Potential *E. coli* Expression Strategies for Production of Soluble Recombinant Streptokinases

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Research Article

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Abstract

The expression of recombinant protein for industrial and therapeutic applications, requires high yielding expression system like *E. coli*, which is easier to handle and satisfactory for various purposes. On other hand, one of the limitations of this system is Inclusion bodies formation. In this study, we optimized the conditions for the expression of the soluble form for three recombinant streptokinases (FSK, KSK, and FSKK). The change in culture conditions, like optical density, post-induction temperature, inducer concentration, media, and expression host had different effects on the expression levels of hybrid streptokinase proteins. The high amount of soluble fraction for FSK protein was in LB medium at 20°C in BL21 (DE3) and GJ1158. The amount of soluble protein was approximately 50%, but the amount of total protein at 20°C was lower than other induction temperatures. The high amount of soluble protein, expressed hybrid streptokinase (KSK) in LB medium at 30°C in BL21 (DE3) hosts, 0.5 O.D. cell densities were induced in 0.5mM IPTG. Approximately 70% of protein is soluble in these conditions. FSKK was never produced as a soluble form irrespective of any change in condition. All expressed FSKK protein appeared as inclusion bodies.

1. Introduction

The production of recombinant proteins (e.g. therapeutic proteins) in the Prokaryotic expression host especially *E. coli*, which is one of the challenging fields of biotechnology (Shrivastava et al. 2013; Jana and Deb 2005). Production of recombinant proteins involves, cloning the suitable gene into an expression vector, under the control of a strong promoter. But the efficient expression of the recombinant gene depends on other factors such as, optimal expression signals (the level of transcription and translation), protein folding properly, and cell growth characteristics.(Chou 2007).

Streptokinase (SK) is a potent plasminogen activator with widespread clinical use as a thrombolytic agent in the treatment of acute myocardial infarction (Collen and Lijnen 1991; Lijnen and Collen 2000). Structurally modified Streptokinases (non-specific fibrin) have been produced in recombinant DNA technology by fused finger and kringle 2 domains from tissue plasminogen activator gene (specific to fibrin) with SK gene to produce clot specific hybrid SK in *E.coli* as an expression host and T7 expression system (Buniya et al. 2014).

*Escherichia coli* has been widely used as an expression host for the production of recombinant proteins. Its popularity lies on various elements like genetics, physiology, and completes genomic sequence which highly facilitates gene cloning and cultivation(Sorensen and Mortensen 2005; Voulgaridou et al. 2013). On another hand, the expression in prokaryotic hosts does have some disadvantages. Protein expressed in massive amounts often precipitates into insoluble aggregates called Inclusion bodies, from which it can only be recovered in an active form by solubilization in denaturing agents followed by careful renaturation (Marston and Hartley 1990).
In general, the recombinant way of expressing a particular protein in *E. coli*, generate higher amounts of protein than their natural counterparts. But in most cases, bulk of protein goes into inclusion bodies. Since a strong promoter (T7) is used, it causes self-crowding of the transcripts. Thus, aggregation of target proteins is one of the major reasons for shifting of interest to choose a eukaryotic expression system, for commercial choice of protein expression (Ventura and Villaverde 2006; Prasad et al. 2011; Singhvi et al. 2020).

The inclusion bodies can be extracted from *E. coli*, by mere rupturing the cell wall by sonication, and subjecting to centrifugal force to enable sedimentation of the aggregated masses (Panda 2003). Hence, expressing proteins into inclusion bodies also aid in the purification of the target protein. There are many therapeutic proteins, that are solubilized from inclusion bodies to yield functional proteins which are successfully commercialized. (Patra et al. 2000; Singh and Panda 2005). Schein and Noteborn (1988) report, that the expression of the recombinant proteins at low growth temperature favors soluble expression. However, this may not be extrapolated for all proteins. The solubilization and extraction of the individual polypeptide from the aggregates by treating with 8M Urea/6M Guanidine HCl is a tedious process which results in losses of the recombinant protein during the process.

In this study, we attempt, for the production of soluble recombinant streptokinases by using different culture conditions.

