CHAPTER 3

RESULTS

The choice of an expression system for the overproduction of heterologous proteins depends on many factors like cell growth characteristics, expression host, medium, post induction time, temperature, post translational modification, mRNA stability and biological activity of protein of interest. We have chosen *E.coli* for the expression of recombinant and modified streptokinase.

The overall outline of the present study is given in the flowchart (Figure 3.1). In this study, many strategies have been followed to produce biologically active recombinant streptokinase. Towards this, various recombinant constructs were developed and their expression kinetics were analysed. To compare the purification efficiency, native and fusion protein strategies were followed simultaneously. Attempts were also made to increase the potency of the recombinant streptokinase by exploiting bacterial lipid modification process. In addition, the effect of RBS in the expression of streptokinase also has been studied.
3.1 OVER EXPRESSION AND PURIFICATION OF STREPTOKINASE

3.1.1 Cloning and Expression of Streptokinase

The pETSK clone was designed without fusion tag in pET-20b(+) vector between Nde I and Eco RI sites. The streptokinase gene was also cloned in pRSETB vector with fusion tag (histidine) between Bam HI and Eco RI restriction sites and it was named as pRSETB-SKH. The pETSK construct was transformed into E.coli BL21 (DE3) for expression studies which were conducted at different temperatures (37°C, 40°C, 42°C and 45°C), different post induction time (3 hrs and 6 hrs) and optical density of the culture (0.6 O.D and 1.0 O.D). In E.coli BL21 (DE3), the therapeutic protein streptokinase was found to be highly expressed upon induction with 1mM IPTG.

Further, the streptokinase protein from pETSK was purified, followed by identification and purification of inclusion body. The same protocol (inclusion body purification and purification of SK) was followed for pRSETB-SKH (with histidine tag) construct also.
Figure 3.1 Outline of the present study
Comparison of expression profile of the recombinant streptokinase (pETSK) at various temperature 37°C, 40°C, 42°C and 45°C was analysed in 10% SDS-PAGE.

UI- Uninduced, IN- Induced.
E.coli BL21 (DE3) cells harboring the recombinant construct pETSK were grown in LB medium until the culture optical density (O.D) reached 0.6 and 1.0 O.D. They were separately induced with 1mM IPTG followed by incubation at various temperatures like 37°C, 40°C, 42°C and 45°C. The samples were collected at different time intervals (0, 3 and 6 hrs) and analysed by 10% SDS-PAGE. The results (Figure 3.2) showed that over-expression was achieved in the samples induced in all conditions. At 40°C, more expression was observed compared to other conditions. Among the induced samples there is no significant difference in expression either at particular temperature or O.D or time intervals.

3.1.2 Inclusion Body Identification

Generally, it is a well known fact that in E.coli the formation of inclusion body is concomittant of over-expression of heterologous proteins. The aggregation of expressed proteins loses their biological activity due to the misfolding or improper folding. The basis of this experiment is to know whether the recombinant streptokinase produced by different constructs ends with inclusion body or not. We have employed a simplified technique; centrifugation after the sonication of the induced and uninduced cell pellet has led to the separation of supernatant and pellet which were subsequently analysed by SDS-PAGE. The supernatant contains soluble proteins whereas the pellet contains aggregated proteins (inclusion body).
Figure 3.3 Flowchart for the expression and purification of recombinant streptokinases
### Figure 3.4 Comparitive analysis for solubility profile of expressed streptokinase

SDS-PAGE analysis for comparing the solubility profile of the recombinant streptokinase (from pETSK) under various parameters such as temperatures (37°C, 40°C, 42°C and 45°C), optical densities (0.6 and 1.0 O.D), post induction time intervals (0, 3 and 6 hrs). SUP- Supernatant, PEL- Pellet, UI- Uninduced, IN- Induced, pET-20b(+) - Vector control.
Table 3.1 Solubility profile of recombinant pETSK construct in BL21 (DE3) under various conditions.

