CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

Fine chemicals were procured from Sigma Chemicals Company, St.Louis, USA and chemicals for media preparation were purchased from Hi-Media, Bombay, India. Restriction enzymes and T4 DNA ligase obtained from New England Biolabs Beverley, USA. Taq DNA polymerase and Hybond nitrocellulose membrane were purchased from Amersham pharmacia biotech, Birmingham, UK. Primers used in this study were synthesized in Microsynth, Switzerland and Bangalore Genei, Bangalore, India.

pRSETB vector, *E.coli* strain BL21 (DE3) and DH5α were purchased from invitrogen Corporation, Sandiego, USA. pET-20b(+) expression vector was purchased from Novegen, Merck Darmstadt, Germany. Large scale purification of plasmids and midi preparation kits were purchased from QIAGEN, Germany. Most of the immunological reagents used in the study were purchased from Pierce, USA or from Sigma, USA.

2.2 CULTURE MEDIA

In all the experiments the *E.coli* culture was grown in the Luria-Bertani (LB) Broth. To prepare LB Broth 10g/L of Tryptone, 10g/L of sodium chloride and 5g/L of yeast extract were dissolved in distilled water and the pH was adjusted to 7.3. And 1.5% agar was added to the liquid broth to prepare solid medium. LBON (LB lacking NaCl) was prepared similarly without
including NaCl). Media was supplemented with 100µg/ml of Ampicillin or 50µg/ml of chloramphenicol wherever required.

2.3 RECOMBINANT CONSTRUCTS USED IN THE PRESENT STUDY

The streptokinase mature sequence was cloned into the pRSETB and pET-20b(+) vectors and the clones were named as pRSETB-SKH (histidine fusion tag) and pETS (no fusion tag) respectively. Similarly the SK gene was cloned in modified pRSETB vector containing the apyrase and Braun’s lipoprotein signal sequences separately resulting in the respective constructs named as LM-SK and LPP-SK. The insertion of (CAT)_3 sequence to the C terminal of LM-SK and LPP-SK named as LM-SKH and LPP-SKH respectively. The cloning of SK in the pRSETB vector was performed and the construct pRSETB-SK thus obtained is used as control for the lipid modification of SK.

The ribosome binding site (RBS) is a sequence on mRNA, which is an important sequence for translation initiation. The influence of RBS in the expression of therapeutic protein SK has been studied by making certain modifications in the RBS of the pRSETB vector such as replacement, partial deletion and complete deletion resulting in the clones SD-REP, SD-parDEL and SD-comDEL respectively. The above constructs were further used for the expression studies.

2.4 PLASMID DNA EXTRACTION

Plasmid DNA was isolated from E.coli by the method of Birnboim and Dolly (1979).
a) A single colony from a plate was picked up and inoculated in 100ml LB medium containing 100µg/ml ampicillin. The cells were grown overnight (O/N) in a shaker at 37°C.

b) The cells were pelleted at 7000 rpm at 4°C for 10 min.

c) The medium was decanted and residual medium was removed by aspiration and pellet was resuspended in 6ml of freshly prepared ice-cold (50mM Tris and 10mM EDTA, 50mM glucose pH 8.0) buffer, incubated in ice for 10 min.

d) Freshly prepared alkaline SDS (12.0ml) was added and mixed by gentle inversion and incubated in ice for 10 min.

e) 7.5ml of 3M (sodium acetate) pH 4.6 was added and mixed by gentle inversion, incubated in ice water for 20 min.

f) The mixture was centrifuged for 15 min at 12000 rpm and the supernatant was transferred to another tube.

g) DNase free RNase A to a final concentration of 20µg/ml was added to the supernatant and incubated at 37°C for 20 min.

h) One volume of buffered phenol: chloroform was added to the supernatant and extracted twice (centrifuged at 12000 rpm for 5 min).

i) After centrifuging at 12000 rpm for 5 min, the upper aqueous phase was transferred to a fresh tube and two volumes of absolute ethanol were added. The mixture was incubated at 20°C for 30 min and centrifuged at 12000 rpm for 20 min.

j) The supernatant was removed and the pellet was washed with 5ml of 70% ethanol (centrifuged at 12000 rpm for 5 min).

k) The pellet was dried under vacuum and the DNA was dissolved in water.
2.5 RESTRICTION DIGESTION AND LIGATION OF DNA

Restriction digestions were performed using Nde I, Eco RI, Pst I, Bam HI and Xba I enzymes from New England Biolabs, MA, USA in the manufacture’s recommended buffer for respective constructs. Restriction digestions were carried out as follows.

