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ORIGINAL ARTICLE

Optimized Expression and Activity Assessment of Bacterial Staphylokinase in *E. coli* BL21(DE3)

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ABSTRACT

Background*: The application of recombinant proteins is rare following the high production costs of expressing proteins with expensive inducers, such as isopropyl-β-D-1-thiogalactopyranoside (IPTG). Staphylokinase (SAK), a fibrinolytic enzyme, is a small bacterial thrombolytic agent that specifically clots and converts plasminogens to plasmins and lysis fibrin clots.* ***Objective****: The primary aim of the present investigation is increasing the yield and lower the cost of staphylokinase production using Escherichia coli BL21 (DE3).* ***Methodology****: The influence of target protein in expression host by different culture medium, culture density, and IPTG concentration on the expression of SAK protein was explored.* ***Results****: The results indicated that only the culture density and concentration of IPTG were significant. This study achieved cost reduction by decreasing the IPTG inducer concentration (1.0 and 0.5mM), which acted as the inducer. The production rate was also maintained or increased in low culture density.* ***Conclusion****: Suitable production conditions, particularly diminished inducer concentration, effectively reduced upstream production costs and yielded high sak gene expression protein as an active form.*

INTRODUCTION

By Castellino[1], fibrinolysis entails the degradation of fibrin blood clots in blood vessels by an enzyme system found in all mammalian blood. A dormant proenzyme designated plasminogen, which can transform into an active enzyme, plasmin, a trypsin-like serine protease that breaks down fibrin into soluble fibrin degradation products, are components of the blood fibrinolytic system[2].

Fibrinolysis is the process of an enzyme system found in the blood of mammalian species, dissolving a fibrin blood clot in the blood arteries. The Fibrinolytic system is an enzyme system that is the natural equivalent of the blood coagulation system. The blood fibrinolytic system consists of plasminogen, an inactive proenzyme that may be transformed into plasmin, a trypsin-like serine protease that degrades fibrin into soluble fibrin degradation products by the various plasminogen activators (PAs). Plasminogen activators as a group of enzymes that are used in treating cardiovascular and cerebrovascular obstructions like Acute myocardial infarction, pulmonary embolism, arterial deep vein thrombosis, and retinal artery thrombosis. Fibrinolytic agents offer a main approach to the arrangement of treatments of thrombotic diseases[3,4].

Inactive plasminogens are converted to fibrinolytic plasmins via a limited proteolytic cleavage mediated by various plasminogen activators (PAs)[5]. PAs are classified as "non-fibrin specific", for example, streptokinase, anisoylated plasminogen streptokinase activator complex (APSAC), and two-chain urokinase-type plasminogen activator (tcu-PA), or "fibrin specific", which includes tissue-type plasminogen activator (t-PA) and single-chain u-PA (scu-PA), and staphylokinase (SAK)[6]. The majority of *Staphylococcus aureus* strains produce a protein SAK. The protein possesses fibrinolytic properties, The SAK is of 15.5 kDa molecular weight, consists of 163 amino acids, and is encoded by the *sak* gene of 489 base pairs[7,8]. Its three-dimensional structure characteristics feature a central alpha-helix lying across a five-stranded beta-sheet[9].

Studies on the SAK protein revealed its action mechanisms as a fibrinolysis factor. The protein is not an enzyme but forms a one-to-one stoichiometric complex with plasmins that activate other plasminogen molecules. SAK reacts poorly with reaction plasminogen in plasma when added to fibrin clot-containing human plasma. Conversely, the protein demonstrated high affinity towards plasmin traces on clot surfaces, converting them into plasmin-SAK complex, which efficiently activates plasminogen to plasmin. The half-life of SAK in plasma is 6.3 min[10,11].

The manufacturing process and purification of SAK employing *sak* gene coding clones across different expression systems, such as *Escherichia coli* (*E. coli*) and yeast, has been extensively investigated[12,13]. SAK has also been utilized to convert streptokinase from non-

specific clots to specific clots by producing staphylokinase- streptokinase recombinant protein[14]. In this study, the *sak* gene was cloned and expressed in varying culture conditions with *E. coli* BL21(DE3) under the T7 expression system to get optimal conditions for low-cost and active-form protein.

