How to Cite:
Hameed, A. K., Al-Hayawi, A. Y., & Buniya, H. K. (2022). Cloning and expression of native streptokinase in E. coli BL21(DE3). *International Journal of Health Sciences*, *6*(S4), 8406–8412. https://doi.org/10.53730/ijhs.v6nS4.10582

Cloning and expression of native streptokinase in E. coli BL21(DE3)

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Abstract*---Streptokinase (SK)*** is a potent plasminogen activator naturally produced by beta-hemolytic *streptococcus* bacteria. *Streptokinase* is one of the most important thrombolytic agents that was frequently employed treatment of myocardial infarction. In this study, we amplified the *SK* gene isolated from *Streptococcus pyogenes*. The sequence of SK was recorded in the NCBI database. The Accession number of the SK sequence was LC625519.1. The SK gene was digested by *EcoR1* and *Hindlll*, ligated with the linear pGEM®-3Zf (+) vector, and then introduced into an expression host E. *coli* BL21(DE3). For the production of the SK protein, we induced by 1mM IPTG. SDS-PAGE was used for the evaluation of the streptokinase synthesis with a molecular weight of around 47 kDa. Using the casein lysis method and the in vitro clot lysis assay, the activity of native SK was assessed. The native *SK* has clot lysis activity and a clear zone in the casein plate.

***Keywords*---**streptokinase, SK protein, E. coli, expression.

Introduction

Clot formation is a crucial procedure that avoids excessive blood vessel bleeding. *Streptokinase* (*SK*) is a thrombolytic medication and enzyme produced by a type of bacteria known as beta-hemolytic streptococci **(**Abed *et al.,* 2019**)**. It is not a plasminogen activator by itself, but it binds to plasmin or free circulating

plasminogen to form a complex that can change other plasminogen into plasmin. **(**Baruah *et al.,* 2006)**.** Many pathogenic strains of hemolytic streptococci naturally release streptokinase (SK), a single chain (47 kDa) extracellular protein with 414 amino acids and a 26 amino acid signal peptide. As a common thrombolytic therapy, this protein is extremely important clinically. (Aghaeepoor *et al.,* 2019**)**.Plasminogen is indirectly activated by streptokinase. It first assembles an I: 1 stoichiometric complex with the zymogen plasminogen, but this complex then transitions, exposing the active site without obvious peptide bond cleavage. This plasminogen streptokinase complex modification functions as a plasminogen activator. (Verstraete and Collen, 1986; Bhattacharya *et al.,* 2012**)**. Other streptokinase-plasminogen molecules can also be subjected to limited proteolysis by the activator complex, which results in the conversion of plasminogen to plasmin. As streptokinase breaks down into smaller fragments, the activator's activity gradually decreases (Joubert, 2021**)**. The present study is to produce native SK in *E. coli* BL21(DE3) and check its biological activity.

Materials and Methods

Strain of bacteria, vector and reagents

The *SK* gene was cloned using pGEM®-3Zf (+), a T7 expression vector (Promega, USA). The native SK was expressed in the *E. coli* BL21 (DE3) strain expression host (NEB, USA). The restriction enzymes *EcoRI* and *HindIII* were used to accomplish restriction digestions from Promega, USA, and NEB, USA in the manufacturer's recommended buffer for respective constructs. Taq DNA polymerase from Bioneer (Korea). T4 DNA ligase was procured from Promega (USA). We bought a PCR purification kit from Geneaid (Taiwan).

Construction and Molecular cloning of the SK gene and vector

Using the gene-specific primers SK F1 and SK R1 (Table1) and an annealing temperature of 58 °C, the 1.3-kb SK gene was amplified by PCR (Buniya *et al.,* 2014). The sequence of SK was recorded in the NCBI database. The Accession number of the SK sequence was LC625519.1. *EcoR1* and *Hindll* restriction sites were present in the forward and reverse primers, respectively. After being digested by the restriction enzymes *EcoRI* and *Hindlll*, the PCR amplicons were ligated into the pGEM®-3Zf (+) expression vector, which had previously been digested by the same enzymes.

Table 1: Oligonucleotides used in this study

Target	Primer	Primer Sequence 5′ ′3 ⟶	Product size (bp)
SK	F1	5′ GGGCGAATTCATTGCTGGACCTGAGTGGC ′3	1300
	R1	5′ CCGAAGCTTTTATTTGTCTTTAGG ′3	

Expression analysis of the constructs

The constructs were transformed into the expression host *E. coli* BL21 (DE3) for SK protein expression following the Mandel and Higa technique (Mandel and Higa, 1970). The Luria-Bertani medium was used to cultivate the transformants (LB: 10 % tryptone, 5 % yeast extract, and 10 % NaCl). Ampicillin at a concentration of 100 µg/ml was added to the media. Shaking the cells at 200 rpm and 37 °C until the culture achieved an O. D600. By adding 1mM IPTG, the expression of the target protein was stimulated. All induced and uninduced cultures reached O. D600 after the induction period had been in place for 3 hours. 12 % SDS-PAGE (Solar Bio, China) was used to assess the expression profiles of the constructs, and Coomassie brilliant blue R-250 was used to stain the gels.