- DNA (3-4 µg) 2 µl
- Buffer (10X) 2 µl
- Enzyme (2-3 units/µg of DNA) 1 µl
- BSA (10X) 2 µl

The total reaction volume was made up to 20 µl with core water and the reaction mixture was incubated at 37°C for 3 hrs. The completion of digestion of DNA was monitored by analyzing the samples on a 1% agarose gel.

For cloning, the restriction enzymes digested DNA fragments were purified by QIA quick PCR purification kit and estimated by measuring the absorbance at 260 nm and stored at -20°C till use. Ligation of digested vector and insert DNA was performed as follows. The ligation mixture consist of

- 10X Ligation buffer 3 µl
- Vector (50 ng) 3 µl
- Insert (20-50 ng) 9 µl
- T4 DNA Ligase 1.0 µl

The total reaction volume was made up to 30 µl with distilled water and ligations were performed overnight (16 hrs) at 16°C. Molar ratios of 3:1 of insert to vector was used in the ligation reaction. The ligated products were
transformed in DH5α *E.coli* host and were screened for the presence of the insert by PCR. For screening of transformants, a small portion of freshly grown transformed colony was picked using a sterile toothpick and resuspended in 50µl of 0.1X TE (1mM Tris and 10mM EDTA, pH 8) buffer. The cells were lysed by boiling for 10 min; snap chilled on ice, centrifuged at 10000 rpm for 10 min and 1µl of the supernatant was used as template in PCR.

### 2.6 *E.Coli* Transformation

The procedure for rendering cells of *E.coli* competent for transformation is based on the method described by Sambrook et al (1989) with some modification as described below.

a) A single colony of the *E.coli* strain was inoculated from a plate into 10ml LB medium and kept in the shaker for overnight at 37°C.

b) 500µl of this preculture was used to inoculate 50ml LB broth contained in a 250ml flask. The culture was allowed to grow at 37°C with shaking until the O.D at 600 nm reaches 0.6.

c) The flask was removed from the shaker and transferred to an ice water bath in which it was agitated so as to cool its contents rapidly.

d) After 30 min, 40ml of the culture was transferred to a sterilized and chilled screw cap centrifuge tubes and the cells were pelleted at 4000 rpm in 12166 rotor of a sigma 4k centrifuge for 10 min at 4°C.
e) The supernatant was discarded and the pellet was gently resuspended in 20ml ice-cold sterile 100mM calcium chloride. The tubes were kept chilled on ice for 30 min.

f) The cells were centrifuged again as in the above step and the pellet was resuspended in 2ml of calcium chloride solution. The cells are now competent enough for transformation.

g) 200µl aliquots of competent cells were taken into sterile eppendorf tubes containing 1-2µg of DNA and mixed by swirling gently.

h) The tubes were incubated on ice for 30 min.

i) The tubes containing the cells were subjected to a heat shock for 90 sec in a 42°C water bath. Immediately the tubes were chilled on ice for 5 min.

j) 0.8ml of pre warmed LB broth was added to each tube and the tubes were kept at shaker for 45 min at 37°C.

k) 100µl of the above mix was plated on to LB agar plates containing ampicillin (100µg/ml).

l) The plates were incubated at 37°C for 14 hrs within which colonies arising from transformed cells appeared on the plates whereas plates receiving control cells (without plasmid) showed no appearance of colonies.

2.7 POLYMERASE CHAIN REACTION

For polymerase chain reaction (PCR) 1µl of the respective DNA prepared as described earlier was added to a PCR reaction mixture consisting of 1X PCR buffer, 2.5mM dNTPs, 5pmol of paired primers, 1.25 units of Taq
DNA polymerase (Bangalore Genei) and core water in a total volume of 50µl. The reaction mixture was placed in a Thermal cycler for cyclic reaction. The PCR reaction was set up as per the nature of primer and size of amplified product. The PCR products were run on 1.2% agarose gel stained with ethidium bromide and documented. The primer sequences are listed out in Table 2.1.