<table>
<thead>
<tr>
<th>O.D</th>
<th>0.6</th>
<th>1.0</th>
<th>3 hrs</th>
<th>6 hrs</th>
<th>0 hr UI</th>
<th>3 hrs</th>
<th>6 hrs</th>
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<tr>
<td></td>
<td></td>
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<td>UI</td>
<td>IN</td>
<td>UI</td>
<td>IN</td>
<td>UI</td>
</tr>
<tr>
<td>TEMP</td>
<td>0 hr UI</td>
<td></td>
<td>UI</td>
<td>IN</td>
<td>UI</td>
<td>IN</td>
<td>UI</td>
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<td>S</td>
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<tr>
<td>37°C</td>
<td>-</td>
<td>+</td>
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<tr>
<td>40°C</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>42°C</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>45°C</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(-) Nil, (+) Less, (++) Moderate, (+++) Higher amount. UI- Uninduced, IN- Induced, S - Supernatant, P- Pellet

The oval shaped rings indicate the completely insoluble form of the recombinant streptokinase (from pETSK) under various conditions.
The main intention of conducting this experiment was to find out under what conditions the maximum amount of inclusion body forms which makes the purification strategies easier. The 0.6 O.D and 1.0 O.D culture of the construct pETSK, grown in LB media, were separately induced with 1mM IPTG followed by incubation at various temperatures like 37°C, 40°C, 42°C and 45°C. The samples were collected at different time intervals (0, 3 and 6 hrs) and sonicated. After centrifugation, the collected supernatants and pellets were analysed by 10% SDS-PAGE. From the above (Figure 3.4) and (Table 3.1) it was found that more than 90% of the inclusion bodies were observed in the samples at 40°C (3 hrs and 6 hrs induced samples at 0.6 and 1.0 O.D), 42°C at (3 hrs and 6 hrs induced samples at 0.6 O.D and 6 hrs induced sample at 1.0 O.D) and 45°C (6 hrs induced sample of 0.6 and 1.0 O.D). The arrows indicate the expression of inclusion body of the recombinant protein streptokinase at 47 kDa. At 37°C both soluble and insoluble forms of the protein were obtained in 3 and 6 hrs induced samples. This trend indicates that as the post-induction temperature increases the soluble protein content decreases (maximum present in inclusion body).

3.1.3 Inclusion Body Purification (pETSK)

The pETSK recombinant construct was transformed into *E.coli* BL21 (DE3), followed by the induction of 1000ml culture with 1mM IPTG at 37°C for 3 hrs and overnight. In another experiment, the above conditions were followed except the post induction temperature which was increased to 40°C. The harvested cell pellets were processed as per the procedure given in the materials and methods section. Streamline of the procedure is outlined in the Figure 3.5.
Inclusion Body Purification Protocol

Figure 3.5 Blue print of the inclusion body purification and assay
Figure 3.6 SDS-PAGE analysis of inclusion body purification of recombinant streptokinase at 37°C for 3hrs and overnight

The samples obtained from various sequential stages (as mentioned in Figure 3.5) were analysed by 10% SDS-PAGE.

WCL- Whole Cell Lysate
S1- Supernatant, after washing with STE buffer
S2- Supernatant, after sonication
S3- Supernatant, after the treatment with 2M urea, Triton X-100 in STE buffer
N- Overnight
Figure 3.7  SDS-PAGE analysis of inclusion body purification of recombinant streptokinase at 40°C for 3hrs and overnight.

The samples obtained from different sequential stages (as mentioned in Figure 3.5) were analysed by 10% SDS-PAGE.

WCL- Whole Cell Lysate
S1- Supernatant, after washing with STE buffer
S2- Supernatant, after sonication
S3- Supernatant, after the treatment with 2M urea, Triton X-100 in STE buffer
ON- Overnight
From the above Figures (3.6 and 3.7) it may be inferred that protein loss was found in the wash samples (S1) at 37°C compared to 40°C. Also there was a considerable loss of protein in the sonicated samples at 37°C compared to 40°C. Among the 3 hrs and overnight samples at 40°C, the protein loss was found in the 3 hrs samples while higher amount of inclusion body without any degradation was obtained in the overnight samples at 40°C. This sample was further subjected to column chromatography.

