2. Materials and Methods

2.1 Materials used in the present study

2.1.1 Bacterial Strains

All the bacterial strains used in this study are listed in Table 2.1. *Escherichia coli* were maintained on LB agar plates, with 30 µg/ml kanamycin or 100 µg/ml ampicillin. For short-term storage of weeks to a month, the strains were maintained on their respective agar plates, supplemented with appropriate antibiotics whenever required, at 4°C. For long-term storage of months to years, the cultures were maintained in 15% glycerol at -70°C.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Source</th>
</tr>
</thead>
</table>
| *E. coli* JM109 | Rec A1, hsd R17 Δ(lac-pro AB)  
[F tra D36 pro AB lacIqZ ÄM15] | Promega |
| *E. coli* BL21(DE3) | F', ompT, rB-mB-prophage carrying T7 RNA polymerase. Has lacUV5 promoter inducible by IPTG | Novagen |

Table 2.1: Host strains used in the present study.

2.1.2 Plasmids

The following vectors and pre-constructs were used in this study:

**pBluescript KS⁺ (pBS KS⁺):** A pUC19 derived phagemid (2.9 kb) carrying ColE1 origin of replication, Amp', an extensive polylinker cloning site and a β-galactosidase with first 14 amino acids deleted. In JM series of *E. coli* strains, α-complementation of the deleted amino acids takes place resulting in a functional β-galactosidase. Thus, in the presence of IPTG (inducer) and X-gal (substrate), cells containing pBS KS⁺ show up as blue colonies. Recombinant colonies can be easily screened on the plate as white colonies due to insertional inactivation; obtained from Stratagene.

**pET9b:** This is a low copy number expression vector (Studier *et al.*, 1990), which is derivative of pBR322 and carries Kan' and a strong T7 promoter which is inducible by IPTG; obtained from Novagen, USA.
2.1.3 Oligonucleotides

Oligonucleotides were obtained from commercial sources e.g. BioBasic Inc., Canada, Sigma-Aldrich, or Integrated DNA Technologies Inc., USA.

2.1.3.1 List of primers used for site directed mutagenesis of SAKPro42-Lys50 region

Substitutional mutagenesis, to introduce changes at various residues from Pro42 to Lys50 of SAK, was carried out using the sets of oligonucleotide primers mentioned in the table 2.1. The primers 1-28 were designed as the internal primers having the desired mutation (no restriction site) indicated by the primer designation, while 29 and 30 were used as terminal primers (NdeI and BamHI restriction sites respectively) for creating these mutants through overlap PCR or site directed mutagenesis kit (Startagene).

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Primer sequence</th>
<th>Primer Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5'-TCCCCTAAATATGTCGAG-3'</td>
<td>His43Lys-NT</td>
</tr>
<tr>
<td>2.</td>
<td>5'-GACATATTTAGGGGATAG-3'</td>
<td>His43Lys-CT</td>
</tr>
<tr>
<td>3.</td>
<td>5'-TCCCCTGAATATGTCGAG-3'</td>
<td>His43Glu-NT</td>
</tr>
<tr>
<td>4.</td>
<td>5'-GACATATTCAGGGGATAG-3'</td>
<td>His43Glu-CT</td>
</tr>
<tr>
<td>5.</td>
<td>5'-TCCCTTTTTATGTCGAG-3'</td>
<td>His43Phe-NT</td>
</tr>
<tr>
<td>6.</td>
<td>5'-GACATAAAAAGGGGATAG-3'</td>
<td>His43Phe-CT</td>
</tr>
<tr>
<td>7.</td>
<td>5'-CTATCCCCTCATTTTGTGAGTTTCC-3'</td>
<td>Tyr44Phe-NT</td>
</tr>
<tr>
<td>8.</td>
<td>5'-TAGGAAACTCGACAAAATGAGGGGA-3'</td>
<td>Tyr44Phe-CT</td>
</tr>
<tr>
<td>9.</td>
<td>5'-CTATCCCCTCATGCAGTGCTGAGTTTCC-3'</td>
<td>Tyr44Ala-NT</td>
</tr>
<tr>
<td>10.</td>
<td>5'-CTCGACTGATGAGGGGA-3'</td>
<td>Tyr44Ala-CT</td>
</tr>
<tr>
<td>11.</td>
<td>5'-GAATTTGCTATCCCTACATTATGTCG-3'</td>
<td>Pro42Leu-NT</td>
</tr>
<tr>
<td>12.</td>
<td>5'-CTCGACATAATGTAAGGATAG-3'</td>
<td>Pro42Leu-CT</td>
</tr>
<tr>
<td>13.</td>
<td>5'-CTATCCCCTCATTATTATGAATTTTCT-3'</td>
<td>Val45Tyr-NT</td>
</tr>
<tr>
<td>14.</td>
<td>5'-AGGAAACTCTAATAATGAGGGGA-3'</td>
<td>Val45Tyr-CT</td>
</tr>
<tr>
<td>15.</td>
<td>5'-CTATCCCCTCATTATGAGCGAATTTTCT-3'</td>
<td>Val45Ala-NT</td>
</tr>
<tr>
<td>16.</td>
<td>5'-AGGAAACTCTGACATAATGAGGGGA-3'</td>
<td>Val45Ala-CT</td>
</tr>
<tr>
<td>17.</td>
<td>5'-CTATCCCCTCATTTTTTTGATTTTCT-3'</td>
<td>Tyr44Phe, Val45Phe-NT</td>
</tr>
<tr>
<td>18.</td>
<td>5'-AGGAAACTCACAAAAATGAGGGGA-3'</td>
<td>Tyr44Phe, Val45Phe-CT</td>
</tr>
<tr>
<td>19.</td>
<td>5'-TTCATTATGTCGCGTTTCTATTAAAC-3'</td>
<td>Glu46Ala-NT</td>
</tr>
<tr>
<td>20.</td>
<td>5'-AATTAGGAAACGCGACATAATGAGG-3'</td>
<td>Glu46Ala-CT</td>
</tr>
</tbody>
</table>
21. 5’-ATTATGTCGAGTATCCTATTAAAC-3’ Phe47Tyr-NT
22. 5’-CCAGGTTTAATAGATACTCGACAT-3’ Phe47Tyr-CT
23. 5’-CATTATGTCGAGTTTGGATTTAAACC-3’ Pro48Gly-NT
24. 5’-GTAGTCCAGGTTAATACCAAACTCGA-3’ Pro48Gly-CT
25. 5’-GTCGAGTTTCCTGTAAACCTGG-3’ Ile49Val-NT
26. 5’-GTGTAAGTCCAGGTTAAACAGGAAC-3’ Ile49Val-CT
27. 5’-TGTCGAGTTTCATTGACCTCGGACTA-3’ Lys50Ala-NT
28. 5’-AAGTGATGTCGAGGTCAATAGGAAC-3’ Lys50Ala-CT
29. 5’-ATACATATGTCAAGTTCCATTGCAACAAGA-3’ SAKNT
30. 5’-TCATAGGATCCTTATTTCTTTATACA-3’ SAKCT