Table 2.1 Oligonucleotide primers for PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKNF</td>
<td>5’GGAATTCATATGATTGCTGGACCT</td>
<td>Nde I</td>
</tr>
<tr>
<td>SKBF</td>
<td>5’GGCCGAATTCATTGCTGGACCTGAG3’</td>
<td>Bam HI</td>
</tr>
<tr>
<td>SDREPF</td>
<td>5’GCTCTAGAATAATTTTGTTTAACTTTAAATAAGATATACATATGATTGCTGGACCT 3’</td>
<td>Xba I</td>
</tr>
<tr>
<td>SDPDEL1F</td>
<td>5’GCTCTAGAAATAATTTTGTTTAACTTTAATAATGATTGCTGGACCTG3’</td>
<td>Xba I</td>
</tr>
<tr>
<td>SDDEL1F</td>
<td>5’GCTCTAGAAATAATTTTGTTTAACTTTACATATGATTGCTGGACCTG3’</td>
<td>Xba I</td>
</tr>
<tr>
<td>SKER</td>
<td>5’CGGAATTCTTATTTGTCGTTATC3’</td>
<td>Eco RI</td>
</tr>
<tr>
<td>SKPR</td>
<td>5’ AACTGCAATTGATTTGCTGTTAGG3’</td>
<td>Pst I</td>
</tr>
<tr>
<td>SKPRH</td>
<td>5’ AACTGCAATTGATTTGCTGTTAGG3’</td>
<td>Pst I</td>
</tr>
<tr>
<td>T7 Forward</td>
<td>5’TAAATCGACTCCTATAGGG3’</td>
<td>-</td>
</tr>
<tr>
<td>T7 Reverse</td>
<td>5’TATTTATTGTCACCGGTGG3’</td>
<td>-</td>
</tr>
</tbody>
</table>

The restriction enzyme sites included in the primers are given in italics.

2.8 CELL GROWTH AND INDUCTION PROTOCOL

The following basic protocol was used for expression and screening of small cultures.

a) Single colony was inoculated into 3ml LB containing 100µg/ml ampicillin, then the cultures was grown overnight.
b) 2ml LB media was inoculated with 20µl of the overnight culture and allowed to grow at 37°C until O.D reaches 0.6 and 1.0. The expression was induced by adding IPTG to a final concentration of 1mM.

c) The cultures were allowed to grow for another 3-4 hrs and then cells were harvested by centrifugation at 8000 rpm for 5 min and the supernatant was discarded.

d) The cells were resuspended in PBS.

e) The protein content was estimated by Bradford method and 20 or 50µg of total protein was loaded onto SDS-PAGE.

NOTE: Host containing vector plasmid alone was processed simultaneously in the same way and used as control.

2.9 SONICATION

The process of disrupting biological materials by use of sound wave energy is sonication. Ultrasonication produces cavitation phenomena when acoustic power inputs are sufficiently high to allow the production of microbubbles at nucleation sites in the fluid.

a) The culture was centrifuged in 1.5ml microfuge tube and to the pellets PBS was added.

b) Clean the sonicator tip with 70% iso propyl alcohol.

c) Set the sonicator to the following:

   i. Time = 1 min 30 sec
   ii. Amplitude = 65%
   iii. Pulse On = 30 sec
   iv. Pulse Off = 30 sec
d) Set up an ice water bath and with the sample immersed in it, place the set up into the sonicator.

e) Lower the sonicator tip to the bottom of the tube and push the start button.