2. Material And Methods

2.1 Preparation of Hybrid Streptokinase Constructs

The hybrid streptokinase FSK (Finger Domain + sk gene), KSK (Kringle 2 + sk gene), and FKS(K (finger Domain + Kringle 2 + sk gene) were cloning in T7 expression vector pRSETB (Invitrogen, USA). The specific primers for PCR were synthesized by Eurofins Genomics India Pvt, Ltd (India). Qiagen PCR purification kit (cat no. 28704, USA). The restriction enzymes (*Nde*I, *Kpn*I, and *Eco*RI), Taq DNA polymerase, and T4 DNA ligase were obtained from NEB (USA). The positive clones were confirmed by DNA sequencing (Buniya et al. 2014).

2.2 Expression soluble protein protocol

2.2.1. **Expression Host**: two *E. coli* strain used to express the hybrid SK: *E. coli* BL21 (DE3) (IPTG inducible) from Invitrogen (SanDiego, USA) and *E. coli* GJ1158 (NaCl inducible) from Genei (Bangalore, India). For recombinant protein expression, the constructs were transformed into an expression host, using the Mandel and Higa protocol (Mandel and Higa 1970).

2.2.2. **Culture Media**: The expression host were cultured in two types of media: LB medium (Luria–Bertani: 10% tryptone, 5% yeast extract,
10% NaCl and 0.8% glucose), and GYE media (Glucose, Yeast Extract: K$_2$HPO$_4$ 6g, KH$_2$PO$_4$ 3 g, NaCl 0.5g, NH$_4$Cl 1 g, Yeast extract 5 g, Glucose 20%, MgSO$_4$ 1M) for BL21(DE3) strain. LBON and GYEON (lacking NaCl was prepared similarly without including NaCl) for the GJ1158 strain. The media was supplemented with 100mg/ml of Ampicillin.

2.2.3. **The Cutler Optical Density**: The cells were grown at 37 °C by shaking at 200 rpm until the culture reached an O.D$_{600}$ of 0.3 and 0.6 then induced to start the target protein expression.

2.2.4. **Post Induction Temperature**: after induction, cutler tube incubated at a different temperature such as, 37, 30, 25, and 20 °C in a shaker incubator.

2.2.5. **Inducer concentration**: For induction, IPTG / NaCl was added to a final concentration of 1mM, and 0.3M respectively for 1$^{st}$ concentration, 0.5 mM and 0.15M for 2$^{nd}$ concentration.

2.3. **Expression Analysis**

The culture was centrifuged at 5000g for 5 minutes. The supernatant was discarded and the bacterial pellet containing the recombinant protein was re-suspended in 3ml of 1X phosphate-buffered saline then sonicated and centrifuged. Both supernatant and pellets were analyze, and equal concentration of total protein was loaded (40 µg) in each. The expression profiles of the constructs were analyzed by 10% SDS–PAGE as per Laemmli protocol (Laemmli 1970), and the gels were stained with Coomassie brilliant blue G-250.

3. Result

In general, the recombinant proteins form an inclusion body, when they are expressed in *E. coli*. In this work, we attempted to find out whether the hybrid streptokinases expressed from various recombinant constructs form inclusion bodies or remain as soluble proteins. To obtain them as soluble proteins, we have changed the various parameters like different growth temperatures after induction, different medium, different optical density and different concentration of inducer. Towards this, all the constructs were expressed in *E. coli* BL21 (DE3) and GJ1158 hosts. The results revealed, that KSK and FSK proteins appeared in soluble form in BL21 (DE3) when the temperature after induction was 30ºC and below, and the solubility increased, when the cultures' optical density and inducer concentration was reduced. Whereas, the expression of the same constructs in GJ1158 led to different solubility profiles. The solubility of FSK and KSK in GJ1158 was lesser than in BL21 (DE3). But the hybrid streptokinase FKS K was expressed as an insoluble form (inclusion bodies) irrespective of host strains, temperature, optical density, and inducer concentration (Table 3.1).

3.1. **Solubility of FSK**

The hybrid streptokinase (FSK) was expressed in BL21 (DE3) as a soluble form when the host was grown at 30, 25, and 20ºC in LB medium. In GJ1158, the soluble form is expressed in 30 and 25ºC.
The reduced optical density for culture, and the concentration of inducer gave the soluble form for FSK protein in 30 and 25°C (Figure 1 A) respectively.