Table 2.1 List of Primers

2.1.3.2 List of primers used for SAK cysteine mutants and SAKβ chimeras
31. 5’-ATAGGATCCTTACGACACGCTTCTCTCTCTACAAC-3’-SAK1CysCT. C-terminus primer for adding one cysteine residue at C-terminus of SAK with BamHI site.
32. 5’-GATACATATGGCGTGTGCTCAAGTTCTCCATTGCAAAAG-3’-SAK1CysNT N-terminus primer for adding one cysteine residue at N-terminus of SAK with Ndel site.
33. 5’-ATAGGATCCTTAAACACATTTCTCTTCTATAAC-3’-SAK2CysCT C-terminus primer for adding two cysteine residues at C-terminus of SAK with BamHI site.
34. 5’-GATACATATGGCGTGTGCTCAAGTTCCATTGCAACAAGA-3’-SAK2CysNT N-terminus primer for adding two cysteine residues at N-terminus of SAK with Ndel site.
35. 5’-TCATACATATGTATAAAGAAAAACCAATA-3’-SKBNTNde N-terminus primer for SK β domain with Ndel site.
36. 5’-ATAGGATCAGCTTTTATGACTGCGATCAAGGG-GSKC MCT C-terminus primer for SK β domain with HindIII site.
37. 5’-GATACATATGGCGTGTGCTCAAGTTCTCCATTGCAACAAGGAAAA-3’ N-terminus primer for SAK domain with NcoI site
38. 5’-TGAGGCGCGGCTTTTCTTTCTATAACAA-3’ SAKCTR GD-1
39. 5’-TCGCTCTCCCGCTTACTGAGGCAGGACTTTT-3’ SAKCTR GD-2
41. 5'-TCCTGAGTCTCCTCCGTCTCCGCGTCCTG-3' SAKCTRGD-3
42. 5'-TCCGTCTCCGCGTCCTGAGTCTCCTCCGT-3' SAKCTRGD-4
43. 5'-GGCTCGTCCTCCGTCTCCGCGTCCT-3' SAKCTRGD-5
The primers from 39-43 were used to add RGD linker to the C-terminus of SAK for making chimeras.

2.1.4 Reagents
All reagents used in this study were of analytical grade. Various fine chemicals were procured from the sources listed below:

- Antibiotics – Amersham Biosciences, Roche, Sigma Chemical Co., MP Biomedicals USA.
- Pfu turbo DNA polymerase, DpnI – Stratagene
- Urea, Acrylamide, Bis-Acrylamide, APS, TEMED, Ethidium Bromide, DTT – USB.
- Tryptone, Yeast Extract, Agar – HiMedia, India.
- IPTG, Lysozyme, Protein molecular weight markers, X-gal, Imidazole, BSA and Bromo Phenol Blue – Sigma Chemical Co.
- Sequencing kit and Miniprep kit – ABI PRISM, Amersham Biosciences.
- Glassware, Plasticware, Microfuge tubes, Tips etc. – Tarson, India.

2.1.5 Media used
- LB medium (Luria Bertani medium)
  Tryptone 10 g
  Yeast extract 5 g
  NaCl 10 g
Materials and Methods

The contents were dissolved in 900 ml of deionized water and pH was adjusted to 7.4 with 10 N NaOH. Final volume was made to 1 L with deionized water. Sterilized by autoclaving for 15 min at 15 lb/in² in liquid cycle.

**LB agar**

15 g/L agar was added to the LB medium and sterilized by autoclaving for 15 min at 15 lb/in² in liquid cycle. Plates were stored at 4 °C for a period of 15-20 days.

**LB agar with antibiotics**

The autoclaved LB medium was allowed to cool to 45 °C before adding the antibiotics.

**X-gal indicator plates**

X-gal indicator plates were made (Sambrook et al., 1989) to screen the recombinant clones by blue-white screening. 40μl of X-gal (20 mg/ml in dimethyl formamide) and 70 μl of IPTG (0.1 M in water, filter sterilized) were spread on agar plates before plating the cells.

2.1.6 Preparation of antibiotics

**Ampicillin**

A 100 mg/ml stock solution of the sodium salt of ampicillin (USB) in water was made and filter sterilized using 0.22 μm Millipore filter. Aliquots were stored at -20 °C. A working solution of 100 μg/ml was used for E. coli.

**Kanamycin**

A 30 mg/ml stock solution of the kanamycin (USB) was prepared and filter sterilized using 0.22 μm Millipore filter. Aliquots were stored at -20 °C. The working solution of 30 μg/ml was used for E. coli.

2.1.7 Preparation of organic reagents

**Phenol**

Tris-saturated phenol (pH 7.5-8.0) was obtained from Bangalore Genei, India.

**Phenol: Chloroform**

A mixture consisting of equal parts of equilibrated phenol and chloroform was used throughout the study to remove proteins from preparation of nucleic acids. Equal
volume of phenol and chloroform was stored in 100 mM Tris.HCl, pH 8.0 in a light-tight bottle.

### 2.1.8 Preparation of commonly used stock solutions

#### 30% Acrylamide

- Acrylamide: 29.2 g
- N,N'-methylene-bis-acrylamide: 0.8 g

The above ingredients were first dissolved in 60 ml of double distilled water by stirring on a magnetic stirrer. The final volume was then adjusted to 100 ml with double distilled water. The solution was kept in a brown container and stored at room temperature.

#### 100 mM ATP

60 mg ATP (Sigma) was dissolved in 0.8 ml of autoclaved water. The pH was then adjusted to 7.0 with 0.1 N NaOH. The volume was finally adjusted to 1 ml with autoclaved water and stored at –20 °C.

#### 1 M CaCl₂

21.90 g of CaCl₂.6H₂O (Sigma) was dissolved in 100 ml water and filter sterilized using 0.22 µm Millipore filter.