Biological activity of streptokinase

Caseinolytic test

Using a caseinolytic plate assay, the native streptokinase's activity was determined. The caseinolytic-agarose base needed for this test was made by melting 90 mg of agarose in 9 ml of a buffer solution (50 mM Tris-HCl, 150 mM NaCl), which also contained 1% plasminogen and 1% skim milk. After the solution was gelled, 5 mm-diameter wells were formed on the caseinolytic-agarose base. The wells were filled with 50 microliters of the native SK and left to incubate for 24 hours at 37 °C. It was demonstrated how the wells' clear zone developed (Buniya *et al.,* 2014).

Fibrinolytic test

This test was done to evaluate the native streptokinase's capacity to lyse clots. Human blood clots were used for the in vitro clot lysis. In order to create human blood clots, 100 µl of fresh blood was incubated in a 1.5ml microfuge tube at 37 °C for 45 minutes. The clots were then cleaned with normal saline two to three times. (Prasad et al., 2006). The native SK was added to the human blood in aliquots totaling 50 µl. The tubes were all then kept at 37 °C. It was discovered how long it took the native protein to perform clot lysis.

Results

Cloning and SDS PAGE analysis of Streptokinase

The 1.3 kb gene that encodes the SK protein was amplified using gene-specific primers which contain *EcoRI* and *Hindlll* restriction sites in the forward and reverse primers respectively (Table 1) (Figure 1). The amplicons were purified and then subjected to restriction digestion with *EcoRI* and *Hindlll* restriction enzymes. Meanwhile, the expression vector, pGEM®-3Zf (+) was prepared for ligation by digestion with *EcoRI* and *Hindlll* enzymes and subsequently purified with gel elution. The purified amplicons were ligated with the linearized vector by using T4 DNA ligase. The ligation products were then transformed into *E. coli* BL21(DE3). The constructs were then induced by IPTG for expression in *E. coli* BL21 (DE3). In

12 % SDS-PAGE, the expression profile of SK (47 kDa) showed overexpression in *E. coli* BL21 (DE3)as in figure (2).

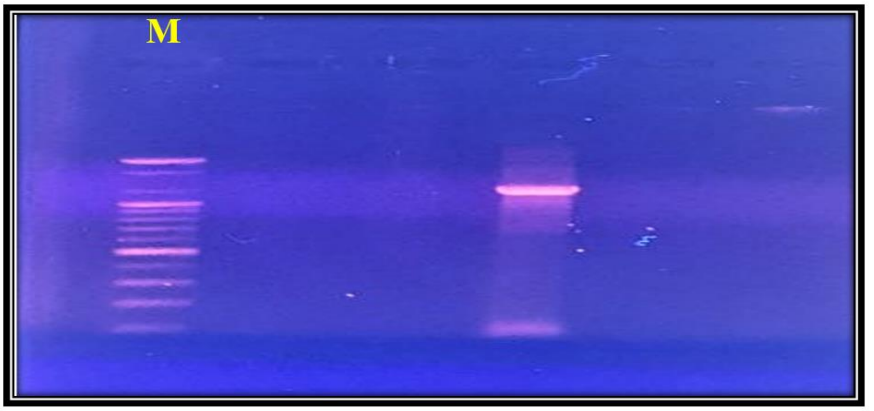

Figure 1: Agarose gel electrophoresis (1%, 90 V/cm) for PCR product of SK gene. M: 100bp DNA Ladder. Annealing temperature 58 °c

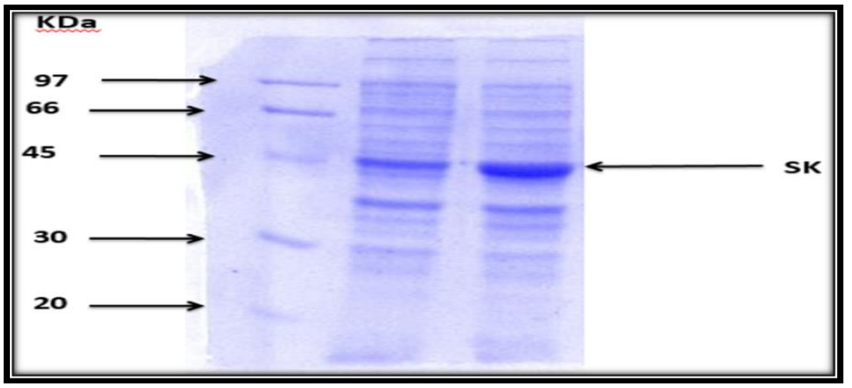

Figure 2: Expression analysis of the native streptokinase protein in *E. coli* BL21 (DE3)

The activity of the native streptokinase protein

Two techniques were used to assess the biological activity of the native streptokinase: a caseinolytic assay and a clot lysis assay with a human blood clot. For native SK protein, the zone of clearance was seen in the caseinolytic assay (Figure 3). The zone of clearance's creation demonstrated the native streptokinase's activity.