### 3.1.4 Purification of Recombinant Streptokinase in Native and Fusion Forms

After inclusion body purification, the recombinant proteins (from pETSK and pRSETB-SKH) were purified by hydrophobic interaction chromatography (HIC) and immobilized metal affinity chromatography respectively (IMAC). In HIC, the solublized pETSK was loaded onto the phenyl sepharose low substituted column. The protein was eluted from 50% to 100% of 20mM phosphate buffer and 37% to 60% ethylene glycol. All the fractions such as flow through, wash and elutions were analysed by 10% SDS-PAGE. In IMAC, the solublized pRSETB-SKH (with histidine tag) was loaded onto the chelating sepharose matrix charged by Ni²⁺. The bound protein was eluted by 20mM phosphate buffer containing 50mM to 250mM imidazole. The detailed methodology for purification is given in the materials and methods section.
Figure 3.8  SDS-PAGE analysis of purified recombinant streptokinase (pETSK) by HIC

Various fractions obtained in different stages of HIC were analysed by 10% SDS-PAGE, which shows the homogenous nature of the purified streptokinase protein by Hydrophobic Interaction Chromatography.

WCL - Whole Cell Lysate (pETSK)
FT I- Flow Through I
FT II- Flow Through II
EG 40% E I- Elution I by 40% Ethylene Glycol
EG 40% E II- Elution II by 40% Ethylene Glycol
EG 40% E III- Elution III by 40% Ethylene Glycol
After complete solubilization of inclusion body, it was purified by HIC using phenyl sepharose low substituted HIC column. The SDS-PAGE analysis revealed that the target protein was not present in the samples, flow through and wash. The elution fractions containing 40% ethylene glycol has yielded the maximum amount of protein with repeated elutions as indicated in the Figure (3.8). This result clearly shows that phenyl sepharose low substitute matrix and elution buffer containing 40% ethylene glycol is an ideal combination for 60% recovery of purified protein. The purified samples were confirmed with western blot analysis (Figure 3.9) and bioassay (Figure 3.10).

![Figure 3.9 Western blot analysis of purified recombinant streptokinase (pETSK) by HIC](image)

Appearance of single band corresponding to the molecular weight of 47 kDa after challenging the blot containing purified sample with antibody (against streptokinase) confirms the homogenous nature of purified protein. No band was found corresponding to streptokinase in pET-20b(+) vector control.
Figure 3.10 Caseinolytic assay of recombinant streptokinase (from pETSK) purified by HIC

EG- Ethylene Glycol, STD SK – Standard streptokinase

The purified samples from HIC were checked for their biological activity by caseinolytic plate assay. No activity was observed in the vector, flow through and wash samples. Zone of clearance was observed in the standard streptokinase and purified samples which confirms that the HIC purified streptokinase is biologically active.
Figure 3.11 SDS-PAGE analysis of recombinant streptokinase (pRSETB-SKH) at 40°C

UI- Uninduced
IN- Induced

The pRSETB-SKH construct was transformed into BL21 (DE3) host and the cells were allowed to grow until 0.6 O.D and 1.0 O.D. was reached. The cells were then induced with 1mM IPTG at 40°C and analyzed by 10% SDS-PAGE as shown in (Figure 3.11). The above mentioned conditions were followed since similar conditions have given hyper expression of the recombinant protein by another construct pETSK.
Figure 3.12 SDS-PAGE analysis of purified recombinant streptokinase (from pRSETB-SKH) by IMAC

The purification of the recombinant protein (from pRSETB-SKH) by Immobilised Metal Affinity Chromatography shows the homogenous nature of the purified streptokinase protein in SDS-PAGE analysis.

WCL - Whole Cell Lysate
FT I- Flow Through I
FT II- Flow Through II
Elution I, II, III- Elution by 250mM imidazole
The complete solubilization of streptokinase from pRSETB-SKH construct was followed by the purification by IMAC using chelating sepharose matrix. The SDS-PAGE analysis revealed that the target protein was not present in the samples flow through and washes. The elution fractions containing 250mM imidazole has yielded the maximum amount of protein with repeated elutions as indicated in the Figure (3.12). This result clearly shows that chelating sepharose matrix and elution buffer containing 250mM imidazole is an ideal combination for 57% recovery of purified protein. The purified protein was further confirmed with western blot analysis (Figure 3.13) and bioassay (Figure 3.14).