#### 0.5 M EDTA

18.61 g of ethylenediamine-tetra-acetic acid disodium salt (Qualigens, India) was added to 80 ml of double distilled water and pH was raised to 8.0 using NaOH pellets. EDTA usually gets dissolved at pH 8.0. The volume was adjusted to 100 ml, sterilized by autoclaving and kept at room temperature.

#### 10 mg/ml Ethidium bromide

1 g of ethidium bromide (Sigma) was added to 100 ml distilled water and dissolved properly by stirring for 2 hr. The solution was stored in a dark bottle at room temperature.

#### IPTG (Isopropyl-thio-β-D-galactoside)

0.2 g of IPTG was dissolved in 0.8 ml of double distilled water. Final volume was adjusted to 1 ml and filter sterilized using 0.22 µm Millipore filter. The solution was stored at –20 °C.

#### X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

20 mg/ml stock solution of X-gal was prepared in dimethyl formamide. Stock was stored in a microcentrifuge tube wrapped in aluminum foil at –20 °C.
Materials and Methods

+ **3 M Sodium acetate**

40.81 g of sodium acetate was dissolved in 80 ml of distilled water. The pH was either adjusted to 5.2 with glacial acetic acid or adjusted to 7.0 using dilute acetic acid. Final volume was adjusted to 100 ml with distilled water and autoclaved.

+ **10% SDS**

10 g of SDS (Sigma) was dissolved in 90 ml of distilled water. It was dissolved by heating at 65 °C. Final volume was adjusted to 100 ml and stored at room temperature.

+ **1 M Tris**

121.1 g of Tris-base (Sigma) was dissolved in 80 ml of distilled water and pH was adjusted to 8.0 by adding 4.2 ml of concentrated HCl. Final volume was made to 1 L by using distilled water. Sterilized by autoclaving and stored at room temperature.

+ **10% Glycerol**

100 ml of glycerol was mixed with 900 ml of water and sterilized by autoclaving.

+ **50% Glycerol**

50 ml of glycerol was mixed with 50 ml of water and sterilized by autoclaving.

+ **1 M Potassium phosphate buffer, pH 7.0**

615 ml of 1 M K$_2$HPO$_4$ was combined with 385 ml of 1 M KH$_2$PO$_4$. Solution was sterilized by autoclaving.

+ **TE (pH 8.0)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris.HCl (pH 8.0)</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

2.1.9 Buffers for plasmid DNA preparation

+ **Solution I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>50 mM</td>
</tr>
<tr>
<td>Tris.HCl (pH 8.0)</td>
<td>25 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>RNase</td>
<td>100 µg/ml</td>
</tr>
</tbody>
</table>

Each component (except RNase) was autoclaved separately and the final solution was reconstituted in an autoclaved container using autoclaved double distilled water. The solution was stored at 4 °C.
Materials and Methods

**Solution II**

- NaOH: 0.2 N
- SDS: 1.0%

Freshly prepared and kept at room temperature.

**Solution III**

- 5 M Potassium acetate (autoclaved): 60.0 ml
- Glacial acetic acid: 11.5 ml
- Distilled water (autoclaved): 28.5 ml

Stored at 4 °C.

**2.1.10 Electrophoresis buffers**

**50X TAE buffer**

- Tris.HCl: 242 g
- Glacial acetic acid: 57.1 ml
- 0.5M EDTA (pH 8.0): 100 ml

Volume was made to 1 L with double distilled water.

**6X DNA Gel loading buffer**

- Bromophenol blue: 0.25%
- Glycerol: 30.0%

**5X Protein sample buffer**

- Tris.HCl (pH 6.8): 125 mM
- SDS: 4%
- Glycerol: 20%
- β-mercaptoethanol: 10%
- Bromophenol blue: 0.006%

**Laemmli buffer (Polyacrylamide gel electrophoresis)**

- Tris.HCl: 3 g
- Glycine: 14.4 g
- SDS: 1 g

The volume was adjusted to 1 L with deionized water.

**Lower Tris (pH 8.8)**

- Tris.HCl: 18.17 g
Volume made to 100 ml with deionized water after adjusting pH 8.8 with 6 N HCl and the solution was then autoclaved.

**Upper Tris (pH 6.8)**

Tris.HCl 6.06 g

Volume made to 100 ml with deionized water after adjusting pH to 6.8 with 6 N HCl and the solution was then autoclaved.

**Gel staining solution**

- Acetic acid 10%
- Methanol 40%
- Coomassie brilliant blue R250 0.25%

Made in deionized water.

**Gel destaining solution**

- Acetic acid 10%
- Methanol 40%

Made in deionized water

### 2.1.11 Solutions for Western blotting

**Transfer buffer**

- Glycine 11.25 g
- Tris.HCl 2.34 g

Dissolved in 600 ml of deionized water. pH was adjusted to 8.3 and volume was made up to 800 ml. 200 ml of methanol was then added.

**10X Phosphate buffered saline (10X PBS, pH 7.4)**

- NaCl 80 g
- KCl 2 g
- Na₂HPO₄ 14.4 g
- KH₂PO₄ 2.4 g

Volume was made to 1 L with deionized water after adjusting the pH to 7.4.

**1X PBST**

0.1% Tween-20 in 1X PBS.

**Blocking agent**

5% Skimmed milk in 1X PBST.
2.2 Methods

2.2.1 Isolation of plasmid DNA

The rapid alkaline lysis method of plasmid isolation described by Sambrook et al. (Sambrook, 1989) was followed. For small-scale preparation of plasmid DNA, 1.5 ml of overnight culture was pelleted and suspended in 0.1 ml of ice-cold Solution I by vigorous vortexing. The cells were lysed by the addition of 0.2 ml of freshly prepared Solution II. The contents of the tube were mixed gently and stored at room temperature till the lysis was complete. After lysis, 0.15 ml of ice-cold Solution III was added; contents were mixed by inverting the tube gently. The tube was kept on ice for 10 min after which it was centrifuged at 10,000 rpm for 10 min at RT. The supernatant was transferred to a fresh tube and extracted with equal volume of chloroform:isoamyl alcohol (24:1). The plasmid DNA was precipitated by adding 2 volumes of ice-cold ethanol and the tube was kept on ice for 15 min. The plasmid was recovered by centrifugation at 12,000 rpm for 15 min at RT and washed with 70% ethanol. The pellet was dried and dissolved in appropriate volume of TE (pH 8.0). The DNA was stored at -20 °C until used.

For large-scale plasmid DNA preparation, plasmid miniprep method was scaled up accordingly, except that after suspending the cell pellet in Solution I, the cells were treated with lysozyme (10 mg/ml) for 15 min at 37 °C.