2.10 INCLUSION BODY PURIFICATION (FLOW CHART IS GIVEN IN RESULT SECTION)

a) Single colony containing recombinant streptokinase insert was picked up and inoculated into 100ml LB medium containing ampicillin (100µg/ml).

b) The cells were allowed to grow overnight at 37°C till 0.6 O.D was reached and the culture was transferred to 1L flask containing LB amp+ medium and allowed to grow at 37°C, 100 rpm till 1.0 OD was reached.

c) Then the sample was induced with 1mM IPTG and allowed to grow at 37°C and 40°C for 3 hrs and overnight.

d) The induced sample was centrifuged at 11000 rpm for 20 min at 4°C and pellet was washed with 30ml of STE buffer (Sodium chloride 5M, Tris HCl, EDTA) at 11000 rpm for 20 min, 4°C.

e) Pellet and supernatant (S1) were separated and pellet was resuspended with 30ml of STE Buffer.

f) The cells were sonicated for 30 min which involved 30 cycles. During each cycle the probe was turned on for 30 sec followed by 30 sec incubation in ice to avoid heat generation.
g) After sonication the sample was centrifuged at 11000 rpm for 20 min at 4°C and supernatant (S2) was collected separately.

h) Then the pellet was resuspended by adding 30ml of buffer containing 2M urea, 0.5% Triton X-100, STE buffer.

i) The sample was then centrifuged at 11000 rpm for 20 min at 4°C and supernatant (S3) was separated.

j) To the pellet 10ml of 8M urea was added and kept in rocker at room temperature for 6 hrs.

k) All the supernatants S1, S2, S3 and urea (8M) treated pellet were analysed by 10% SDS-PAGE

### 2.11 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Proteins are separated by hydrophobic interaction on columns with hydrophobic groups attached (e.g. phenyl, octyl groups). Proteins are separated on the basis of the differing strengths of their hydrophobic interactions with a gel matrix that contains uncharged hydrophobic groups.

The principle for protein adsorption to HIC media is complementary to ion exchange chromatography and gel filtration. HIC is even sensitive enough to be influenced by non-polar groups normally buried within the tertiary structure of proteins but exposed if the polypeptide chain is incorrectly folded or damaged (e.g. by proteases). This sensitivity can be useful for separating the pure native protein from other forms.

**Buffers**

Buffer A: 20mM sodium phosphate, 1M ammonium sulphate, pH 7.2

Buffer B: 20mM sodium phosphate, pH 7.2
**Procedure**

a) Pack the matrix (2ml) in suitable columns with a bed volume of 10ml.

b) The column was washed with five bed volumes of millipore water.

c) 1.5ml of 0.25N NaOH was added and washed after a standing time of 5 min. Then washed with millipore water.

d) Equilibrate the column with 5ml of the equilibration Buffer A.

e) 100µl of sample was loaded into the column along with 1900µl of buffer A.

f) Two flow through of 1ml each were collected separately and labeled as FT I and FT II.

g) 5ml of Buffer A was added to the column and allowed to stand for 10 min. Five flow through of one ml each were collected and separately labeled as wash I-wash V respectively.

h) The bound fraction was eluted using descending salt gradient from 60% to 100% Buffer B (20mM sodium phosphate buffer, pH 7.2). Two eluates of 1ml each were collected and stored.

i) 3ml of 40% Ethylene Glycol was added and after five min three eluates of 1ml each were collected and labeled as EG 40%I to EG 40%III.

j) The above step was repeated for 50% Ethylene glycol.

k) The collected samples were analysed by 10% SDS-PAGE.
2.12 IMMOBILIZED METAL ION AFFINITY CHROMATOGRAPHY

Chelating Sepharose charged with nickel ions will selectively retain proteins containing a histidine-tag. Histidine tagged protein is then eluted using buffers containing imidazole. Proteins with affinity to metal ions can be bound to the column and subsequently eluted by change of conditions.