Figure 3.13  Western blot analysis of purified recombinant streptokinase (pRSETB-SKH) by IMAC
Appearance of single band after challenging the blot containing purified sample with antibody (against streptokinase) confirms the homogenous nature of purified protein. No band was found corresponding to streptokinase in pRSETB vector control.

Figure 3.14 Caseinolytic assay of recombinant streptokinase (pRSETB-SKH) purified by IMAC
STD SK – Standard streptokinase
Elution I, II, III- Elution by 250mM imidazole

The purified samples from IMAC were checked for their biological activity by caseinolytic plate assay. No activity was observed in the vector, flow through and wash samples. Zone of clearance was observed in the standard streptokinase and purified samples which confirms that the IMAC purified streptokinase is biologically active.
Figure 3.15 Comparison of the efficiency of purification strategies for streptokinase

Table 3.2 Recovery of purified streptokinase

<table>
<thead>
<tr>
<th>S.No</th>
<th>Construct</th>
<th>Method</th>
<th>Amount of protein loaded</th>
<th>Amount of protein eluted</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pETSK</td>
<td>HIC</td>
<td>410µg</td>
<td>250 µg</td>
<td>60%</td>
</tr>
<tr>
<td>2</td>
<td>pRSETB-SKH</td>
<td>IMAC</td>
<td>430 µg</td>
<td>220 µg</td>
<td>57%</td>
</tr>
</tbody>
</table>

This (Table 3.2) shows the recovery of purification of streptokinase by HIC and IMAC (protein expressed from pETSK and pRSETB-SKH respectively). The quantitative comparison is shown in the above graph (Figure 3.15). Protein estimation has confirmed 60% recovery in HIC and 57% in IMAC.
3.2 LIPID MODIFICATION OF STREPTOKINASE

By considering the applications of lipid modification (refer introduction section), we tried to modify native streptokinase, into lipid modified one. Streptokinase, a cytoplasmic and non-lipoprotein is subjected to lipid modification by fusion of modified apyrase signal sequence of *Shigella* and Braun’s lipoprotein from *E.coli* with the streptokinase mature sequence. The signal sequence was recognized and the protein was targeted to the membrane with lipid moiety. The flow chart (Figure 3.16) is explaining the strategies followed for lipid modification studies.

3.2.1 Development of Recombinant Constructs LM-SK, LM-SKH, LPP-SK and LPP-SKH

Cloning of lipid modified streptokinase (LM-SK) with and without C- terminal histidine tag (LM-SKH) was done in LM vector which is a modified pRSETB vector consisting of apyrase signal sequence. The LPP-SK and LPP-SKH clones were obtained by manipulating the LPP vector, in which the pRSETB vector was, restricted at *Nde* I and *Bam HI* sites and ligated with Braun’s lipoprotein signal sequence. Both restricted vectors and the inserts were ligated and the ligation mixture was transformed in *E.coli*. Then the transformants were screened for having the recombinant constructs by lysate PCR (Figure 3.17).
Figure 3.16 Strategies for lipid modification of streptokinase constructs
Figure 3.17 Screening (lysate PCR) of LM-SK and LPP-SK recombinant streptokinase gene containing transformants

The ligation mixture was transformed into *E. coli* DH5α. The transformants were subjected to lysate PCR using insert specific primers. These amplified products were electrophoresed on 0.8% agarose gel. Bands above 1.2 kb corresponding to the marker lane confirm the presence of the streptokinase gene in the transformants.

NEG- Negative control, POS- Positive control

3.2.2 Expression Kinetics of Lipid Modified Constructs

The recombinant SK constructs (LM-SK, LM-SKH, LPP-SK and LPP-SKH) were transformed into the expression hosts BL21 (DE3) and GJ1158 and were allowed to grow in various media such as LB, LBON, NB, NBON, GYE, and GYEON. Then 1.0 O.D. of the culture was induced with either 1mM IPTG or 0.3M NaCl and kept at various temperatures, 25°C, 30°C, and 37°C for 3 hrs and 6 hrs. After induction, the samples were analysed by 10% SDS-PAGE.
The LM-SK and LPP-SK constructs, that were grown in LB medium till 1.0 O.D were induced with 1mM IPTG and incubated at 30°C and 37°C for 3 hrs. The SDS-PAGE analysis showed that the characteristic expression of lipid modified streptokinase at 48.5 kDa was seen (in the Figure 3.18) at 30°C in lane 8, while mild expression was seen at 37°C in lane 7. But no expression was observed in the construct LPP-SK induced at both 30°C and 37°C.