METHODOLOGY

Bacteria, a vector, and reagents

A competent *E. coli* BL21 (DE3) strain [NEB, USA - C2527H) was employed as the expression host in this study. Either a 1.5% Luria Bertani agar supplemented with 100 mg/mL ampicillin or a Luria Bertani broth (LB) comprising 10% tryptone, 5% yeast extract, and 10% sodium chloride (NaCl) have been employed to grow the bacteria.

The *sak* gene has been cloned in the current study by employing pGEM®-3Zf (+) expression vector from Promega, USA. Restriction enzymes *Bam*HI and *Eco*RI (Promega, USA) were also utilized to restrict digestions in the buffers recommended by the manufacturer for the respective recombinant DNA.

Bioneer (Korea) furnished the AccuPower® polymerase chain reaction (PCR) Premix that was employed in the present study, and Promega (USA) contributed the T4 DNA ligase. Geneaid (Taiwan) contributed the PCR purification kit, and Eurofins Genomics India Pvt. Ltd. (India) generated the PCR oligos.

Constructing and molecular cloning the *sak* gene

The present study employed the SAK F1 (5'-CGC*GAATTC*TCAAGTTCATTCGAC -'3) and SAK R1 (5'- CCG*GGATCC*TTTCCTTTCCTATAACAAC –'3) gene-specific primers to amplify the 489 bp *sak* gene at 56°C [15], before recording its sequence in the National Centre for Biotechnology Information (NCBI) database. The *Eco*RI and *Bam*HI restriction sites were present in the forward and reverse primers, respectively. The PCR amplicons were thereafter ligated into the pGEM®-3Zf (+) expression vector after having been digested by the same enzymes.

Expression analysis of the constructs

According to Mandel and Higa's position[16], the constructs accomplished in this investigation were inserted into the protein's expression host, *E. coli* BL21 (DE3), for SAK protein expression. The transformants were first cultivated in a Luria-Bertani (LB) medium at 37°C and shaken at 200 rpm. After that, the culture was incubated for 4 hours containing isopropyl-D-1-thiogalactopyranoside (IPTG) to promote the expression of the target protein. The expression sequences of the constructs were evaluated using a 12% SDS-PAGE (Solar Bio, China), and the gels were then stained with Coomassie brilliant blue R-250.

Protein protocol expression

Culture medium

The expression host was cultured in LB (10% tryptone, 5% yeast extract,10% NaCl, and 0.8% glucose) and GYE (6 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 5 g yeast extract, 20%glucose, and $MgSO_4$ 1 M) media. Both media were supplemented with 100µg/ml of ampicillin.

Optical density

To obtain 0.3 and 0.6 absorbance and O.D600 optical density (T80 Visible/UV Spectrophotometer; PG Instruments Limited), the cells were grown in the current investigation at 37°C and 200 rpm/ min. The cells were then stimulated to start expressing the target protein.

Inducer concentration

For induction in this investigation, IPTG was administered to final concentrations of 1 and 0.5 mM.

Biological activity

Activities of the expressed SAK were determined through a caseinolytic plate assay[17]. The caseinolytic-agarose base for the investigation was developed by melting 90 mg of agarose in 9 ml of a buffer solution consisting 50 mMTris-HCl, 150 mMNaCl, 1% plasminogen, and 1% skim milk. Subsequently, the solution gelled, and 5 mm-diameter wells were formed on the caseinolytic-agarose base. The wells were filled with 50 µL of the lysis-induced cells. Cells lysis done by sonication (Intelligent Ultrasonic processor [FAITHFUL], China. Then incubated for 24 h at 37°C before observing the development of clear zones in the wells.

RESULTS

Generally, *sak* genes are expressed in different expression hosts, including *E. coli*. The current study assessed whether SAK could be expressed under various conditions before evaluating the activities of the expressed protein. The 489 bp *sak* gene, which generates the SAK protein, was successfully amplified in this work using certain primers (Figure 1). The PCR products were then confirmed via sequence alignments in the NCBI database before being submitted to NCBI with the accession number LC626454.1.