Buffers

Start buffer: 20 mM Na$_2$HPO$_4$, 0.5 M NaCl, 10mM imidazole pH 7.4
Elution buffer: 20mM Na$_2$HPO$_4$, 0.5 M NaCl, 250mM imidazole pH 7.4

Procedure

a) Pack the chelating sepharose matrix in suitable columns with a bed volume of 10ml.

b) The column was incubated with 0.25N nickel chloride for about 15 min and washed with millipore water.

c) 1.5ml of 0.25N NaOH was added and washed after a standing time of 5 min and washed with millipore water.

d) Equilibrate the column with 2 bed volumes of the start buffer.

e) 100µl of sample was loaded into the column along with 1900µl of start buffer.

f) Two flow through of 1ml each were collected separately and labeled as FT I and FT II.
g) 5ml of start buffer was added to the column and allowed to stand for 10 min.

h) Five flow through of 1ml each were collected and separately labeled as wash I-wash V respectively.

i) The bound fraction was eluted using elution buffer containing various concentration of imidazole ranging from 50mM to 250mM.

j) Three eluates of 1ml each were collected and stored.

k) The collected samples were analysed by 10% SDS-PAGE.

2.13 Precipitation of Protein by Trichloroacetic Acid

a) 1ml of eluted protein sample was taken and 10µl of sodium deoxy cholic acid was added to the protein sample and incubated at room temperature for 10 min.

b) 150µl of 50% Trichloroacetic acid was added and the solution was mixed gently by inverting the tube and incubated in ice for 15 min.

c) The mixture was centrifuged at 13000 rpm at room temperature for 15 min.

d) Supernatant was discarded and to the pellet 40µl of 0.25N NaOH was added and the pellet was dissolved by brief spin.

e) The sample was then analysed by loading into 10% SDS-PAGE.
2.14 GEL ELECTROPHORESIS

2.14.1 Agarose Gel Electrophoresis of DNA (Sambrook et al 1989)

Horizontal submerged gels were employed in this study. The buffer for electrophoresis was TBE (89mM Tris, 89 mM Boric acid and 2mM EDTA, pH 8.0). The gel loading buffer was a solution of 20% sucrose and 0.01% bromophenol blue in TE buffer. Samples containing an appropriate quantity of DNA in TE buffer were mixed with a 1/4 volume of gel loading buffer and loaded onto the gel.

Depending on the size of the fragments to be separated, 0.7-1.2% agarose gels were run. The gels were 10cm long and 3mm thick. Electrophoresis was performed at 10 V/cm until the dye reached the bottom of the gel. Gels were stained in a 1 µg/ml solution of ethidium bromide in water for 5 min and viewed under illumination of 300nm. Photographs were taken for a permanent documentation.

2.14.2 SDS-Polyacrylamide Gel Electrophoresis

Proteins present in cell extracts were analysed by SDS-PAGE according to the method of Laemmli (1970) with some modifications. The composition of the systems is described below and also in appendix.

a) Monomer solution: 30% acrylamide and 0.8% bisacrylamide.
b) Separating gel buffer: 1.5M Tris, pH 8.8.
c) Stacking gel buffer: 1.5M Tris, pH 6.8.
d) Ammonium per sulphate (APS) – 140 mg/ml.
e) SDS 10% solution.
f) TEMED.
For a 12% w/v separating gel, 6ml of monomer solution, 3.8ml separating gel buffer, 5ml water, 150µl 10% SDS, 75µl APS and 5µl TEMED were used.

For a 4% w/v stacking gel, 1ml acrylamide solution, 1.5ml of stacking gel buffer 60µl SDS, 3.4ml distilled water, 60µl of APS and 3µl of TEMED were used.

The samples were mixed with the sample buffer (1.25ml 0.5 M Tris pH 6.8, 4ml 10% SDS, 4ml glycerol, made upto 10ml with distilled water and a small pinch of bromophenol blue was added to give blue colour, β-mercaptoethanol (0.5µl/ml) was added freshly before use).

Electrophoresis was performed at room temperature at a constant current of 30mA. The electrophoresis buffer used was 3.1g Tris base, 14.4g glycine, 1g SDS dissolved in 1L of distilled water. When the dye reached 1cm from the bottom of the gel, electrophoresis was stopped and transferred to fixer solution. The gel was removed and soaked in staining solution (coomassie blue) and later excess stain was removed by destaining. The composition of the stainer and the destainer are given in the appendix.

2.14.3 Tricine SDS- Polyacrylamide Gel Electrophoresis

Proteins present in cell extracts were analysed by TRICINE SDS-PAGE. The composition of the systems is described below and also in appendix.

a) Monomer solution: 30% acrylamide and 0.8% bisacrylamide.

b) Separating gel buffer: 1.5M Tris Cl, pH 8.8.

c) Stacking gel buffer: 1.5M Tris Cl, pH 6.8.

d) Ammonium per sulphate (APS) – 140mg/ml.
e) SDS 10% solution.

e) TEMED.