The recombinant construct LM-SK was studied for the expression under different conditions as mentioned above and analysed by SDS-PAGE. From the analysis it was found that more expression was observed at 30°C for 6 hrs in LBON medium. All the other media and E.coli strains showed similar level of expression. The SDS-PAGE analysis of the expression of recombinant streptokinase (LM-SK) in NBON medium at 30°C and 37°C for 3 and 6 hrs has been illustrated in the (Figure 3.19). Characteristic expression of lipid modified streptokinase at 48.5 kDa was seen at both 30°C and 37°C but over expression was observed in the culture induced at 30°C compared to the culture induced at 37°C.

The expression studies were conducted similarly for the recombinant constructs (LPP-SKH and LM-SKH) and analysed by 10% SDS-PAGE. Characteristic expression of lipid modified streptokinase at 48.5 kDa was seen at 30°C for 3 and 6 hrs in lane 7 and lane 8 of the (Figure 3.20), but no characteristic expression was observed in the recombinant construct LPP-SKH induced at 30°C. Since the expression analysis and bioassay profile of LM-SKH was similar to the whole cell lysate and sub-cellular fraction of LM-SK, further studies were continued with the comparison between native SK and LM-SK.
Figure 3.18  SDS-PAGE analysis of recombinant streptokinase (LPP-SK and LM-SK) expressed in BL21 (DE3) in LB medium at 30°C and 37°C
UI- Uninduced
IN- Induced
Figure 3.19 SDS-PAGE analysis of recombinant streptokinase (LM-SK) expressed in GJ1158 in NBON medium at 30°C and 37°C
UI- Uninduced
IN- Induced
3.2.3 Confirmation of Lipid Modified Streptokinase

The lipid modified streptokinases expressed from LM-SK and LM-SK constructs were confirmed by Tricine SDS-PAGE, sub cellular fractionation, bioassay and radiolabeling study.
3.2.3.1 Mobility shift in tricine SDS-PAGE

The induced samples (whole cell lysate) of native streptokinase and lipid modified streptokinases were analysed in Tricine SDS-PAGE (12%). The mobility shift was observed between native and lipid modified streptokinases. The native streptokinase migrated in the gel corresponding to 47 kDa whereas lipid modified streptokinase moved at 48.5 kDa. The mobility difference of about 1.5 kDa was seen distinctly between the lipid modified (LM-SK and LM-SKH) and native streptokinase in the (Figure 3.21) which confirmed that the protein has undergone modification resulting in the increase in size of the protein. The molecular weight increase has confirmed the occurrence of lipid modification in the recombinant streptokinase.

Figure 3.21 TRICINE SDS-PAGE analysis of recombinant streptokinase (LM-SK)

IN- Induced
3.2.3.2 Sub-cellular fractionation studies of *E.coli*

The samples collected from sub-cellular fractionation of BL21 (DE3) harboring lipid modified constructs (LM-SK and LM-SKH) after induction with 1mM IPTG were subjected to Tricine SDS-PAGE analysis. The results have revealed that the lipid modified streptokinase was found to be present only in membrane but not in the cytoplasm or periplasm.

![Figure 3.22 Tricine SDS-PAGE analysis of recombinant streptokinase (LM-SK and LM-SKH) by cell fractionation at 30°C.](image)

WCL- Whole Cell Lysate, IN- Induced
The samples from different cellular fractions were loaded and analysed by Tricine SDS-PAGE (Figure 3.22). Expression of LM-SK and LM-SKH protein was observed in lanes 3, 6 and 8, 10 respectively. Mobility difference of about 1.5 kDa was seen between lane 2 and 3 for LM-SK, 7 and 8 for LM-SKH. No bands corresponding to lipid modified streptokinase above 47 kDa was observed in periplasmic and cytoplasmic fraction. Hence, this result confirmed that the lipid modified streptokinases (LM-SK, LM-SKH) were targeted to the membrane of *E.coli*.