2.2.2 DNA purification and analysis

The plasmid DNA was purified by equilibrium centrifugation in a CsCl-Ethidium Bromide gradient. For every ml of DNA solution, exactly 1 g of solid CsCl was added and dissolved by gentle mixing. To this DNA/CsCl solution, 0.8 ml of ethidium bromide (10 mg/ml) solution was added for every 10 ml. The solution was centrifuged at 10,000 rpm for 5 min at room temperature. The clear red solution was carefully transferred to ultracentrifugation tubes. The remaining space was filled with paraffin oil and sealed properly. The tubes were centrifuged at 45,000 rpm for 48 hr in Ti50 rotor at 22 °C. Lower band constituting circular form of plasmid DNA was taken out using 21-gauge needle and extracted with isoamyl alcohol to remove ethidium bromide (Sambrook, 1989). The clean solution of DNA was dialysed against TE (pH 8.0) for at least 12 hr and quantitated spectrophotometrically at 260 nm and 280 nm. The DNA solution was stored at 4 °C or -20 °C.
2.2.3 Polymerase chain reaction (PCR)

This technique was utilized to carry out site-directed mutagenesis of SAK. Appropriate oligonucleotides were used as listed in materials. PCR was performed according to the method developed by Mullis (Mullis and Faloona, 1987). For normal reaction, 20 pmol of primers and nearly 50 ng of template DNA (genomic or plasmid), 500 μM of each dNTP, 1X Thermopol buffer (NEB, UK) and two units of Deep Vent Polymerase (NEB, UK) were added. The final volume of the reaction mix was made to 100 μl with autoclaved double distilled water and the contents were mixed gently. The PCR cycle consisted of: Denaturation at 95 °C for 1 min, annealing at 45 °C to 50 °C for 1 min depending on Tm of the primer used, followed by extension at 72 °C for 1 min or more depending on the size of the DNA fragment being amplified. These reaction conditions were repeated for 30 cycles followed by a final extension at 72 °C for 10 min. Amplification was verified by agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis

DNA fragments were fractionated on 0.8% (w/v) agarose gels as a routine. 1% - 2% gels were run for analyzing very small fragments (<500bp). 6X gel loading buffer was added to DNA samples at a final concentration of 1X prior to loading onto the gel. Electrophoresis was carried out in 1X TAE buffer at 10 V/cm for the resolution of restricted plasmid DNA. Ethidium bromide (0.5 mg/ml) was supplemented in the agarose gel for visualizing DNA on a UV transilluminator (Spectroline, USA).

2.2.5 Restriction endonuclease digestion

The restriction enzyme digestion of DNA samples (4-6 μg) was carried out according to the manufacturer’s instructions (NEB, UK). After incubation at appropriate temperature, the reaction mixture was heated at 75 °C for 10 min to inactivate the enzyme and was then immediately quenched on ice. The digested DNA was mixed with DNA gel loading buffer and fractionated on agarose slab gel electrophoresis. Lambda DNA fragments generated by HindIII digestion, 100bp DNA ladder and 1kb DNA ladder were used as molecular size markers for calculating the size of unknown DNA fragments from their relative mobility.
2.2.6 Purification of DNA fragment from agarose gel

After electrophoresis, DNA was visualized using a UV transilluminator, the fragment(s) of interest was located and cut out from gel and purified using GFX PCR DNA and Gel Band Purification Kit from Amersham Biosciences, UK. The purified DNA was used for further applications.

2.2.7 Ligation

The vector and insert DNA were mixed in an 1:3 ratio in a total volume of 20 µl at a final concentration of approximately 250 µg/ml DNA. Blunt end ligation was carried out at 18 °C by adding 2 µl of 10X T4 DNA ligase buffer, 1 µl of (1-2 Weiss units) of T4 DNA ligase (NEB, UK). In case of sticky ends, 2 µl of 10 mM ATP was also included and the reaction was incubated at 16 °C for 16-18 hr.

2.2.8 DNA sequencing

Sequencing grade DNA was prepared using ABI PRISM miniprep kit. Briefly, the cell pellet was treated with alkaline lysis solutions. The contents were centrifuged and the supernatant obtained, was mixed with binding buffer. The entire solution was poured onto a miniprep column, the resin of which preferentially binds supercoiled plasmid DNA. The resin was washed with wash buffer and the DNA was finally eluted in TE (pH 8.0). Both commercially available as well as custom synthesized primers (3.2 pmol) were used in PCR reactions. 200-500 ng of template DNA was taken for sequencing PCR in a total volume of 10 µl. The following PCR steps were used:

1. 96 °C, 5 min
2. 96 °C, 30 sec
3. 50 °C, 15 sec
4. 60 °C, 4 min
5. Go to step 2, 23 cycles
6. 4 °C hold

The PCR samples were further processed for sequencing. DNA was precipitated by adding 2 µl of 125 mM EDTA, pH 8.0 and 50 µl of absolute ethanol. The contents were mixed and incubated at RT for 15 min. The tubes were centrifuged at 13,000 rpm for 20 min and the pellet was washed twice with 70% ethanol and air-dried. Just prior to submission of samples for sequencing, the DNA pellet was resuspended in 12 µl of HiDi foriformamide and heated at 95 °C for 3 min. The samples were immediately chilled on ice.
Sequencing was carried out on an automated sequencer (ABI PRISM 377 DNA Sequencer, Perkin Elmer Applied Biosystems).

2.2.9 Preparation of competent cells and transformation in *E. coli*

Single colony of *E. coli* was inoculated in 10 ml of LB or superbroth medium and grown to saturation. The culture was re-inoculated in fresh medium at a dilution of 1:100 and grown to mid log phase (OD$_{600\text{nm}}$ of 0.3-0.5) at 37 °C with shaking. The cells were harvested by centrifugation at 5,000 rpm at 4 °C and processed further for the preparation of competent cells and transformation by any of the following methods.

2.2.9.1 Calcium chloride method

CaCl$_2$ competent *E. coli* cells were prepared by the method of Cohen *et al.* (Cohen *et al.*, 1972). The harvested cells were resuspended in 0.1 volume of ice-cold 0.1 M CaCl$_2$ and incubated on ice for 30 min. Following centrifugation, the pellet was resuspended in 0.02 volumes of the buffer containing 0.1 M CaCl$_2$ and 15% glycerol. The aliquots of 200 μl were made on ice and stored at −70 °C for later use. For transformation, ligation mix (10 μl containing ~100 ng of DNA) or plasmid DNA (100 ng) was added to an aliquot and incubated on ice for 30 min. Following heat shock treatment at 42 °C for 90 sec, the cells were kept on ice for 5 min. The transformed cells were incubated at 37 °C for 1 hr after the addition of 800 μl of LB broth to allow the expression of antibiotic marker. Cells were then plated on LB agar plates supplemented with appropriate antibiotic. For blue-white screening, 40 μl of X-gal (20 mg/ml) and 70 μl of IPTG (0.1 M) were spread on the plates prior to plating the cells.

