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Production of Recombinant Streptokinase in *E. coli* and Reactivity with Immunized Mice

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Abstract: Streptokinase (SK) is a potent plasminogen activator with widespread clinical use as a thrombolytic agent. In this study, we produce high level expression of recombinant streptokinase in *E. coli* by expression vector pET32a. Genomic DNA of streptokinase gene (SKC) was extracted, then amplified by polymerase chain reaction (PCR) method and sub-cloned to prokaryotic expression vector pET32a. *Escherichia coli* BL21 (DE3) pLysS were transformed with pET32a-skc and gene expression was induced by IPTG. The expressed protein was purified by affinity chromatography by Ni-NTA resin. High concentration of the recombinant protein obtained from the single-step purification by affinity-chromatography (Ni-NTA). The yield of recombinant streptokinase was nearly 470 mg L^{-1} of initial culture. Our data showed that production of recombinant streptokinase improved by pET32a in *Escherichia coli*.

Key words: Streptokinase, cloning, recombinant, pET32a vector, *Escherichia coli*

INTRODUCTION

Thrombosis, the blockage of blood vessels with clots, can lead to acute myocardial infarction and ischemic stroke, both leading causes of death. Other than surgical interventions to remove or by pass the blockage, or the generation of collateral vessels to provide a new blood supply, the only treatment available is the administration of thrombolytic agents to dissolve the blood clot (Kunamneni and Ellaiah, 2007).

The choice of a thrombolytic agent during therapy is dictated by a number of factors, which depends essentially upon the relative merits and demerits of individual PG activators. These include the cost of the drug, the side-effects and their severity, *in vivo* stability and specificity towards fibrin clots and immunological reactivity (Menon *et al*., 2009).

Streptokinase (SK) is a potent plasminogen activator with widespread clinical use as a thrombolytic agent. It is naturally secreted by several strains of beta-haemolytic streptococci. The enzyme streptokinase has been most frequently associated with hemolytic streptococci of the Lancefield group A, human C and G, with the C group being preferred. In particular, the strain H46A (identified by the American Type Culture Collection, Rockville, Md. As No. 12449, Rebecca C. Lancefield strain H46A, 1956) is the most frequently employed strain. Native streptokinase is a single chain polypeptide with a molecular weight of 47 kDa. The protein consists of 415 amino acid residues in a single polypeptide chain.

The gene coding for streptokinase skc, from *S. equisimilis* H46A was expressed in several heterologous gram positive and gram negative bacteria (Steiner and Malke, 2002; Kaur *et al*., 2007).

Some studies have focused on recombinant production or optimizing the vector and the level of expression of recombinant streptokinase with low level (Jafari and Mirshahi, 2007; Reza *et al*., 2007). Therefore, in this study, we produced recombinant streptokinase with some modification in method.

MATERIALS AND METHODS

Bacterial strains and plasmids: *Streptococcus dysgalactiae* subsp. *equisimilis* used as the source of chromosomal DNA for the Polymerase Chain Reaction (PCR). *E. coli* DH5α (Stratagene) (f-gyr A96 Nalr, recA1 relA1 Thi-1 hsdR17 r'k m+k) was used as the primary host for the construction and propagation of plasmids. For recombinant protein production, a prokaryotic expression

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vector pET32a (Novagene) was used. This vector enables to expresses a fusion protein with a six histidines tag, a thrombin recognition site and a T7 tag at the N-terminus. These additional amino acids increase the size of expressed protein near 15 kDa. The recombinant pET32a (pET32a-SKC) is transformed in *E. coli*, BL21 (DE3) pLysS (f ompt hsdB, rB⁻ mB⁻, dcm gal, DE3, pLYsS cmr) as host strain. LB agar and broth used for routine bacterial culture. The required antibiotics were added to media according to references recommendation.

Isolation of chromosomal DNA: After overnight incubation of *Streptococcus dysgalactiae* subsp. *equisimilis* of the group C in BHI broth at 37 °C, Bacterial cells were centrifuged at 5000 rpm for 2 min and the pellet was re suspended in 567 μL of TE buffer. Chromosomal DNA prepared according to standard CTAB/NaCl method. Briefly, after resuspend the pellet of 1.5 mL overnight bacterial culture in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8), the bacterial cell was lysed by SDS and proteinase K, the chromosomal DNA was extracted by CTAB/NaCl solution (10% CTAB And 0.7 M NaCl). Remove the cell debris and proteins by two times phenol/chloroform/isoamylalcohole (25:24:1) mixture. DNA was precipitated by isopropanol and washed in ethanol (70%), air dried and then resuspend in TE buffer. Quality and quantity of purified genomic DNA was assayed by 0.8% Agarose gel electrophoresis in 1x TBE buffer and spectrophotometrically (260/280 nm), respectively (Sambrook *et al*., 2001).