The same clone in BL21 (DE3) and GJ1158 were cultured in GYE and GYEON medium, and used the same previous temperature. The hybrid streptokinase appeared in BL21 (DE3) in 30°C only as a soluble form (Figure 1 B), whereas the GJ1158 host did not express soluble form in same medium and temperatures (data not shown).

The reduced optical density for culture, for induction to O.D. 600 0.3 and temperature after induction to 30 and 25°C, and reduced the concentration of inducer given soluble form for hybrid streptokinase FSK, but could not find any significant change in solubility before and after reducing (Figure 1 C).

### 3.2. Solubility of KSK

The construct KSK is expressed in BL21 (DE3) and GJ1158 in LB and LBON medium as a soluble form in 30 and 25°C (Figure 2 A). Both hosts expressed soluble KSK in 30°C only when GYE medium (Figure 2 B) is used. At 20 °C, all expressed protein appeared in insoluble form (data not shown).

The change in culture conditions like culture optical density and inducer concentration in these constructs gave a high amount of soluble protein. The soluble part in the supernatant was higher, than the insoluble in the pellet, and the KSK protein was expressed as a soluble form better than FSK and FKSK (Figure 2 C). when checked the protein activity, KSK has high activity in colt lysis assay (Buniya et al. 2014).

### 3.3. Solubility of FKSK

The construct FKSK expressed in BL21 (DE3) and GJ1158 with LB and GYE medium at 37, 30, 25, and 20°C. In both hosts, and in all temperatures and mediums, FKSK fail to produce a soluble form. All expressed proteins appeared as inclusion bodies (Figure 3 A and B). The change in culture conditions did not lead to any change in solubility of hybrid streptokinase FKSK. It reduced the culture optical density, inducer concentration, and decreased temperature results insoluble protein.

### 4. Discussion

In this study, we have three recombinant proteins with different culture conditions. The changes in some parameters in culture conditions like rise in temperature after induction, optical density, and concentration of inducer, showed effects in soluble expression levels.
Inclusion bodies formation can be decreased by various methods. One of it is, decreased culture temperature after induction effect in the production of soluble protein (Schein 1989). The effect of lowering temperature is hard to pinpoint, but can be useful by permitting slower production. In some cases, this had a positive effects on solubility, especially when using the proper promoters/inducers, such as the T7 promoter (Weickert et al. 1996; Baneyx and Mujacic 2004). Many recombinant proteins from different sources are more soluble, when expressed in \textit{E. coli} at lower growth temperature (25-30 C) (Shaw and Ingraham 1967; Chesshyre and Hipkiss 1989), because the cells will grow quite slowly after induction (Schein 1990).

Generally, at higher temperatures inclusion body formation is favored in \textit{E. coli} because the high temperature increases the hydrophobic interactions, leading to aggregation (Kiefhaber et al. 1991). Lowering the cultivation temperature reduces the expression of heat shock proteases, which in turn increases the recombinant protein stability and solubility (Chesshyre and Hipkiss 1989).

In this study, reducing the inducer concentration also plays a major role in producing soluble form in recombinant protein. Several studies, refers that, high yield of soluble protein can be attained, if IPTG inducer concentration is reduced to less than 1 mM. Rabhi-Essafi et al. (2007) and Soleymani and Mostafaie (2019) demonstrated that reducing IPTG concentration with low post-induction temperature increase the production of soluble recombinant IFN\textalpha{}2B protein and recombinant bovine SRY protein in \textit{E. coli} BL21 cells, respectively.

IPTG induces T7 RNA polymerase and protein production in \textit{E. coli}. In the pRSET system with the strong T7 promoter and high inducer concentrations, product yield high proteins, this leads to an increase in misfolding and inclusion body formation (Baneyx and Mujacic 2004). Low IPTG concentration probably the strong affinity of the T7 promoter and \textit{lac} repressor occurred in a suitable form; Therefore, the production during cultivation also occurred slowly, which increases the solubility form of protein. One possible reason for this increased solubility at low IPTG concentration is the slow production which eventually results increase in solubility.

