2 protein and their cleavage products on the Western blot membrane were quantified using the ImageJ v.1.43u software[13].

Proteins activity assay and kinetic assay

To determine the effect of Lys to Gln substitutions on the activity for PG activation, SKA/SKA2 was each mixed with Glu-PG and subjected to colorimetric and radial caseinolysis assays. In the colorimetric assay, 7.5 µl of 10 µM PG was added to reaction mixture of 2.5 µl of 10 µM SKA/SKA2 and 7.5 µl of 4 M S225. The generation of Pm was measured at λ415 nm, at 28°C, and using S-2251™ (Instrumentation Lab. Co, Milano, Italy) as a substrate[14,15]. The caseinolysis method was done according to a previous publication with slight modification. The 12.5, 25, and 50 µmol of SKA/SKA2 was each added into disc paper on caseinolysis medium containing 1% of agarose, 30% of pooled human plasma from the Indonesian Red Cross, and 2% of skim milk. The plates were incubated at 28°C for 12 h[16]. Kinetic constants of PG activation were each calculated from Lineweaver-Burk plots[8]. In this assay, 5 µl of 500 nM SKA/SKA2 was each mixed with 1-10 µl of 1.0-10 µM PG and the generation of Pm was measured as described above.

RESULTS

Gene cloning and site directed mutagenesis

The ska ORF was amplified by PCR using chromosomal DNA of S. pyogenes CS24 as the template. The PCR product was 1254 base pairs (bp) in size (data not shown) and after the purification from agarose gel was cloned into pGEM-T vector in *E. coli* JM109. Recombinant plasmids were isolated from several randomly selected ampicillin resistant white transformants and analyzed. The nucleotide sequence analysis of the DNA insert showed that the recombinant pGEM-T contained a ska ORF flanked by BamHI and XhoI sites at both sides (data not shown). The nucleotide sequences of ska ORF obtained from this work showed 99% homology to those of a ska ORF and the deduced amino acid sequence displayed 100% identity to SKA from a type M12 *S. pyogenes* (EMBL, accession number: ABF36759). The insert DNA from a recombinant pGEM-T (pGEMTska) was subcloned into pET32b and the plasmid recombinant was transformed into *E. coli* Top10. After an extensive characterization using migration and restriction analyses, and the confirmation of the DNA insert by two-directional nucleotide sequencing, a recombinant plasmid pET32bska was transformed into *E. coli* BL21(DE3). The result of the single digestion analysis of the pET32bska from *E. coli* BL21(DE3) displayed the presence of one DNA band of 7100 bp (Fig 1, lane 2) corresponding with the theoretical size of linear recombinant plasmid (7141 bp). The two DNA bands obtained from double digestion analysis were 5974 bp and 1151 bp in size (Fig. 1, lane 3) and in agreement with the theoretical size of pET32b vector (5899 bp) and *ska* ORF (1242 bp). These data together with the results of nucleotide sequencing (data not shown) confirmed the identity of the insert. Based on these results, a clone of pET32b harboring a ska ORF from *S. pyogenes* CS24 was obtained.

The Lys59 and Lys386 are both conserved in SKA and SKC and have been shown to be two key sites for Pm cleavage in SKC[6]. This present work was aimed to investigate the role of both lysine residues on the stability of SKA against Pm cleavage. Two steps of site directed mutagenesis was done to change AAA to CAA codons i.e. the first was to alter the codon at position 59 and the second to alter the codon at position 386. Several transformants of *E. coli* Top10 from the first mutagenesis were randomly selected and two directional sequencing of the DNA insert revealed that the AAA59 CAA mutation was present in the ska ORF without any other mutation (data not shown). The sequencing analysis of the second mutagenesis result showed that both mutations, AAA59 CAA and AAA386 CAA mutations occurred in *ska*2 ORF (Fig. 2) and there was no other unwanted mutation (data not shown). Therefore, a pETska2 was obtained and confirmed to be correct. The pETska2 was transformed to *E. coli* BL21(DE3) for protein overproduction.