2.2.9.2 Electrotransformation

Electrocompetent *E. coli* cells were prepared according to the method of Dower *et al.* (Dower *et al.*, 1988) whenever high frequency of transformation was required. The cells were first washed with equal volume and then with half the volume of ice-cold 1 mM HEPES buffer (pH 7.0). The cells were then washed with 0.04 volumes of ice-cold 10% glycerol. Finally, these were suspended in 0.002 volume of ice-cold 10% glycerol and 50 μl aliquots were made on ice that were stored in −70 °C for later use. For electroporation, ligation mix (1 μl containing ~20 ng DNA) or 50 ng of plasmid DNA was added to an aliquot and put in 0.2 cm cuvettes (Bio Rad, USA). Care was taken to
avoid formation of air bubbles. The cuvette was placed in the cuvette holder and electric pulse was given using a Bio-Rad Gene Pulser at a setting of 2.5 kV, 25 μF and 100Ω. Immediately, 1 ml LB broth was added to the transformed cells and properly mixed. The contents of the cuvette were transferred to fresh tube and incubated at 37 °C for 1 hr. Cells (50-100 μl) were plated on LB agar plates supplemented with appropriate antibiotics. X-gal and IPTG were included at this step whenever blue-white selection was required.

2.2.10 Intracellular production of SAK derivatives and their recovery from recombinant E. coli

*E. coli* cells transformed with recombinant plasmid carrying SAK/SAK mutant gene were streaked on a Luria Bertani (LB) agar plate containing 50 μg/ml kanamycin and grown at 37°C for overnight. Individual colonies appearing on the plate were used to raise seed culture in a 10 ml liquid LB medium containing 50 μg/ml growth medium supplemented with 50 μg/ml kanamycin and grown at 37°C for 8-10 hr. This primary seed culture (1% v/v) was used to inoculate 1 liter of LB medium allowed to grow at 37 °C till its OD₆₀₀ nm reaches to 0.4 -0.5. The culture was then induced for SAK production by adding IPTG and further grown at 37 °C for another 6-8 hr. Cells were then harvested by centrifugation at 6000 rpm and the cell pellet was resuspended in 10 ml of Tris.Cl buffer. The cells were lysed by sonication and the cell lysate was ultracentrifuged at 40000 rpm. The clear lysates were diluted fourfold with 10 mM tris buffer and loaded on the column of SP-sepharose (Sigma), equilibrated with 10 mM tris buffer (pH 6.2). The column was washed with 10 mM tris buffer, pH 6.2 and eluted with step gradient of 0.1 to 0.5 M NaCl. Each fraction was analyzed on 15% SDS-PAGE to examine the relative purity of the eluted protein.

2.2.11 Purification of SAK chimeras through DEAE sepharose chromatography

*E. coli* cells carrying recombinant plasmids for expression of respective target genes were induced with 0.1 mM isopropyl β-D-thiogalactoside during the mid-exponential phase of growth (OD₆₀₀ = 0.6). Cells were harvested after 7-10 hr of induction by centrifugation at 6000 rpm for 10 min at 4°C and resuspended in 20 mM Tris.HCl, pH 7.5. Cells were lysed by sonication and then subjected to ultracentrifugation (45,000 rpm, 4 °C, 2 hr). The clear supernatant, thus obtained, was
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loaded on ion-exchange column (DEAE-Sephrose CL-6B, Sigma), pre-equilibrated with 20 mM Tris.HCl, pH 7.5 and eluted using 30-50 mM NaCl. It resulted in about 95% pure preparation of protein. SAK protein preparation was loaded on to Superdex G-75 column (Pharmacia, 30 cm x 10 cm) and eluted in 10 mM Tris.HCl, pH 7.5 at a flow rate of 0.3 ml/min.

2.2.12 Protein estimation

The protein concentration was estimated according to the BCA (Bicinchoninic acid) method (Smith et al., 1985) using BSA as the standard. Solution A and B were mixed in 50:1 ratio and 200 µl of it was added in the wells of microtitre plate. To each well, 25 µl of test sample was added and kept at 37 °C for 30 min. The absorbance was monitored at 562 nm in Biotek Powerwave X elisa plate reader.

2.2.13 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out essentially according to Laemmli (Laemmli, 1970). Briefly, protein samples were prepared by mixing with 5X sample buffer at a final concentration of 1X. The samples were boiled for 10 min in water bath and centrifuged at 13,000 rpm for 15 min prior to loading onto the gel. The discontinuous gel system was usually used with 10-15% resolving gel (depending on the size of the protein) in 0.375 M Tris.HCl, pH 8.8 and a 4% stacking gel in 0.125 M Tris.HCl, pH 6.8. Electrophoresis was carried out in Laemmli buffer at a constant current of 20 mA till samples entered the resolving gel and then at 30 mA till the completion of gel run. Protein molecular weight markers were also run concurrently on the gels till completion.

2.2.14 Coomassie Blue staining

On completion of electrophoresis, the gel was immersed in 0.25% Coomassie Brilliant Blue R250 (Sigma) in methanol:water:acetic acid (4:5:1) with gentle shaking and was then destained in destaining solution, methanol: water: acetic acid (4:5:1) till the background was clear.

2.2.15 Silver Staining

In this method, after electrophoresis, the gel was fixed for 20 min in a mixture of methanol: acetic acid: water (4:1:5) and then washed in distilled water for 10 min. The gel was washed in washing solution (Methanol: water, 8:1) for 10 min. Then, the gel
was immersed in sensitivity solution (Sodium thiosulphate = 20 mg in 100 ml water) for 10 min. The gel was stained with prechilled staining solution (silver nitrate = 0.2 g, formaldehyde = 76 μl in 100 ml water) for 10 min and then developed by developing solution (Sodium thiosulphate = 0.4 mg, sodium carbonate = 6 g, formaldehyde = 50 μl). The developing was stopped by adding acetic acid (10 ml in 90 ml water) solution.