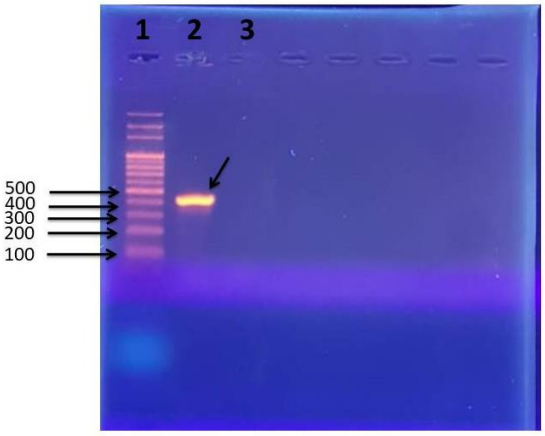

Fig. 1: Agarose gel electrophoresis of PCR products for *sak* gene (1.5%, 70 V/cm^2, 1 hour). lane 1- 100bp DNA ladder, lane 2- *sak* gene, lane 3- Negative control.

In advance of being put through restriction digestion with *Eco*RI and *Bam*HI, the amplicons were purified. Complementary enzymes were employed to digest and linearize the pGEM®-3Zf (+) expression vector beforehand it was ligated with a T4 DNA ligase. The ligation products were subsequently incorporated into *E. coli* BL21 (DE3) before IPTG was applied to stimulate the constructs for expression. The expression profile of the SAK protein (16 kDa) in *E. coli* BL21 (DE3) observed in a 12% SDS-PAGE is illustrated in Figure 2 a, b.

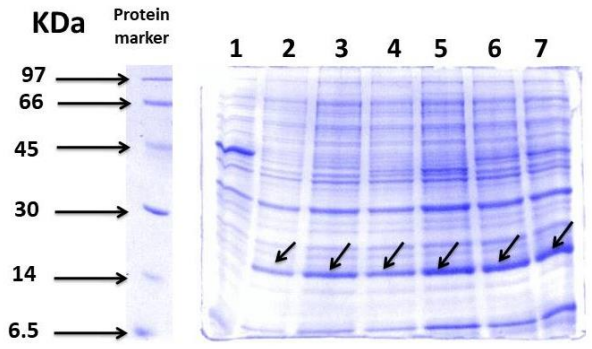

Fig. 2 a. Staphylokinase protein expression analysis in *E. coli* BL21 (DE3) in LB medium. The cell lysate samples were examined using Coomassie brilliant blue R250 and 12 % SDS-PAGE. **Lanes:** 1-*E coli* BL21(DE3) without clone, 2- 7 SAK protein in various concentration of IPTG and Culture Optical density at OD_{600} (2- Un-induced culture, 3- 1mM+0.6, 4- 1mM+0.3, 5- 0.5mM+0.6, 6 and 7- 0.5mM+0.3).

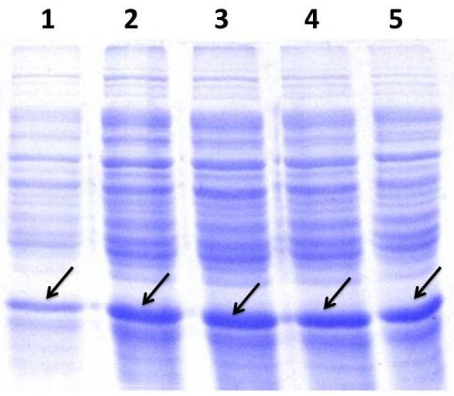

Fig. 2 b. Staphylokinase protein expression analysis in *E. coli* BL21 (DE3) in GYE medium. The cell lysate samples were examined using Coomassie brilliant blue R250 and a 12 % SDS-PAGE**,** and various concentration of IPTG and Culture Optical density at OD_{600} (1- Un-induced culture, 2- 1mM+0.6, 3- 1mM+0.3, 4- 0.5mM+0.6, 5- 0.5mM+0.3).

Protein expression was measured via protein profile in SDS-PAGE (figure 2 a, b) and biological activities for expressed protein by caseinolytic plate assay (Figure 3).