Protein overproduction, purification and confirmation

E. coli BL21(DE3) containing pETska or pETska2 was each cultured in selective LB broth to produce the wild type SKA (SKA) and SKA2. An intense protein band of apparent size of 64.5 kDa was produced both in *E. coli* pETska or

pETska2 after IPTG induction (Fig. 3, lanes 1 and 3). The size was in agreement with the theoretical size of SKA protein fusion (64.4 kDa) consisting of SKA fragment of 47 kDa and amino terminal tag of 17.8 kDa indicating that both SKA and SKA2 were produced by the recombinant E. coli. Without IPTG induction, the 64.4 kDa protein was not produced (data not shown). The SKA and SKA2 proteins were each purified into homogeneity using Nickel column (Fig. 3, lanes 2 and 4). The result of MALDI-TOF/TOF analysis of the purified 64.4 kDa band predicted to be SKA fusion protein was displayed in Table 2. The analysis identified seven tryptic fragments that matched with the amino sequences of SKA of a type M12 *S. pyogenes* (EMBL, accession number: ABF36759) with 35.99 % coverage (Table 2). Therefore, this analysis strongly confirmed the identity of the 64.4 kDa protein to be SKA.

Protein stability assay

To study the stability of SKA and SKA2 against Pm cleavage, a time course experiment was done to an equimolar mixture of purified SKA or SKA2 with PG. The formation of the SKA and SKA2 cleaved products was monitored by Western blot analysis. As pointed out in Fig. 5A, both SKA and SKA2 started to be degraded immediately. SKA was cleaved immediately at 0 min to give a weak band of 46 kDa fragment, which was presumably a cleaved product at Arg401 (lane 5). At 10 min the 37 kDa fragment started to appear as weak band (lane 6). At 20 min the 46 kDa fragment was cleaved to a 44 kDa fragment, a product presumably processed at Lys386 (lane 7). These fragment sizes were in agreement with the theoretical size of fragments when Pm cleaved SKA at Arg401 and at Lys386 at C terminus, and at Lys residue at enterokinase active site of fusion protein at N terminus that theoretically produced two fragments of 45.96 and 44.01 kDa. The 44-46 kDa fragments were almost totally degraded at 30 min to give the smaller product (lane 8). At 20 min, the 37 kDa fragment appeared as a dominant product, the fragment produced by Pm cleavage at Lys59 and Lys386 and was degraded almost 50% after 30 min (lanes 7-8).

The first degradation product of SKA2 at 0 min was a 46 kDa fragment that appeared as a weak band (Fig. 5A, lane 1). At 10 min, this fragment was presumably cleaved at Lys386 to produce a 44 kDa fragment (lane 2). Both proteins became dominant product at 20 min, even at 30 min (lane 3-4). The 37 kDa protein appeared immediately at 0 min as a faint band, and at 30 min it was almost totally degraded (lanes 1-4). Fig. 5B showed the percentage of intact SKA and SKA2 and their

Table 1. Nucleotide sequences of oligonucleotide used in ORF ska assembly, site-directed mutagenesis, and sequencing

Primers	Sequence (5'→3')
Primers for amplification of ska ORF[a]	
Fska	GGATCCATTGCTGGGCCTGAAT
Rska	CTCGAGTTATTTGTCTTTAGGGT
Primers for sequencing of *ska* ORF	
900_up	GTCAACACCAACACCTTGCTAAA
900_low	GCAATTTGTTGGTGTTGACATCA
Primers for mutagenesis[b]	
$Fska_{K59Q}$	GGCTTAAGTCCACAATCAAAA
$Rska_{K59Q}$	GGTTTTGATTGTGGAGTTAAGCCTT
$Fska_{K386Q}$	TAGCTTATGATCAAGATCGTTATAC
$Rska_{K386Q}$	GTATAACGATCTTTGATCATAAGC
Primers for sequencing of mutant *ska*	
450_up ska	CCTGTCCAAGAATTTTTGCTAAG
450_low ska	CGGCTTTAGGTTGATACGGTTT
T7 Promoter pET	AAATTAATACGACTCACTATAGGG
T7 Terminator pET	GCTAGTTATTGCTCAGCGG

[a]The bold letters of Fska and Rska primers are BamHI and XhoI recognition sites, respectively;
[b]The underlined letters of primers for mutagenesis were the codon encoding Gln

cleaved products during 30 min of incubation.