2.2.16 Antibody production

Antigen was purified from polyacrylamide gels for inducing good antibody response. After electrophoresis of total cell lysate from the recombinant strain or partially purified protein, the protein band of interest was located by staining with 0.25% Coomassie Brilliant Blue R250. The gel with the protein band was excised and cut into small pieces with the help of surgical blade and was then transferred to a homogenizer. The gel pieces were crushed by adding 1 ml of 1X PBS. The whole suspension was then transferred to a microcentrifuge tube and an equal volume of Freund's Complete Adjuvant (Sigma) was added to it. A fine emulsion was made using a syringe. For raising the polyclonal antibodies, rabbits were immunized according to Harlow and Lane (Harlow and Lane, 1988) using the above-mentioned emulsion. The emulsion was injected subcutaneously at multiple sites on the rabbit's lateral side. The first booster injection was given with the same amount of antigen mixed with Freund's Incomplete Adjuvant (Sigma) at 3 weeks interval. The second and third boosters were given with a relatively smaller amount of antigen mixed with Freund’s Incomplete Adjuvant (Sigma) at 2 and one week interval, respectively. After a week of last injection, blood samples were collected by puncturing the ear vein and the titer and specificity of the antibodies produced was determined by western blotting.

2.2.17 Western Blotting and Immunoassay

Western blotting of the cell lysate of E coli cells expressing recombinant proteins was carried out as described by Towbin et al. (Towbin et al., 1979). The cell lysate in 6X sample buffer was resolved on SDS-PAGE gel. The dimensions of the gel were measured and two sheets of Whatman paper and one sheet of nitrocellulose membrane of dimensions equal to that of the gel were cut. The gel along with the electro-blotting pads, whatman paper and nitrocellulose membrane (USB, UK) was equilibrated with the transfer buffer. In the electro-blotting apparatus (Bio Rad, USA), the gel cassette was arranged on the negative side (black colored) in the following
sequence from bottom to top: gel pads, whattman sheet, gel, nitrocellulose membrane, whattman sheet and finally gel pad. The cassette was closed and blotting was done electrophoretically onto nitrocellulose membrane at 30 mA for 2 hr. The nitrocellulose membrane (blot) was carefully removed from the cassette and put in blocking solution (5% skimmed milk in 1X PBST) for 12-16 hr at 4 °C. The blot was then washed with 1X PBST and incubated with primary antibodies, raised in rabbit against the recombinant protein blotted, at appropriate dilution in 5% skimmed milk in 1X PBST for 3 hr at RT with gentle shaking. The blot was washed thrice with 1X PBST followed by 3 washings with 1X PBS, each for 15 min. The blot was then incubated with HRP-conjugated goat anti-rabbit antibody (Sigma) at a dilution of 1:6000 in 1X PBST at RT with gentle shaking. It was then washed with 1X PBST three times, each for 15 min. The blot was now developed by either of the following two methods.

2.2.17.1 By DAB (Diamino benzidine)

The color reaction for HRP-linked secondary antibody using DAB was carried out by immersing the blot in 10 ml of reaction buffer solution having 10 mg each of DAB (Diamino benzidine, Sigma) and imidazole (Sigma) and 3 µl of H₂O₂. The reaction was terminated by washing with distilled water.

2.2.17.2 By Chemiluminescence

The blot was developed using ECL plus Western Blotting Detection System Kit from Amersham Biosciences, UK according to the manufacturer’s instructions. Briefly, detection reagents A and B, provided in the kit, were mixed in a ratio of 40:1, respectively and poured as a thin film on the blot. The blot was kept at RT for 5 min and the excess detection reagent was drained off by holding the membrane gently in forceps and touching the edge against tissue. The blot was wrapped in a fresh piece of saran wrap and X-ray film was exposed in dark for 30 sec to 1 min depending on the signal strength. The X-ray film was developed by putting in developer for 4-5 min, washing with water and then putting in fixer for 5 min. The X-ray film was washed under running tap water for 15 min before drying.
2.2.18 PG activation assay of SAK

The specific PG-activating activities of SAK and its mutants were determined with a Pg-coupled chromogenic assay (Jackson et al., 1981, Trieu et al., 1993). Briefly, a mixture of Pg (1.5 μM) and SAK or its mutants (final concentration 0.8 μM) was incubated at 37 °C for 5 min in 50 mM Tris.HCl, pH 7.5. The mixture was diluted 10-fold and the change in absorption at 405 nm was recorded after the addition of chromozyme PL as a function of time in a Shimadzu (UV-1601) spectrophotometer at 25 °C. Purified native SAK (obtained from Professor Patrick J. Gaffney) was used as standard to determine Pg activator activity in unknown protein samples.

Single step assay

To check the plasminogen activation properties of SAK mutants, 5 nM SAK or SAK mutants were mixed with 1.5 μM Pg in assay buffer (50 mM Tris.HCl, pH 7.5, containing 0.1% BSA, 100 mM NaCl and 0.01% Tween 80) containing 1mM chromozyme in 96 well microtitre plate and generation of Pm was measured as a function of time at 405 nm at 25 °C in BioTEK Powerwave X 96 wells microtitre plate reader (Jackson et al., 1981, Trieu et al., 1993, Rajamohan and Dikshit, 2000).

Two step assay

To see the Pg activation by preformed complexes of SAK or SAK mutants with Pm, equimolar mixtures of SAK or SAK mutants (0.5 μM) and Pm (0.5 μM) were preincubated in assay buffer at 37 °C for 5 min to generate the SAK-Pm bimolecular complex. These preformed activator complexes (5 nM) were then mixed with substrate Pg (1.5 μM) and generation of Pm was measured spectrophotometrically at 25 °C using Chromozyme PL (1 μM) (Rajamohan and Dikshit, 2000, Rajamohan et al., 2002).

2.2.19 Steady state kinetics of Pg activation by SAK/SAK mutants

To calculate the steady-state kinetic constants, the preformed complex of SAK-Pm (5 nM) were mixed with different concentrations of Pg (0.5-5 μM) and 1.0 mM Chromozyme PL. The change in absorbance at 405 nm was measured as a function of time at 25 °C as described above. The kinetic constants were determined through Michaelis Menten plot and Lineweaver-Burk plot (Shibata et al., 1994, Wohl et al., 1980, Rajamohan et al., 2002).
2.2.20 Amidolysis activity of SAK-Pm bimolecular complex

To see the specificity of the active site generated by SAK/SAK mutants with Pm, an equimolar mixture of Pm (0.5 μM) with SAK/SAK mutant (0.5 μM) in the reaction buffer (50 mM Tris.HCl, pH 7.5, containing 0.1% BSA, 100 mM NaCl) was incubated for 5 min and then 50 nM of the SAK/SAKmutant-Pm complex was added to the reaction buffer containing 1.5 μM Chromozyme in 96 wells microtitre plate. The amidolysis profile of SAK or SAK mutants was measured spectrophotometrically at 405 nm at 22 °C as a function of time in Biotek Power Wave X microtitre plate reader (Arai et al., 1995, Aneja et al., 2009).