Primers design: Primers were designed according to published sequence for ribosomal protein of *Streptococcus dysgalactiae* subsp. *equisimilis* (accession No.: K02986). The forward, primer (TCG GAT CCA TTG CTG GAC CTG AGT G -3') starts from the beginning of the gene and contain BamHI site. Reverse primer, (5'-GAC TCG AGG TTA TTT GTC GTT-3') contain recognition site for XhoI Streptokinase (SK) is a potent plasminogen activator with widespread clinical use as a thrombolytic agent. It is naturally secreted by several strains of beta-haemolytic streptococci. The enzyme streptokinase has been most frequently associated with hemolytic streptococci of the Lancefield group A, human C and G, with the C group being preferred. In particular, the strain H46A (identified by the American Type Culture Collection, Rockville, Md. As No. 12449, Rebecca C. Lancefield strain H46A, 1956) is the most frequently employed strain. Native streptokinase is a single chain polypeptide with a molecular weight of 47 kDa. The protein consists of 415 amino acid residues in a single polypeptide chain.

The restriction enzyme sites (underlined) added to the primers according to restriction enzyme pattern of the gene (SKC) and multiple cloning site of the cloning and expression vectors for subsequent cloning procedure.

Gene amplification of SKC: PCR was performed in a 50 μL total volume containing 500 ng of template DNA, 1 μM of each primers, 2.5 mM Mg^{2+}, 200 μM (each) deoxynucleoside triphosphates, 1X PCR buffer and 2.5 unit of pwo DNA polymerase (Roche). The following conditions were used for amplification: hot start at 94°C for 5 min, followed by thirty cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min and extension at 72°C for 1 min. The program followed by a final extension at 72°C for 5 min. The PCR product was analyzed by electrophoresis in 1% Agarose gel in 1x TBE buffer and visualized by Ethidium bromide staining on UV transilluminator. The PCR product was purified from the Agarose gel by high pure PCR product purification kit (Roche) according to manufacturer recommendation. Purity of eluted PCR product checked by electrophoresis in 1% agarose gel in 1× TBE buffer.

Cloning of SKC gene in bacterial expression vector: The PCR product was digested with BamHI and XhoI and ligated to pET32a, which digested by the same restriction enzymes, by T4 DNA ligase (Cinagen) at 16°C over night. *E. coli* DH5α and *E. coli* BL21 (DE3) plysS competent cells prepared by Calcium chloride method and were used for transformation of pET32a-SKC plasmid.

The transformed bacteria were selected by screening the Colonies on antibiotic containing media and plasmid purification. The suspected colony further analyzed by restriction enzyme digestion and PCR.

Expression and purification of recombinant SKC: *E. coli* BL21 (DE3) pLysS was transformed with pET32a-SKC and grown in 2 mL LB broth supplemented with Ampicillin (100 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}) at 37°C with agitation.

A colony contained recombinant plasmid was cultured on shaking incubator for overnight at 37°C in 2 mL LB medium containing 100 μg mL^{-1} Ampicillin and 34 μg mL^{-1} chloramphenicol. The next day, 500 μL of culture was removed and inoculated in 50 mL Luria-Bertani broth (per litre: 10 g yeast extract (Difco), 20 g Bactotryptone broth (Difco), 0.2% (mass/vol.) glucose,

10 g NaCl, 1 g KCl, 0.5 g MgCl, 0.5 g CaCl, 100 mg ampicillin) and incubated at 37°C, shaking at 200 rpm with vigorous agitation to an absorbance of 0.5-0.8 at 600 nm.

Expression of the SKC protein was then induced by the addition of isopropyl-thio-P-D-galactoside (IPTG) to a final concentration of 1 mM and incubation was continued for a further 4 h.

The expressed protein was purified using Ni-NTA column according to Manufacture instruction (Qia- gene). The purified protein was dialyzed twice against PBS (pH 7.2) at 4°C over night. The quality and quantity of purified recombinant SKC Protein was analyzed by SDS-PAGE (15%) and Bradford methods, respectively (Sambrook *et al*., 2001).