### 3.2.3.3 Bioassay

The *in vitro* biological activity of the thrombolytic agent was performed in caseinolytic assay as described in materials and methods section. The sonicated samples were subjected to caseinolytic assay. In the (Figure 3.23) the standard streptokinase showed activity by binding with the human plasminogen present in the plasma but no zone of clearance was observed in both uninduced and induced LPP-SK samples. 

The standard streptokinase (pRSETB-SK) and LM-SK showed activity by binding with the human plasminogen as shown in the (Figure 3.24). In comparison, more activity was observed in the lipid modified streptokinase at 30°C than any other temperature conditions.

The samples obtained from sub-cellular fractionation were subjected to caseinolytic assay. The standard streptokinase and all the cell fractions showed activity by binding with the human plasminogen present in the plasma. By comparing the activities of various cell fractions, membrane fraction showed more activity than periplasmic and cytoplasmic fraction (Figure 3.25).

The results obtained in SDS-PAGE did not show any visible band corresponding to the lipid modified protein in periplasmic and cytoplasmic fractions, whereas in bioassay a very small zone of clearance was observed.
This might be due to the contamination during the sub cellular fractionation process.

**Figure 3.23** Caseinolytic assay for recombinant Streptokinase (LPP-SK)

UI- Uninduced, IN- Induced, SK- Streptokinase

**Figure 3.24** Caseinolytic assay for recombinant Streptokinase (LM-SK)

UI- Uninduced, IN- Induced
3.2.3.4 Radiolabeling

At 0.6 O.D the culture containing lipid modified streptokinase was incubated with [9, 10 $^{3}$H] palmitate for 20 min and induced with 1mM IPTG. The desired protein band was excised from SDS-PAGE by KCl staining. After drying, the radioactivity was measured in scintillation counter.

**Table 3.3 Scintillation counting**

<table>
<thead>
<tr>
<th>S.No</th>
<th>O.D</th>
<th>Scintillation Counting (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LM vector</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>30</td>
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</table>
The above (Table 3.3) shows the radioactivity of pRSETB-SK, LM vector, LM-SK and LM-SKH in cpm. The recombinant clones were inoculated in LBON medium and induced at different O.D. using 0.3M NaCl. The induced samples were analysed by 10% SDS-PAGE. An unmodified streptokinase and vector was also treated as same procedure. The radioactivity of lipid modified construct was found to be higher than the control. This showed that the radiolabeled palmitate was incorporated into the lipid moiety fused with the streptokinase mature sequence which confirmed that the protein was subjected to lipid modification. The purified proteins have also been subjected to bioassay and the result has been observed as given in (Figure 3.26).

Figure 3.26  Comparative biological activity study of native and lipid modified streptokinase

2µg of native and lipid modified streptokinase were loaded in (assay plate) well 1 and well 2 respectively. After 8 hrs incubation at 37°C, the zone of clearance was observed to be more in lipid modified streptokinase.
3.3 STUDIES ON THE INFLUENCE OF THE RIBOSOME BINDING SITE IN HETEROLOGOUS PROTEIN EXPRESSION

The ribosomal binding site (RBS) is a sequence on mRNA namely Shine Dalgarno sequence that is bound by ribosome to initiate translation process. The effect of modifications in the ribosome binding site of pRSETB vector in the expression of therapeutic protein streptokinase has been studied.

3.3.1 Construction of Streptokinase with the Replacement, Partial Deletion and Complete Deletion of SD Sequence in pRSETB Vector

The streptokinase gene (1.25 Kb) was cloned into pRSETB vector by amplifying the gene with the corresponding forward and reverse primers followed by digestion with Xba I and Pst I restriction enzymes. The replacement of RBS in the pRSETB vector was done by substituting different nucleotides ‘AAAATAA’ for the SD sequence ‘AGAAGGA’. Deletion of the RBS sequence ‘AGAAGGA’ has been done in SD-parDEL clone, while in complete deletion of SD sequence, the deleted nucleotides consist of ‘AGAAGGAGATATA’ resulting in SD-comDEL clone. The modified pRSETB vector with replaced, partially deleted and completely deleted SD sequence was restricted at Xba I and Pst I sites and ligated with the purified SK fragments, which resulted in the clones SD-REP, SD-parDEL and SD-comDEL.
Figure 3.27 Strategies for RBS modifications and protein expression (streptokinase)
Figure 3.28 Screening (lysate PCR) of SD-REP, SD-parDEL and SD-comDEL clones