2.2.21 Time-course acylation in the bimolecular complex of SAK-Pm

Time-course acylation in the SAK-Pm bimolecular complex was monitored after titration with active site inhibitor of serine proteases i.e. p-nitrophenol-p'-guanidinobenzoate (NPGB) (100 μM) in 10 mM sodium phosphate buffer, pH 7.5 at room temperature (Rajamohan et al., 2002, Aneja et al., 2009). Preincubated mixture of Pg (4 μM) and SAK/SAK mutant (4.5 μM) (for 5 min) was added to an assay cuvette containing 100 μM NPGB in 10mM sodium phosphate buffer and p-nitrophenol burst was monitored spectrophotometrically at 410 nm as a function of time.

2.2.22 Determination of the binding affinity of SAK/SAK mutants for Pg by surface plasmon resonance

The kinetic constants for binding of SAK/SAK mutants with Pg were determined by Surface Plasmon resonance (SPR) (Sakharov et al., 1996) using Sensor Chip SA in a BIACORE 3000 biosensor (Hutsell et al.). Pg was purified from human plasma and biotinylated with Sulpho-NHS-LC-Biotin using minimum biotinylation conditions (Papalia and Myszka). Free biotin was removed from the biotinylated protein by gel filtration through Sephadex G-25 column. Approximately 1500 resonance units of Pg were immobilized on Sensor Chip SA. To calculate the rate and equilibrium binding constants, the different concentrations of SAK/ SAK mutants (1-20 μM or 10-100 μM) were passed over the immobilized Pg. All the experiments were performed in HBS running buffer (20 mM HEPES, 1 mM EDTA, 0.15 M NaCl, and 0.005% surfactant P20, pH 7.2) at 22 °C. The running buffer was also supplemented with 50 μM NPGB i.e. active site inhibitor of serine proteases to prevent any Pg/Pm mediated
proteolysis (Aneja et al., 2009). The inhibition of proteolytic activity of SAK-Pg/Pm complex was also checked by running the preincubated mixtures of SAK/SAK mutants with Pg (for 10 min) in the presence of NPGB on SDS-PAGE. The $k_{on}$ (association rate constant), $k_{off}$ (dissociation rate constant) and $K_D$ (equilibrium binding constant) values were calculated by non linear fitting of the association and dissociation curves using 1:1 Langmuir binding model in BIACORE 3000 evaluation software.

2.2.23 Structure analysis of SAK-µPm complex and molecular modeling of SAK mutants

Interface of SAK-µPm complex was analyzed by retrieving the coordinates of the µPm-SAK-µPm ternary complex (PDB ID: 1BUI) and SK-µPm complex (PDB ID: 1BML) from the Protein Data Bank (Research Collaboratory for Structural Bioinformatics Consortium). Computational analysis of molecular surfaces and electrostatic complementarities of residues present at the interface of activator complex in the ternary structure of µPm-SAK-µPm was carried out using PyMOL (http://www.pymol.org.) (DeLano, 2002). Energy minimization was carried out using Newton software in the Tinker suite of programs (Hodsdon et al., 1996, P. Ren and J. W. Ponder, 2003). The calculations were carried out employing implicit dielectric of 80, vdw-cutoff of 15 Å and force-field parameters of optimized parameters for liquid simulation united-atom (OPLSUA) to simulate continuum solvation model to affect the electrostatic interactions. Coordinates were minimized to an RMSD cut-off of 0.01 kcal/mol/Å. Mutants for different rotameric conformations were generated using SPDB viewer program (Guex and Peitsch, 1997). Total and interaction energies were estimated using Analyze program. Normal mode analysis (NMA) of the ternary complexes was carried out using Elnemo server (http://igs-server.cnrs-rs.fr/elnemo/index.html) which employs elastic network model and computes collective motions of coordinates at lower frequencies. In this work, the DQ was set at ±100 and 20 structures were computed to represent each mode.

2.2.24 Chemical cross linking of cysteine mutants of SAK with PEG (Polyethylene Glycol): PEGylation

The highly purified preparation of cysteine mutants of SAK were used for generating PEGylated derivatives. The cysteine side chain was targeted with maleimide-activated linear PEG (Mal–PEG) molecules with a molecular weight of 5, 10 and 20
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kDa. mPEG carries a maleimide group that reacts specifically with thiol under mild conditions. Derivatization was achieved by reduction with dithiothreitol (DTT) to reduce the disulphide linkages, desalting on Sephadex G-25, and incubating the molecule with a 3-fold molar excess of Mal–PEG in 50 mmol phosphate buffer, pH 7.9, at room temperature. The PEGylation reaction was standardized at different temperatures, different PEG: protein ratio and different time intervals so that we could get minimum PEG to protein ratio and the most of the PEGylated molecules in a single population for its easy purification. The PEGylated protein was purified on Superdex G-75 gel filtration column on AKTA Prime. The purified fractions were run on 10% SDS-PAGE and were stained with coomassie staining to check the purity levels.

2.2.25 Iodine staining of PEGylated proteins

After SDS-PAGE, gel was fixed in 5% glutardehyde solution for 15 min and then immersed in 20 ml of 0.1 M perchloric acid for 15 min. After these washes 5 ml of 5% BaCl₂ solution was added (to gel in perchloric acid directly), followed by 2 ml of 0.1 M iodine solution addition. The reaction was stopped by washing with water.