The type of host strains used to produce recombinant proteins also plays an important role in protein expression yield, and solubility. Several \textit{E. coli} strains that strongly improve membrane protein production have been engineered. \textit{E. coli} BL21 and its derivatives are widely used for the production of recombinant protein in \textit{E. coli}. These strains are deficient in the proteases Lon and OmpT, which can increase the stability of the protein. The strain BL21(DE3) contains a chromosomal copy of the T7 RNA polymerase gene for the simple and efficient expression of genes under the control of the T7 promoter (Long et al. 2014; Joseph et al. 2015). \textit{E. coli} GJ1158 strain is a simple, effective, and generally applicable method for the production of recombinant proteins in \textit{E. coli} that makes use of NaCl as an inducer replaces IPTG (Bhandari and Gowrishankar 1997).

The media composition is affected in solubility for expressed protein. LB medium is most commonly used for culturing expression hosts. \textit{E. coli} grows in low density because its low amounts of
carbohydrates contain and divalent cations (Sezonov et al. 2007). Compatible co-solvents are add during culturing, to maintain protein solubility (Schein 1999; Saluta and Bell 1999).

However, these protocols for the production of soluble forms of recombinant protein, have not yielded successful results for all recombinant streptokinases. Hence, as there is no universal strategy, for production of soluble form for all proteins, therefore, a trial-and-error method is pursued.

Declarations

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- **Funding**: No funds, grants, or other support was received.

- **Non-financial interests**: none.

- **Ethical approval**: this study has not been granted ethics committee approval prior to commencing, retrospective ethics approval cannot be obtained because we started in 2006 and ended 2010.

- **RRID**:
  - Plasmid: pRSETB: (invetrogen, USA)
  - E. coli BL21(DE3): (invetrogen, USA)
  - E. coli GJ1158: Genei (India)

References


Tables

**Table 3.1** Consolidated data of solubility profile for expression of hybrid streptokinase in BL21 (DE3) and GJ1158 with different conditions
<table>
<thead>
<tr>
<th>Clone</th>
<th>Host</th>
<th>Medium</th>
<th>GYE medium</th>
<th>LB medium</th>
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(+): soluble protein
(-): insoluble protein

**Figures**

**Figure 1**

A


B

SDS-PAGE analysis of FSK protein in BL21 (Lysis cells) in GYE medium at 30, 25 and 20º C, 1- FSK Induced (whole cells), 2- Induced sup in 30º C, 3- Induced pellet in 30º C, 4- Induced sup. In 25º C, 5- Induced pellet in 25º C, 6- Induced sup. In 20º C, 7- Induced pellet in 20º C.
C

Analysis for solubility profile of hybrid streptokinase FSK in BL21 (DE3) in LB medium at 30 and 25º C (0.3 O.D. And 0.5 mM IPTG). 1- FSK induced, 2- FSK supernatant at 30° C, 3- FSK pellet. at 30° C, 4- FSK induced, 5- FSK supernatant at 25° C, 6- FSK pellet. at 25° C.

Figure 2

A


B

Analysis for solubility profile of hybrid streptokinase KSK in BL21 (DE3) and GJ1158 in GYE medium at 30, 25 and 20ºC.


C

Analysis of KSK protein in BL21(Induced, sup. And pellet), 0.3 OD, 0.5 + 1.0 mM IPTG LB medium at 30 and 25º C.

1- KSK sup. 0.5 mM IPTG at 30 C, 2- KSK pellet 0.5 mM IPTG at 30 C, 3- KSK sup. 1.0 mM IPTG at 30 C, 4- KSK pellet 1.0 mM IPTG at 30 C, 5- KSK sup. 0.5mM IPTG at 25 C, 6- KSK pellet 0.5 mM IPTG at 25 C.

Figure 3

Analysis of solubility profile for hybrid streptokinase FKS in BL21 (DE3) and GJ1158 in LB medium (A), GYE medium (B)

1- BL21 sup. 30 C, 2- BL21 pellet 30 C, 3- GJ1158 sup. 30º C, 4- GJ1158 pellet 30º C, 5- BL21 sup. 25º C, 6- BL21 pellet 25º C, 7- GJ1158 sup. 25º C, 8- GJ1158 pellet 25º C, 9- BL21 Induced sup. 20º C, 10- BL21 Induced pellet 20º C, 11- GJ1158 Induced sup. 20º C, 12- GJ1158 Induced pellet 20º C.