Activity and kinetic constants of SKA and SKA2 for PG activation

The changes of Lys59 and Lys386 to be Gln altered the profiles of cleavage product of SKA and SKA2. It was important to investigate the effect of the amino acid substitutions on SKA/ SKA2 activity for PG activation. The generated Pm was measured at different time intervals in the presence of chromogenic substrate. As shown in Fig. 6, the amount of Pm produced by SKA and SKA2 at early phase was the same and slowly increased up to 8 min. Afterwards, during the period of 8 - 42 min, the amount of Pm generated by SKA2 was higher (3.5-62%) than that by SKA. At 38 min, the amount of Pm produced by both SKAs was almost similar. To study the effects of Lys to Gln subsitutions to binding affinity and catalytic efficiency of SKA and SKA2, kinetic parameters of both proteins for PG activation were determined. Based on the results depicted in Table 3, the Km values of SKA and SKA2 was comparable, indicating that these amino acid changes did not affect the binding affinity of PG. Meanwhile, although the Kcat and catalytic efficiency values of SKA2 were 1.23-fold and 1.55-fold higher, respectively than those of SKA, but not statistically different.

Measurement of activity of SKA or SKA2 by radial caseinolysis assay

Radial caseinolysis assay was done to examine whether Lys Gln amino acid changes at positions 59 and 386 affected the function of both proteins as PG activator. In this assay, SKA or SKA2 was applied in equal quantity (confirmed by SDS PAGE) to disc paper on an agarose gel containing skim milk and human plasma. Compared to SKA, SKA2 showed a better total activity as a PG activator (Fig. 7A and 7B). SKA2 produced higher clear zone diameter (23-38%) than SKA did. This result reflected that the amino acid alterations significantly ($p<0.05$) increased the total activity of SKA2 in activating human PG.

DISCUSSION

SKs are produced by many group A and C streptococci. However, the most studied SK regarding the Pm cleavage is from group C streptococci. A previous study demonstrated the importance of two lysines located at the amino (position 59) and at the carboxyl (386) termini of

Table 2. Results of MALDI TOF/TOF analysis of purified 64.4 kDa SKA fusion protein

Sequence	Positions	Mr (observed)	% by Mr	Mr (expected)	Mr (calculated)	% by AA's
KFFEIDLTSRPAHGGKT	36-52	1674.8555	3.56	1673.8482	1673.8526	4.11
KVYFADRDDSVTLPTQP VQEFLLSGHVRV	115-143	3089.5771	6.57	3088.5698	3088.5618	7.00
RPYQPKA	144-150	887.5144	1.89	886.5071	886.5025	1.69
RVNVNYEVSFVSETGN LDFTPSLKERY	157-183	2844.3933	6.05	2843.3860	2843.3978	6.52
RDSSIVTHDNDIFRT	219-233	1518.7018	3.23	1517.6945	1517.7110	3.62
KYVDVDTNELLKSE QLLTASERN	298-320	2423.2073	5.16	2422.2000	2422.2227	5.56
KLLYNNLDAFGIMDYT LTGKVEDNHNDTNRI	334-364	3356.6267	7.14	3355.6194	3355.5799	7.49
Total	149	15794.8761	33.61	15787.8250	15787.8283	35.99

Table 3. Kinetic parameters for the plasminogen activation by SKA and SKA2

Streptokinase	Km (μM)	Kcat ((μM.min^{-1})	Kcat/Km (min^{-1}.μM^{-1})
SKA	0.36 ± 0.05	15.79 ± 2.16	44.50
SKA2	0.29 ± 0.05	19.40 ± 1.19	68.98