Antigenicity of recombinant streptokinase and Immunoblot analysis: We injected subcutaneous 100 μL of an emulsion containing 50 μg of streptokinase (Behring) and equal volume of complete Freund's adjuvant (Sigma, St. Louis, Ma) at 3 week intervals to five mice. Sera separated and used as primary antibody.

For Western blot analyses, 0.5 μg of purified recombinant SKC protein was used per well. The gel were blotted on to Polyvinylidine difluoride (PVDF Membrane, Roche) membrane using transfer buffer containing 25 mM Tris (pH = 8.3), 192 mM glycine and 20% methanol at 90 volts for 1.5 h at 4°C. The blotted membrane was blocked with 2.5% (w/v) BSA in TBST buffer (0.5 M NaCl, 0.02 M Tris pH 8.5, 0.05% tween 20) for 1 h at room temperature. Membranes were incubated for 2 h at room temperature with diluted mouse sera, 1:100 from immunized and normal mice. After reactions with the primary antibody, the blots were washed three times with TBST and incubated with peroxidase conjugated goat anti-mouse IgG (Bioscience, Cat No. GM5201-5) at a 1:2500 dilution in TBST. The blots were then washed three times with TBST and reactions were developed by diamino Banzidine (DAB, Roche) Solution (Abtahi *et al*., 2004).

RESULTS

DNA preparation and amplification: The chromosomal DNA of *Streptococcus dysgalactiae* subsp. *equisimilis* was prepared and the concentration was adjusted to 500 μg mL^{-1} which was used as template for amplification of the gene encoded SKC. The Amplified fragment had the expected size of 1323 bp comparing to 100 bp DNA ladder.

The recombinant Plasmid (pET32a-skc) was sequenced by standard T7 primer and dideoxy chain termination method. The sequencing result was confirmed by comparing with databases and Using basic local alignment search tool (BLAST) Software (data not shown).

Expression and purification of recombinant SKC: pET32a-SKC in *E. coli* BL21 pLysS was induced and the expressed protein was Purified by Ni-NTA column (Fig. 1). SDS-PAGE analyses, showed the expected molecular Mass of near 60 kDa recombinant protein.

The concentration of recombinant protein was assayed and calculated to 470 mg purified protein per liter of the initial culture.

Immunoblotting analysis: To determine the antigenicity of recombinant SKC in mice that immunized with Commercial streptokinase (Bohring), the recombinant SKC was assayed by western-blotting. The anti streptokinase mice sera were used. Figure 2 shows the specific interaction between standard mice antibody and purified recombinant SKC protein. A normal serum from mouse used as a negative control.

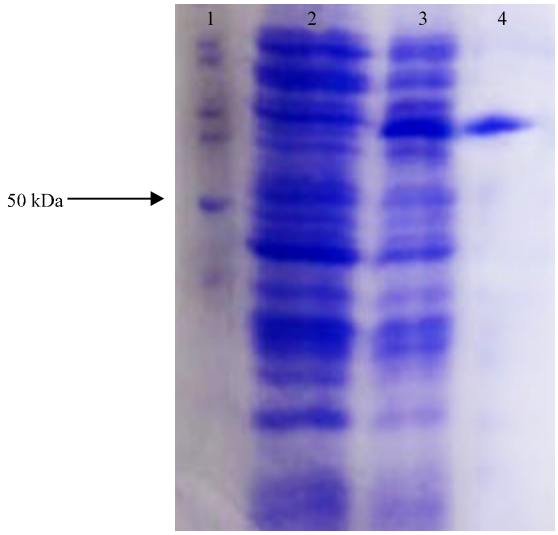

Fig. 1: Ni-NTA purification of recombinant streptokinase produced in *E. coli* BL21(DE3)plysS; Proteins were resolved by SDS-PAGE on 15% of polyacrylamide gel and stained with Coomassie brillant blue. Lane 1: Marker; lane 2: Uninduced cells carrying expression vector; lane 3: Iinduced cells with IPTG; lanes 4: Extract proteins after Ni-NTA affinity chromatography