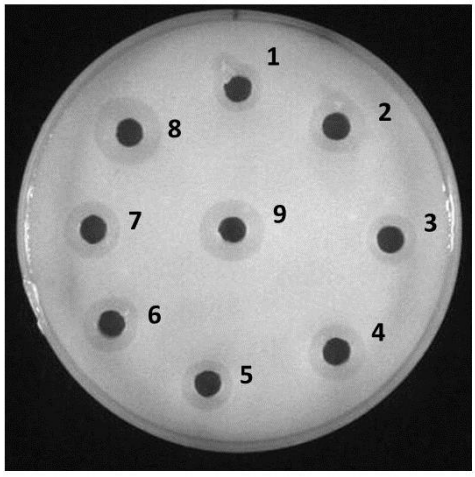

Fig. 3: Caseinolytic test. 1-4 GYE medium (1- 1mM+0.6, 2- 1mM+0.3, 3- 0.5mM+0.6, 4- 0.5mM+0.3), 5-9 LB Medium (5- 1mM+0.6, 6- 1mM+0.3, 7- 0.5mM+0.6, 8- and 9- 0.5mM+0.3)

Alterations in the parameters applied resulted in *sak* gene expression level changes. Low culture optical density (0.3 $O.D_{600}$) and low inducer concentration (0.5 mM and 1 mM IPTG) led to increased SAK protein levels with high activity (Figures 2, a, b and 3). Conversely, different cultural media produced no effects on the expression levels.

DISCUSSION

Staphylokinase (SAK) is one such factor that is secreted by most lysogenic *S. aureus* strains. In the blood, SAK protein achieves its function primarily by forming a plasminogen activating complex together with plasmin itself, initiating the fibrinolytic cascade to help the invading bacterium move deeper into tissues. Consequently, SAK has been intensively studied and it has been developed as a novel thrombolytic drug to treat myocardial infarctions and thrombosis[9].

The *sak* gene has been cloned and expressed in different expression systems, including *E. coli* BL21, *E. coli* GJ1158, *E. coli* DH5α, *Bacillus subtilis*, and *Pichia pastoris* yeast to varied levels[17-21]. SAK is one of the third-generation thrombolytic agents, which is specific to clotting. Moreover, the protein possesses the potential as a cost-effective thrombolytic agent with the least side effects[22].

Figure 3 shows the ability of the SAK protein to activate the plasminogen, which in turn cleaves the casein to form the zone of clearance. In the plate, each well-loaded 50µl of total cell protein samples, and then kept at 37ºC for 24 hours. The SAK protein was able to create a clearance zone in the caseinolytic assay by activating the plasminogen. The diameter of the zone depended on the amount of expressed active protein.

Selecting a suitable expression system to manufacture recombinant protein is essential in therapeutic protein production. A bacterial expression system is optimal for producing numerous recombinant proteins, as it allows the manipulation of several parameters to obtain a high target protein yield[23]. Typically, easily expressed proteins are assembled in *E coli*.

Numerous studies reported the effects of IPTG concentration on gene expression in *E. coli* hosts with a T7 expression system. This study obtained similar results as when normal induction concentration was utilized. The finding could lead to cost-effectiveness in research. Nonetheless, the conditions to manufacture soluble SAK proteins are required in future studies.

Reducing inducer concentration increased the expression rate for recombinant protein in its active form[24-26]. Furthermore, low IPTG concentration might result in the strong affinity of T7 promoters and *lac* repressors, thus producing an appropriate form. Consequently, production during cultivation occurred slowly, which improved with culture density. The high inducer level also led to the high protein expressed, which formed inclusion bodies, hence inactivating the proteins[27].

Culture media are necessary to optimize protein expression levels, The compositions for culture growth are easier to manipulate[28]. Different types of nutritional factors influence the production of recombinant SAK variants[18]. Some components could improve the concentration of desired recombinant proteins, which is essential for the correct folding and for preventing the inclusion bodies formations[29,30].

CONCLUSION

The present study successfully demonstrated that reduced inducer concentration produced similar results as employing the normal concentration for induction. The findings could lead to cost-effectiveness in future studies. Nevertheless, the conditions to obtain soluble *sak* protein still require investigation in future work.

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Declarations

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

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