SKC as major cleavage sites for Pm attack. The Lys59 and Lys386 located in α and β domains respectively are conserved in all SKAs of S. pyogenes. The study on the role of these lysines as primary target for Pm degradation in SKA has not been previously investigated. The results of site-directed mutagenesis and protein stability experiment using Western blot analysis showed for the first time that two lysine residues positioned at 59 and 386 are important for SKA stability against Pm attack and the alteration of Lys Gln residues at both positions render the mutant SKA more resistant to Pm cleavage at both lysines. In vitro activity assays revealed that the SKA2 was catalytically active with slight increase in its ability to activate PG and kinetic results demonstrated that it retained its Km but increased its Kcat and Efficiency values.

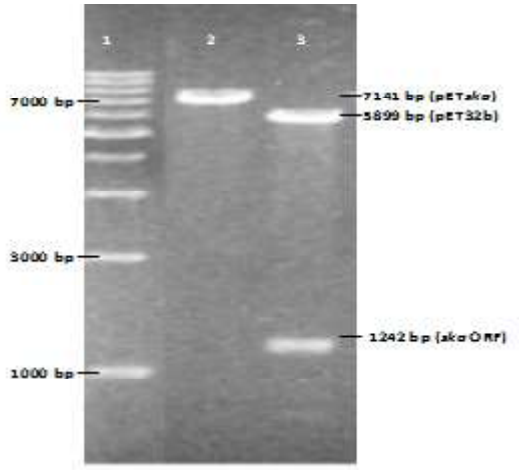


Fig. 1. Characterization of pETska using *Bam*HI dan *Xho*I enzyme digestion. Lane 1: 1 kb DNA ladder, lane 2: pETska (7141 bp) digested with *Bam*HI, lane 3 : pETska digested with *Bam*HI and *Xho*I to give pET32b (5899 bp) and *ska* ORF (1242 bp) bands.

In the present work, we cloned a ska ORF and to study the role of two major Pm cleavage sites in SKA protein, codons 59 and 386 of the ska ORF were altered by site-directed mutagenesis to substitute Lys to Gln at both positions. Both SKA and mutant SKA (SKA2) were each overproduced in *E. coli* and affinity purified as a fusion protein with thioredoxin and histidine tags at its amino terminus. Both of them were shown to be biologically active in chromogenic assays and radial caseinolysis. A previous study showed that SKC exhibited rapid Pm-mediated degradation to