2.2.26 In vivo plasma half life of SAK derivatives and their PEG conjugated forms

Since native SAK and SAK derivatives were expressed in a bacterial host, E. coli, presence of some endotoxins in the purified protein could be expected. To remove endotoxins from the protein preparation, each SAK derivative as well as native SAK protein was passed twice through a Polymyxin B Agarose column and then used for animal studies. Endotoxin free preparation of SAK derivative as well as native SAK was radiolabelled with I-125. Unincorporated free iodine was removed by passing through Sephadex G-25 column. The protein fractions with high radioactivity counts were then precipitated with TCA and counts of supernatant and pellet were taken to check the percentage incorporation of iodine. The iodinated samples were then dialyzed and again TCA precipitable counts of pellet as well supernatant were taken to rule out the possibility of any free iodine. The radiolabelled preparation of SAK derivatives and native SAK were then used for studying their pharmacological characteristics by injecting into mice to test their in vivo stability. Swiss mice were used for testing various radiolabelled SAK derivatives. Mice were injected with nearly 7–8 μg of iodinated protein via the tail vein. About 50 μl of blood sample was collected immediately after injection and after different time intervals from tail vein. In vivo plasma half life of SAK
derivatives was determined by checking the level of residual level of radioactivity left in the plasma processed from the collected samples at different time points. Withdrawn samples were TCA precipitated after adding equal volume of 20% TCA into each sample and incubating on ice for 20 min. The samples were then centrifuged at 5000 rpm for 10 min and supernatant was removed to separate free radioactivity and precipitate was analyzed for the radioactivity associated with the plasma protein by checking radioactive counts left in the precipitate. Two independent sets of experiments were conducted to conclude the results. The results were further confirmed by running the samples on SDS PAGE and visualization by autoradiography.

2.2.27 Immunogenicity of PEGylated cysteine derivatives of SAK

To check the immunogenicity of various SAK cysteine derivatives, polyclonal antisera raised against SAK in rabbit was used to check the reactivity with various PEGylated SAK derivatives by ELISA method. Titre of antibodies was determined by ELISA according to method described by Hay et al, 1976.

The microtitre plate was incubated at 4 °C overnight with 100 µl/well of SAK and PEGylated cysteine mutants containing 1 µg of protein in coating buffer i.e. 0.2 M bicarbonate buffer pH 9.2. The coated wells were washed with 200 µl PBS-T (PBS, pH 7.5, containing 0.05% v/v Tween 20) three times (10 min each). The unoccupied sites were blocked with 5% skim milk in PBS-T and kept for 2 hr at 37 °C. The wells were washed with 200 µl PBS-T three times. 100 µl of diluted (1:40000 in PBS) primary antibody against SAK was added to each well and incubated at 37 °C for 4 hr. After three washings, 100 µl of HRP conjugated antibody was added in 1:5000 dilutions and kept for 1 hr at 37 °C. The wells were washed three times with PBS. 100 µl of TMB substrate was added to each well and incubated for 20-30 min at 37 °C. Finally 50 µl of H₂SO₄ was added to stop the reaction and the plate was read at 450 nm. The immunoreactivity of SAK and different PEGylated derivatives was evaluated by comparing the absorbance values.
STRUCTURE-FUNCTION STUDIES OF STAPHYLOKINASE

(SAK): INTERMOLECULAR INTERACTIONS OF SAK WITH PLASMIN (OGEN)

Chapter 3 Role of His43 and Tyr44 of Staphylokinase in making bimolecular complex with Plasmin and changing its substrate specificity.

Chapter 4 Role of Pro42 and Val45 of SAK in modulating the specificity switch generated by its His43-Tyr44 pair at SAK-Pm binary activator complex.

Chapter 5 Dual participation of Phe47-Lys50 region of Staphylokinase in partner binding and substrate catalysis during plasminogen activation
Introduction

Staphylokinase (SAK), a 15.5 kDa protein secreted by the bacterium *Staphylococcus aureus* (Sako *et al.*, 1983, Sako and Tsuchida, 1983) has been identified as a potential thrombolytic drug for the treatment of cardiovascular diseases. The abilities of SAK to induce highly fibrin specific thrombolysis in human plasma, achieve early perfusion in myocardial infarction and high efficiency to dissolve platelet rich clot have established its immense potentiality as a clot buster (Collen and Van de Werf, 1993, Collen *et al.*, 1993a, Vanderschueren *et al.*, 1994, Vanderschueren *et al.*, 1995b, Vanderschueren *et al.*, 1995a). Unlike some other Pg activators e.g. tissue-plasminogen activator (t-PA) (Renatus *et al.*, 1997a) and urokinase, SAK does not have any proteolytic activity of its own, very similar to its molecular cousin, streptokinase (SK). Like SK (McCintock and Bell, 1971, Reddy and Markus, 1972), SAK forms 1:1 stoichiometric complex with plasminogen (Pg)/plasmin(Pm) and changes its substrate specificity so that it becomes highly specific for activation loop of Pg. Both of these bacterial Pg activators serve as a cofactor with Pg/Pm that specifically cleaves Arg561-Val562 bond of Pg activation loop to convert it into Pm (Trieu *et al.*, 1993, Collen *et al.*, 1993b, Silence *et al.*, 1993a, Shibata *et al.*, 1994, Lijnen *et al.*, 1994, Okada *et al.*, 1994). However, there are certain key differences between SAK and SK in their mechanism of action for Pg activation. Unlike SAK, SK can form a functional activator complex with Pg by modifying its active site without any proteolytic cleavage in Pg molecule while SAK requires the conversion of SAK-Pg complex to SAK-Pm to form a functional activator complex (Grella and Castellino, 1997). Pm-mediated removal of the first ten N-terminal residues of SAK is required for Pg activation process (Schlott *et al.*, 1997, Rajamohan and Dikshit, 2000). Despite these differences in their mechanism of action, SAK shares a close structural homology with SK (Parry *et al.*, 2000), specifically with its α domain. Structural comparison of SAK-μPm interface with that of SKα-μPm, shows the presence of similar type of intermolecular contacts at the respective interfaces (Rabijns *et al.*, 1997, Parry *et al.*, 1998, Wang *et al.*, 1998) suggesting that these contact residues have been well conserved in the evolutionary process for their crucial role in Pg binding and activation (Parry *et al.*, 2000). Presence of these SAK residues at the interface near the active site of μPm shows their involvement in generating the specificity of the active site of Pm for the efficient cleavage of substrate Pg. However, the complete understanding of the fine adjustment of these interactions at the interface in stabilizing binary enzymatic complex and modulating its specificity is still unclear. It is
therefore important to understand how SAK establishes intermolecular interactions at the interface near the active site of Pm to generate the specificity. Moreover, the region of SAK adjacent to the interface of binary activator complex has been found to be interacting with substrate Pg. Overall, there are two major aspects of SAK mediated Pg activation i.e. formation of SAK-Pm binary activator complex and recruitment of the substrate Pg onto this complex for its catalysis. Whether this region of SAK adjacent to the interface of SAK, has any contribution in the function of SAK-Pm activator complex, is not known. Therefore, molecular mechanism by which individual residues of SAK interact with partner Pm to create binary activator complex and provide docking sites to substrate for its catalysis, will provide a detailed understanding of the cofactor function of SAK in Pg activation.