NEG- Negative control, POS- Positive control

The ligation mixture was transformed into the E. coli DH5α and the colonies obtained were screened for the presence of insert by performing colony PCR with the insert specific primers. The amplified products were electrophoresed on 1% agarose gel. The appearance of 1.2 kb PCR product has confirmed the recombinant constructs (Figure 3.28). These constructs were further subjected for expression studies.

3.3.2 Expression and Confirmation of Recombinant Protein Streptokinase with the Modified SD Sequence

The expression of the recombinant constructs (SD-REP, SD-parDEL, SD-comDEL) was checked in different E. coli hosts (BL21 (DE3), GJ1158 and BL21 (DE3) pLysS), different media (LB, NB, GYE, LBON,
GYEON and NBON), at different temperatures (25°C, 30°C and 37°C) and at different time intervals (3 and 6 hrs). The more expression of streptokinase was observed at 30°C, compared to other temperatures in the constructs (SD-REP and SD-parDEL) in the hosts (BL21 (DE3) and GJ1158).

The (Figure 3.29) illustrates the SDS-PAGE analysis of the expression of the streptokinase constructs SD-REP, SD-parDEL and pRSETB-SK (control) in BL21 (DE3) host induced at 30°C followed by incubation for 3 hrs and 6 hrs. It was observed that similar level of expression in the induced samples of SD-REP and pRSETB-SK constructs, while mild expression was noticed in the SD-parDEL samples.

The expression levels of the replaced, partially deleted and completely deleted SK constructs was analysed by 10% SDS-PAGE in the (Figure 3.30). No expression was found in the SD-comDEL construct.

The recombinant constructs SD-REP, SD-parDEL and SD-comDEL were transformed into BL21 (DE3) pLysS host and studied for their expression under various conditions as mentioned above. From the (Figure 3.31), it was noted that there was no expression of streptokinase observed in uninduced and induced samples of all the constructs in the BL21 (DE3) pLysS host.

The SK constructs (SD-REP, SD-parDEL and SD-comDEL) were further confirmed by Western blot analysis (Figure 3.32) using specific antibodies. Then caseinolytic assay (Figure 3.33) was performed for the SK clones to confirm the activity of streptokinase.
Figure 3.29  SDS-PAGE analysis of recombinant streptokinase (SD-REP, SD-parDEL and pRSETB-SK) in BL21 (DE3) at 30°C

UI- Uninduced
IN- Induced
Figure 3.30  SDS-PAGE analysis of SD-REP, SD-parDEL and SD-comDEL constructs of the recombinant SK

UI- Uninduced
IN- Induced
Figure 3.31 SDS-PAGE analysis of SD-REP, SD-parDEL and SD-comDEL constructs of the recombinant SK in BL21 (DE3) pLysS

UI- Uninduced
IN- Induced
Western blot analysis

Figure 3.32 Western blot analysis of SD-REP, SD-parDEL and SD-comDEL constructs of the recombinant protein SK

Appearance of a band corresponding to the molecular weight of 47 kDa after challenging the blot containing the sample with antibody (against streptokinase) confirms the presence of streptokinase. No band was found corresponding to streptokinase in pRSETB vector control.
Bioassay

![Image of bioassay results]

**Figure 3.33** Caseinolytic assay of SD-REP, SD-parDEL and SD-comDEL constructs of the recombinant SK

UI- Uninduced
IN- Induced

The proteins were estimated using Bradford’s assay and about 5µg of the samples were loaded into the wells and kept at 37°C. Clear zones were observed around the wells after few hours of incubation. The zones around the wells containing the uninduced samples of SD-parDEL and SD-REP constructs indicate the presence of leaky expression. Equal amount of activity was observed in the replaced and control constructs of SK. Even though no expression was observed through SDS-PAGE analysis for SD-comDEL construct, its activity was confirmed by bioassay.