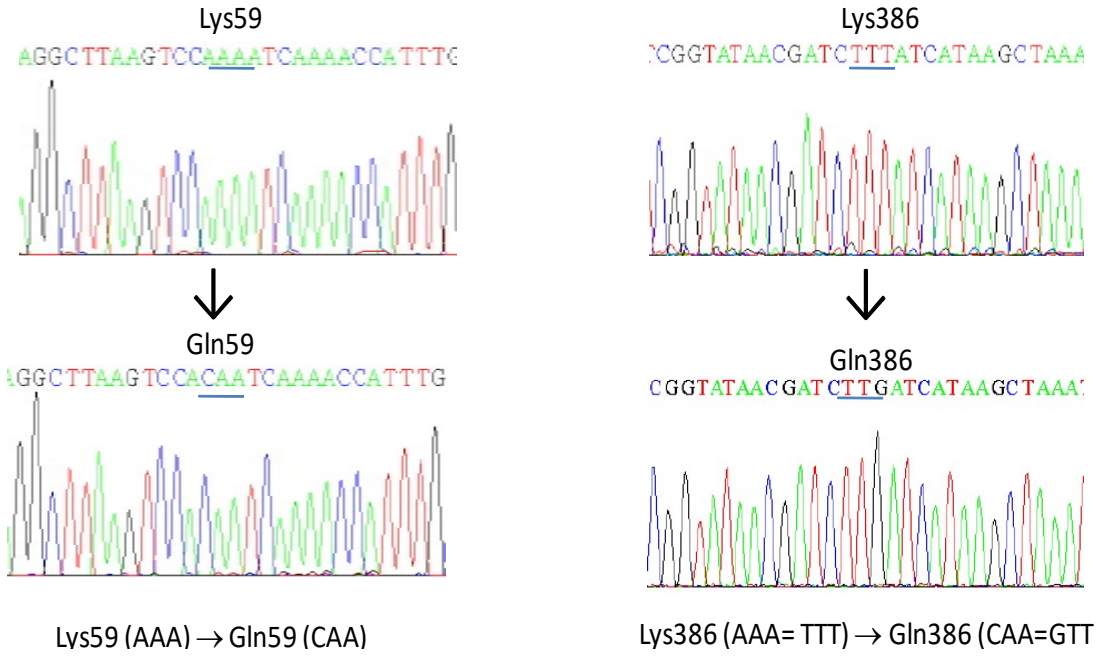


Fig. 2. Nucleotide sequences of mutated codons in ska2 ORF. Codons for Lys59 (AAA) and Lys 386 (TTT, complementary sequence) in ska ORF was changed to Gln59 (CAA) and Gln386 (GTT, complementary sequence) in ska2 ORF.

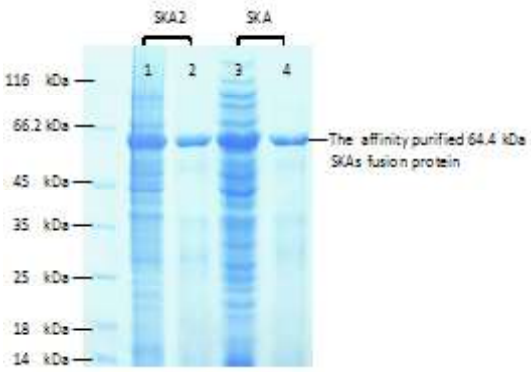
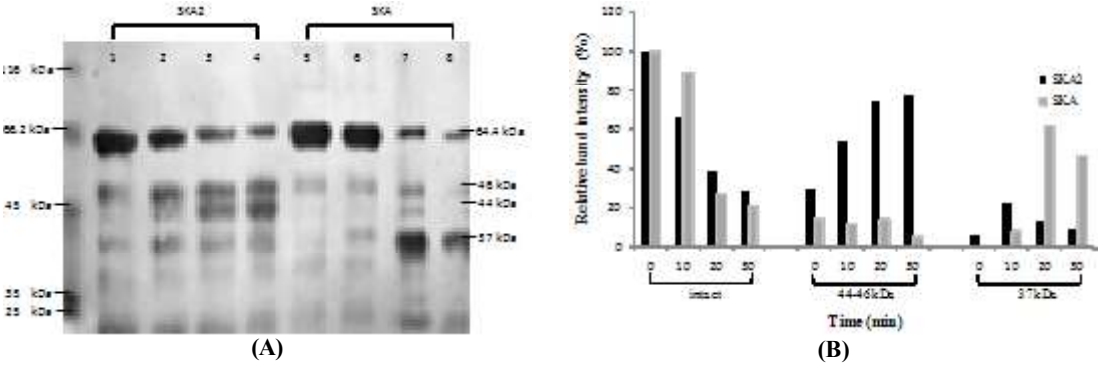


Fig. 3. Overproduction and purification of SKA and SKA2 fusion proteins. Lanes 1 and 3: total intracellular proteins of E. coli (pETska) and E. coli (pETska2) cells induced by IPTG, respectively, lanes 2 and 4: affinity-purified of 64.4 kDa SKA and SKA2 fusion proteins, respectively

form various smaller proteolytic fragments[6]. The 44 and 46 kDa fragments were produced by processing at Lys386 and Arg401, respectively and were shown to be stable until 60 min of reaction time. The 37 kDa intermediate was found as a dominant product after 10 min and was formed by Pm cleavage at Lys59 and Lys3866. A SKC fusion protein with maltose-binding protein (MBP) at its terminus displayed different degradation characteristics with its natural SKC. The cleavage rate of SKC fusion version was slower than that of natural SKC, showing that MBP protected SKC from Pm cleavage[17]. The 37 kDa fragment produced by SKC protein fusion was found to be dominant after 5 min. In this study, we showed that our SKA was more stable than SKC protein fusion against Pm cleavage at Lys59 and Lys386. This is based on the appearance of the 37 kDa fragment which took longer (20 min) in SKA than in SKC fusion protein (5 min). This phenomenon was more likely due to different tags used in previous and current