For a 12% w/v separating gel, 6ml of monomer solution, 3.8ml separating gel buffer, 5ml distilled water, 150µl 10% SDS, 75µl APS and 5µl TEMED were used.

For a 4% w/v stacking gel, 1ml acrylamide solution, 1.5ml of stacking gel buffer 60µl SDS, 3.4ml distilled water, 60µl APS and 3µl of TEMED were used.

The samples were mixed with the sample buffer (1.25 ml -0.5 M Tris Cl, pH 6.8, 4ml-10% SDS, 4ml glycerol, made upto 10ml with distilled water and a small pinch of bromophenol blue was added to give blue colour, β-mercaptoethanol (0.5µl/ml) was added freshly before use).

Electrophoresis was performed at room temperature at a constant current of 30mA. The electrophoresis buffer used was 3.1g Tris base, 14.4g glycine, 1.0 g SDS dissolved in 1L of water. When the dye reached 1cm from the bottom of the gel, electrophoresis was stopped. The gel was removed and soaked in staining solution (coomassie blue) and later excess stain was removed by destaining. The composition of the stainer and the destainer are given in the appendix.

2.15 COOMASSIE BLUE STAINING (G-250)

After electrophoresis, the gel was transferred to fixer solution and kept for about 20 min in the rocker.

The fixer solution was removed and the gel was transferred to Coomassie Brilliant Blue staining solution containing 0.01%(w/v) CBB G-250 dye, 10% acetic acid and kept in the rocker for 10 min.
After staining, the gel was destained overnight in solution containing 10% acetic acid.

2.16 WESTERN BLOTTING

In order to find out the location or presence of specific proteins in the SDS-PAGE, the protein pattern were electro transferred to a nitrocellulose membrane as described (Towbin et al 1979). After the electrophoresis was complete, the gel was incubated for 10-15 min in the transfer buffer to eliminate swelling. In the meantime, the NCP/Hybond cut to the desired size was incubated for 5-10 min in transfer buffer (Tris 25mM, glycine 192mM, methanol 20%, SDS 1%). The membrane was over laid on the gel (by avoiding air bubble) and sandwiched between the filter paper and scotch brite pads. The gel was placed ‘cathodic’ to the membrane. The transfer was carried out at 120 mA, 20V for one and half hours by using LKB transphor 2005 electroblotting apparatus. After the transfer was complete, the molecular weight marker lane was stained with amido black (100 mg amido black in 45ml methanol with distilled water) for 2-3 min and then destained until the background stain was eliminated or prestained protein marker was used. The rest of the membrane was blocked for 1hr at room temperature with 3% skimmed milk in PBS.

The membrane was washed in wash buffer for three times of 5 min duration. The membrane was incubated for 1 hr with the mouse monoclonal anti-Streptokinase antibody (primary) diluted in PBS (1:1000). After extensive washing in the wash buffer, the membrane was incubated for the anti-mouse secondary antibody (1:1000 dilutions) conjugated with alkaline phosphatase.

For alkaline phosphatase staining, the blots were incubated in the buffer (100mM Tris HCl (pH 9.5), 100mM NaCl, 5mM MgCl2) for 10 min
and the colour development was carried out by using 30µl of nitroblue tetrazolium (NBT) (50mg/ml in 70% dimethyl formamide) and 16.5µl of bromo-chloro-indolyl phosphate (BCIP) (50mg/ml in 100% diethyl formamide).