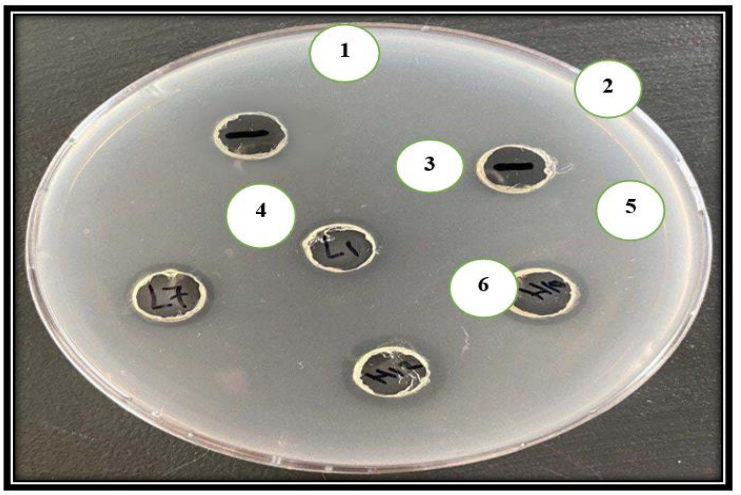

Figure 3: Biological activity of SK protein- Caseinolysis assay**.** 1-2 Uninduced cells, 3-4 Native SK**,** 5-6 Hybrid SK.

The clot lysis assay was then carried out to determine whether the native streptokinase has the ability to dissolve clots. The native streptokinase (50 µl) was added to the microcentrifuge tubes containing the human blood clots and incubated at 37 °C. We routinely checked on the reaction tubes every 2 hours. The native SK's ability to lysis the human blood clots are seen in Figure 4. The results after 24 hours showed that native SK could only partially dissolve the clots and that native Sk had little activity.

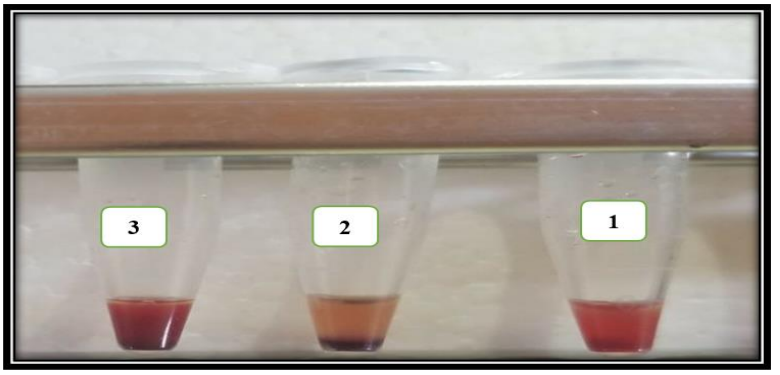

Figure 4: Biological activity of native SK protein- Fibrinolysis assay: Tube 1- Normal saline, Tube 2- Uninduced cells, Tube 3- Native SK.

Discussion

One of the promising blood-clot-dissolving agents for the treatment of heart attack patients is SK. In order to characterize this enzyme biochemically and conduct clinical studies, it is essential to manufacture it in high amounts (Adivitiya and Khasa, 2017). Streptokinase, urokinase, or tissue plasminogen activator must be used in a clinical setting to prevent thrombus development. Streptokinase continues to play a vital role in the treatment of cardiovascular disorders

regardless of the presence of another plasminogen activator. Due to its low cost, streptokinase is crucial in the treatment of heart disorders in developing nations (Zia, 2020). An antigenic thrombolytic agent (streptokinase) is used to treat acute myocardial infarction. In most infarct patients, it lowers mortality just as much as nonantigenic alteplase while also being significantly less expensive. Given the prevalence of cardiac reinfarction and the high prevalence of patients who require fibrinolytic therapy, this financial impact is crucial (Lee,1995).

In treating acute myocardial infarction, streptokinase is just as efficient as tissue plasminogen activator (tPA), and it is unquestionably more affordable. The continued viability of streptokinase therapy is being questioned in view of the relatively recent release of the competitor recombinant Tpa (Astrup, 2018). Despite this, streptokinase research continues, and it is still an important and reasonably priced therapy, particularly in the world's less-developed healthcare systems (Murugami, 2021). The native SK protein was able to create a clearance zone in the caseinolytic assay by activating the plasminogen. The native SK was effective in dissolving the blood clots in the blood clot lysis assay. However, the native SK without the ability to attach to fibrin was unable to activate the plasminogen linked to fibrin, leading to a limited amount of clot lysis**.** In this study, the production of native streptokinase by cloning it into the expression vector pGEM®-3Zf (+) and transforming it into *Escherichia coli* BL21 (DE3) was important and had the majority favor compared with the production in pathogenic bacteria.

Acknowledgments

We sincerely thank the Department of Biology, College of Education for pure sciences, Anbar university for providing the laboratory facilities to carry out this research work.

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