IAGPEWLLDRPSVNNSQLVVSVAGTVEGTNQEISLKFFEIDLTSRPAHGGKTEQGLSPKSKPFATNSSAM
PHKLEKADLLKAIQEQLIANVHSNDGYFEVIDFASDATITDRNGKVYFADRDDSVTLPTQPVQEFLLSG
HVRVRPYQPKAVHNSAERVNVNYEVSFVSETGNLDFTPSLKERYHLTTLAVGDSLSSQELAAIAQFILS
KEHPDYIITKRDSSIVTHDNDIFRTILPMDQEFTYHIKDREQAYKANSKTGIVEKTNNTDLISEKYYVLK
KGEEPYDPFDRSHLKLFTIKYVDVDTNELLKSEQLLTASERNLDFRDLYDPRDKAKLLYNNLDAFGIM
DYTLTGKVEDNHNDTNRIITVYMGKRPEGENASYHLAYDKDRYTEEERELVYSYLRYTGTPIPDNPKDK

Fig. 4. Amino acid sequences of SKA as determined by MALDI-TOF/TOF.
The underlined letters are residues identified in the analysis.



(A) **(B)**

Fig. 5. The stability of SKA and SKA2 to Pm cleavage. Intact SKA and SKA2 and their cleavage products were monitored by Western blot analysis using SKA-specific polyclonal antibody (A). Lanes 1 & 5 = 0 min, lanes 2 & 6 = 10 min, lanes 3 & 7 = 20 min, and lanes 4 & 8 = 30 min. Relative band intensity (%) of intact SKA and SKA2 (B).

studies and not due to different amino acid sequences located around Lys59 and Lys386 which are highly conserved in both SKC and SKA.

Similar to previous results in natural SKC, Lys59 to Gln and Lys59 to Gln substitutions rendered SKA more resistant to Pm at both major Pm cleavage sites demonstrated by the appearance of low level of 37 kDa fragment in mutant SKA (SKA2). In SKA, the 37 kDa fragment became dominant cleavage fragments, at 20 and 30 min. However, in SKA2, at the same reaction time, the 37 kDa fragment was present in small amount. The 44-46 kDa proteolytic fragments turned out to be the main products in SKA2 at 20 and 30 min, whereas in SKA, the fragments were present in small amount. Our data provide strong evidence that Lys 59 and 386 are major sites for Pm cleavage in SKA and probably in all SKs.

To study the effect of Pm cleavage to SK activity, amidolytic and radial caseinolysis assays were used to monitor the amount of Pm generated from PG activation. In both assays, SKA2 was higher in its PG activation than SKA. The increase of activity was probably due to the presence of stable and catalytically active 44-46 kDa fragments during the reaction and the small amount of low activity 37 kDa fragment in SKA2. Prior work on the SKC revealed that the 37 kDa product comprising residues 60-386 containing partial α and β domains and complete β domain retained only 16% of the full activity in SKC. However, due to the high variability in amino acid sequences of domain which is responsible for PG activation, the activity of the 37 kDa fragment of SKA is unknown and awaits to be determined in future study. In SKC, a region comprising residues 332-386 that includes Lys386 demonstrated to be responsible for mediating tight binding of SK to PG[8,18,19,20,21]. Another work in SKC revealed that a loop spanning 45-70 residues that includes Lys59 was demonstrated to be crucial for substrate PG recognition[22]. The alteration of Lys to Gln performed in this study might also affect above mentioned activities and consequently increase the SKA2 activity. The evidence showing that SKA2 was catalytically active and this demonstrated that there was no major conformational changes caused by the amino acid substitutions.