2.17 SUB-CELLULAR FRACTIONATION

Sub-cellular fractionation is a technique in which proteins in the different compartments such as periplasmic, cytoplasmic and membrane fractions are segregated sequentially and thus helpful in the targeting studies.

a) The induced *E.coli* culture containing the plasmid was centrifuged at 5000 rpm for 10 min at room temperature.

b) The pellet was washed twice either using PBS or 0.9% saline at 5000 rpm for 10 min.

c) The pellet was resuspended in mixture of lysis buffer (50mM Tris, 10mM EDTA and 20% Sucrose) and lysozyme (1mg/ml) and incubated at 37°C for 45 min.

d) The mixture was centrifuged at 13000 rpm for 15 min at 4°C.

e) The supernatant containing the periplasmic fraction was separated and stored at -20°C for further use.

f) The pellet obtained was suspended in core water and kept for incubation at 37°C for 10 min.

g) After incubation, the suspension was centrifuged at 13000 rpm for 15 min.

h) The supernatant containing the cytoplasmic fraction was collected and stored at -20°C for future use.

i) The pellet obtained was resuspended in phosphate buffered saline and used as membrane fraction.
2.18 RADIOLABELING

a) The clone containing lipid modified streptokinase was inoculated and allowed to grow till 0.4 and 0.5 O.D.

b) To the culture, tritium labeled palmitate was added and allowed to shake for about 30 min at 30°C.

c) Then the culture was induced either with 1M IPTG or 0.3 M NaCl and kept at 30°C in the shaker for 5 hrs.

d) The culture was centrifuged and the pellet obtained was suspended in phosphate buffer saline.

e) The suspension was then loaded in SDS-PAGE and the desired protein bands were excised using KCl staining.

f) The gel pieces were treated with 1M NaCl till the gel pieces became transparent.

g) To the gel pieces absolute alcohol was added and kept at 37°C in static incubator till the gel pieces dried completely.

h) The dried pieces were taken in the OPTI plate and 100µl of scintillation fluid was added and the radioactivity was counted as cpm (counts per min) in the scintillation counter.

2.19 PROTEIN ELUTION BY POTASSIUM CHLORIDE STAINING

a) The induced sample containing the desired protein was resolved by SDS polyacrylamide gel electrophoretic method.

b) The gel was then washed in distilled water.
c) To the gel 1M KCl was added. KCl stains the proteins and the proteins were visualized as white bands.

d) Protein bands of desired kDa were excised from the gel and cut into small pieces.

e) The gel pieces were treated with 1M NaCl to remove KCl.

f) The above step was repeated till the gel pieces become transparent.

g) Then phosphate buffer saline was added to the gel pieces and refrigerated for 5 hrs.

h) After incubation, the sample was analysed for the single protein band of desired size using SDS-PAGE.

2.20 PROTEIN ESTIMATION BY BRADFORD METHOD

Bradford assay is a colorimetric protein assay which is based on the absorbance shift in the coomassie dye. Coomassie red dye donates its free proton to the protein which exposes the hydrophobic pocket and thus forms a non covalent bond with the non polar region of the dye via van der Waals force. This bonding stabilizes the blue colour of the dye and the absorbance of the dye at 595nm is directly proportional to the amount of protein present.

a) Standard was prepared by adding known concentration of BSA (1mg/ml) at various amounts ranging from 0µl to 10µl. 0µl serves as blank.

b) 10µl of protein whose concentration to be known was taken

c) The well was made upto 20µl with millipore water.

d) Then 180µl of 1X Bradford reagent was added and the plate was incubated at 37°C for 10 min.
2.21 CASEINOLYTIC ASSAY

The biological activity of streptokinase can be detected *in vitro* by taking advantage of the caseinolytic activity of activated plasminogen. The plasma in the assay medium serves as the source of plasminogen, with which the streptokinase in the lysate of expressed culture binds and forms an activator complex. The plasminogen moiety in a SK:Plg activator complex expresses proteolytic activity catalyzing the conversion of other plasminogen molecules to plasmin. The active plasmin hydrolyses casein present in milk producing clear zones of caseinolysis around the well.

a) 10 mg agarose/ml was dissolved in 50mM Tris, pH 8.0, 150mM NaCl and mixed with 20 volume percent boiled milk.

b) 5 volume percent of plasma was added to the cooled mixture, mixed thoroughly and poured into a sterile plate.

c) Wells were bored in the assay medium after solidification.

d) Lysate of expressed culture was added to the well.

e) The diameter of zone of caseinolysis was measured after incubation for 3-5 hrs at 37°C