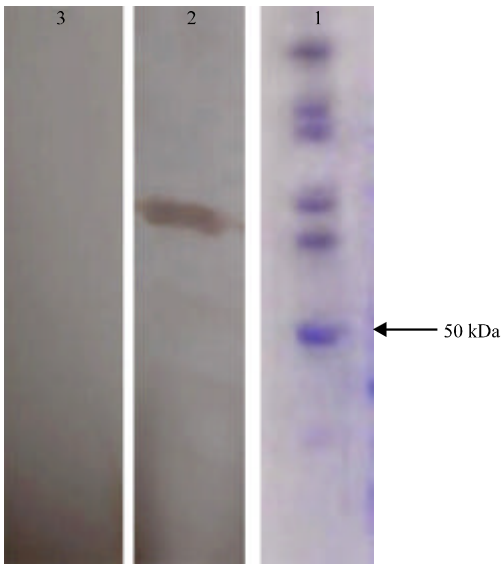

Fig. 2: Western blot analysis result, lane1: Marker, lane 2: Western blot result of purified streptokinase, lane 3: Control negative

DISCUSSION

A failure of hemostasis and consequent formation of blood clots in the circulatory system can produce severe outcomes such as stroke and myocardial infarction. Pathological development of blood clots requires clinical intervention with fibrinolytic agents such as urokinase, tissue plasminogen activator and streptokinase (Banerjee *et al*., 2004).

The low yields obtained in SK production and the pathogenesis of its natural host has been the principal reasons to search for a recombinant source for this important therapeutic protein.

In several study, recombinant streptokinase were produced by different vectors and hosts. The expression and subsequent secretion of SK have been studied in several heterologous hosts like *Escherichia coli*, Bacillus subtilis and Pichia pastoris (Baneyx, 1999; Baneyx and Mujacic, 2004).

Selection of an appropriate expression system is dependent on the characteristics and intended application of the recombinant protein and is essential to produce sufficient quantities of the protein. Over the last 30 years, there have been considerable advances in the technologies for expressing recombinant proteins (Brondyk, 2009).

Escherichia coli is widely used as an expression host for the production of recombinant proteins, both in research and industry (Baneyx, 1999).

In the present study we used *E. coli* BL21 (DE3) plysS; this strain is deficient in the known cytoplasmic protease gene products, such as Lon, OmpT, DegP or HtpR, in order to give high level expression of fusion proteins (Sugimura and Higashi, 1988). Therefore the highest expression of streptokinase in *E. coli* BL21 (DE3) plysS could be due to protease deficiency in this strain. The pET system has been recognized as one of the most powerful methods for producing recombinant proteins in *E. coli* and the significant advantages of this system have been widely discussed. All pET vectors are available in three reading frames. The plasmid contains the f1 origin of replication and the T7 lac promoter using IPTG as the inducer (Terpe, 2006).

The maximum amount of expression reported has been approximately 150 mg of pure recombinant protein under the control of Ptac promoter (pMAL) system per liter of initial bacterial culture (Jafari and Mirshahi, 2007). In this study, we have sub cloned and expressed skc gene that encodes streptokinasel protein under the control of T7 promoter and optimized condition. The expression level was increased to 470 mg of purified protein per liter of initial bacterial culture. In agreement with other researcher our results also indicates that the highly regulated expression vector and powerful T7 promoter (pET32a,), suitable host cell (*E. coli*, BL21, DE3, pLysS), optimizing the growth condition and controlling the parameters which influence the induction are the most important factors in increasing the expression of this protein.

Expressions of protein in pET system add several amino acids such as 6xHis tag and T7 tag to the C or N terminal of recombinant peptide. These additional amino acids cause increasing near 13 kDa to the size of synthesized peptide, as seen in Fig. 1, these additional aminoacids may interfere with the subsequent immunological analyses also. In order to investigate the effect of additional amino acids in immunological analyses, an empty vector in *E. coli* (BL21, DE3, pLysS) also induced by IPTG and further analyzed by Western blotting (Abtahi *et al*., 2004). The results showed that there is no interfere related to fused amino acids.

In other studies, SKC was produced by plasmids that have fusion proteins. These plasmids (such as pGEX and pMAL) have sequences that inserted 22 -25 kDa proteins to rSKC. These fusion proteins effect on production and antigenicity of rSKC (Jafari and Mirshahi, 2007; Reza *et al*., 2007).

Present data showed that recombinant SKC protein can be detected as an antigen by serum in immunized mouse. Therefore, Recombinant SKC has same epitopes with natural form of this antigen. Recombinant SKC also

seemed to be a promising antigen for the serologic diagnosis of animal streptococci group C infections.

CONCLUSION

The results of this study indicate the T7 lac promoter is might stronger than other promoters can induce production of rSKC. Vectors have not fusion protein or have little weight fusion protein might product recombinant protein further than vectors that have high weight fusion protein. Recombinant SKC is immunogenic antigen, thus, we can use it in diagnosis of animal streptococci infections.

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