The kinetic data showed the Km values of both SKA and SKA2 were comparable indicating the amino acid substitutions did not change the affinity of activator complex to substrate PG. This is expected to occur since both Lys59 and Lys386 are located in α and β domains, respectively, and

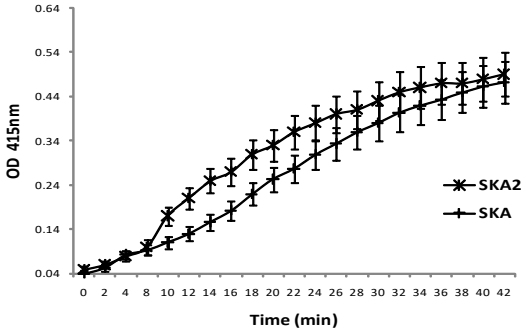
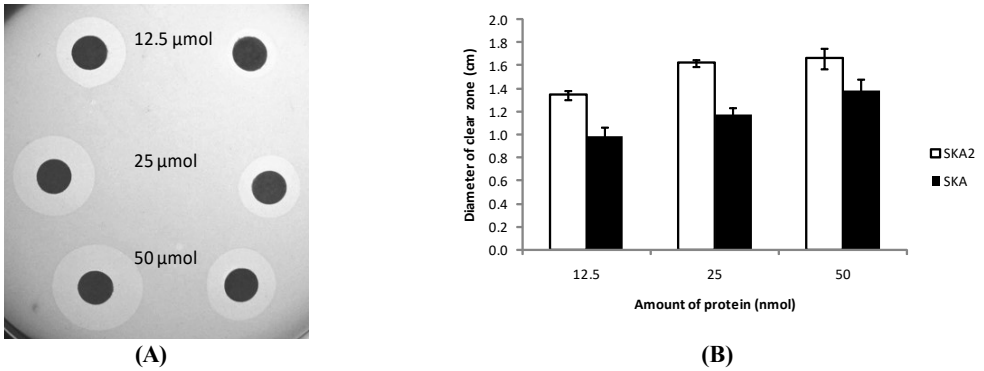


Fig. 6. PG activation by SKA and SKA2 using colorimetric assay. OD at 415 nm represented the amount of Pm produced during the PG activation.



(A) **(B)**

Fig. 7. PG activation by SKA and SKA2 as determined by the radial caseinolysis assay (A) and quantification of diameter of clear zone (B).

therefore they do not participate in PG binding or docking which occurs in domain[19]. It also means that there was no altered mode of substrate PG binding to β domain of SKA2. Although the Km values remained relatively the same after amino substitutions, the catalytic turnover rates of SKA2 slightly increased, demonstrated by the higher values of Kcat and Kcat/Km (Efficiency). This indicates that the Lys substitutions at positions 59 and 386 increased the ability of SKA for substrate processivity in activator complex and for PG activation. The phenomenon can be explained by the dominant appearance of the 37 kDa fragment in SKA but not in SKA2, where it was previously shown that the fragment retained PG binding activity but exhibited barely PG activation activity[8].

In a study conducted earlier, it showed that SKC protein fusion displayed three-fold longer lag phase for PG activation than its natural version[17]. In the present work, both SKA fusion proteins exhibited a similar fashion with about 150 sec lengths for their lag phase. The presence of tag portion in SKA and SKA2 proteins probably sterically hinders the interaction with PG or Pm in the catalytic complex formation that would explain the longer lag phase. It was shown previously that a SKC fused to MBP had decreased Km and therefore, lowered its affinity of an activator complex to substrate PG[17].

In conclusion, in the current work we present evidence for the first time that Lys59 and Lys386 are definitely two main sites for Pm recognition in SK from *S. pyogenes*. The substitution of Lys to Gln residues at positions 59 and 386 in SK from S. pyogenes renderes SK more stable against Pm cleavage. The changes do not affect its PG affinity but slightly increases its catalytic activity in activator complex formation, substrate PG processivity and its rate for PG activation.

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