



Production of Staphylokinase from Locally Isolated Lysogenic *Staphylococcus aureus*

Tarek A. Elbashiti

Department of Biology & Biotechnology, The Islamic University-Gaza, P.O. Box. 108, Rimal, Gaza Strip, Palestine

Abstract

Staphylokinase (SAK) is a bacterial kinase produced by certain strains of *Staphylococcus spp.* It is a 14.5 kDa, consisting of 136 amino acids single polypeptide chain protein, which activates plasminogen to form plasmin and digest fibrin clots. SAK is a promising thrombolytic agent for the treatment of myocardial infarction. The present research was carried out to produce SAK from locally isolated lysogenic *Staphylococcus aureus*. The main focus of this work was to isolate and characterize lysogenic *S. aureus* from various environmental samples involving non-pathogenic *Staphylococcus spp.*, while the earlier isolation was done using pathogenic samples.

Keywords Staphylokinase, Thrombolytic agent, Lysogenic, *Staphylococcus aureus*

Introduction

Thrombosis, the blockage of blood vessels with clots, can lead to acute myocardial infarction and ischemic stroke both are leading causes of death. The only treatment available is the administration of thrombolytic agents to dissolve the blood clot [1-2].

Staphylokinase (SAK) is a 136-amino acid extracellular protein produced during the late of exponential growth phase, produced by lysogenic strains of *Staphylococcus aureus* as one of virulence factor [3-4]. It activates plasminogen to form plasmin, which digest fibrin clots [5]. This disrupts the fibrin meshwork that can stop the spread of infection [6]. SAK lacks fibrin-binding and thrombin inhibitor activities, two functions which would supplement and potentially improve its thrombolytic potency [7]. SAK, when produced from non-pathogenic samples reduces the chances of cross contamination by any other microbe present in the sample, makes the production process safe, reduces downstream processing steps and even reduces the cost of purification, in turn making the production cheap and easy [2].

In this present study, we provided a rapid method for isolating staphylokinase producing strain based on lysogenic *S. aureus* phenotypes and give more efficient treatment for patients suffer from thrombolytic disorders.

Materials and Methods

Sample Collections

The samples were collected from several areas including clinical pure culture from ALshifa Hospital and non-clinical including Bovine milk samples, tap water and soil samples.

Isolation and Identification of lysogenic *S. aureus*

Isolated samples were inoculated on Blood agar medium at 37 °C for 18-24 hours to observed for hemolysis. The colonies grown on the blood agar plates and exhibited β -hemolysis were streaked on Mannitol Salt Agar (MSA) for overnight incubation at 37 °C. Gram staining, coagulase test, catalase test and DNase test were performed to characterize the *Staphylococcus spp.* [8].



Production and Partial Purification of Staphylokinase:

Production SAK by *S. aureus* was carried out by growing them in a medium (Sato's medium) containing 10g/L nutrient broth, 3 g/L yeast extract, 5g/L NaCl, and 10ml/L glycerol, at 30° C, at 100 rpm shaking rate for 24 h. The pH of the medium was adjusted to pH 6.8 before sterilization. The culture was centrifuged at 10000 rpm, at 4 °C for 10 min. The supernatant was filtered and collected as crude enzyme. Then the enzyme was purified by Ammonium salt precipitation method and the collected pellets were resuspended with 1X PBS buffer [9].

Screening for Staphylokinase Activity

The enzyme produced from the isolated *Staphylococcus spp.* was screened by casein hydrolysis assay and heated plasma agar assay. Twenty five microliter of bacterial suspension samples were loaded on the wells made in casein and plasma agar plates and incubated overnight [10].

Determination of Staphylokinase Activity

Thrombolytic activity of the purified SAK enzyme was determined according to the modified Holmstrom method. This is one of the most important methods to test the thrombolytic activity of an enzyme. In this method both crude and ammonium sulphate precipitated samples were used. 1 ml of human blood were taken in Eppendorf tubes and allowed to clot. After the blood clotted completely, enzyme was added at a concentration of 10-100 µl [11-12].

Results

Isolation of lysogenic *Staphylococcus aureus*

▪ Blood Agar

After the incubation colonies developed on the blood agar and exhibited β-hemolysis (colonies around which clear, colorless zones) was considered as *S. aureus* as shown in Table 1.

Table 1: The growth of the different isolates on Blood Agar

	Soil sample				Tap water				Milk			
Dilutions	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
β-hemolysis	-ve	+ve	+ve	-ve	-ve							

▪ Mannitol Salt Agar Medium

The colonies which were able to grow on MSA, confirmed the presence of *Staphylococcus spp.* On the mannitol salt agar plates some of the *Staphylococcus spp.* showed complete fermentation of mannitol, as the color of the media was changed from orange to yellow. The golden yellow colored colonies obtained on the MSA were supposed to be *S. aureus* whereas other color colonies were expected to be related to other species as shown in Table 2.

Table 2: Samples with positive mannitol fermentation

Sample	10 ⁻¹ bovine milk	10 ⁻² bovine milk	Clinical sample
Mannitol fermentation	Yellow color (+ve)	Yellow color (+ve)	Yellow color (+ve)

Identification of Lysogenic *S. aureus*

The lysogenic strains distinguished from the host strains by the absence of coagulase and deoxyribonuclease activities.

A. Catalase Test

Hydrogen peroxide (H₂O₂) is used to determine if bacteria produce the enzyme catalase. Bubbles appear positive result for *S. aureus* Table 3 and Figure 1.

Table 3: Catalase test

10 ⁻¹ bovine milk	10 ⁻² bovine milk	Clinical sample
bubble appear (+ve)	bubble appear (+ve)	bubble appear (+ve)





Figure 1: Positive catalase test

B. Coagulase Test

Specific test for isolation of lysogenic strain of *S. aureus*. Lysogenic strain exhibited coagulase negative, this distinguish lysogenic strain from other *S. aureus* strains. Coagulase, which can clot plasma into gel in tube or agglutinate cocci inslide. There are two types of coagulase, free coagulase and bound coagulase. Free coagulase is an enzyme that is secreted extracellularly, while bound coagulase is a cell wall associated protein. Free coagulase detected in tube coagulase test and bound coagulase detected in slide coagulase test Table 4 and Figure 2.

Table 4: Coagulase test

Sample	10 ⁻¹ bovine milk	10 ⁻² bovine milk	Clinical sample
Coagulase	-ve(no clot)	-ve (no clot)	+ve (clotting)



Figure 2a: Tube coagulase negative test

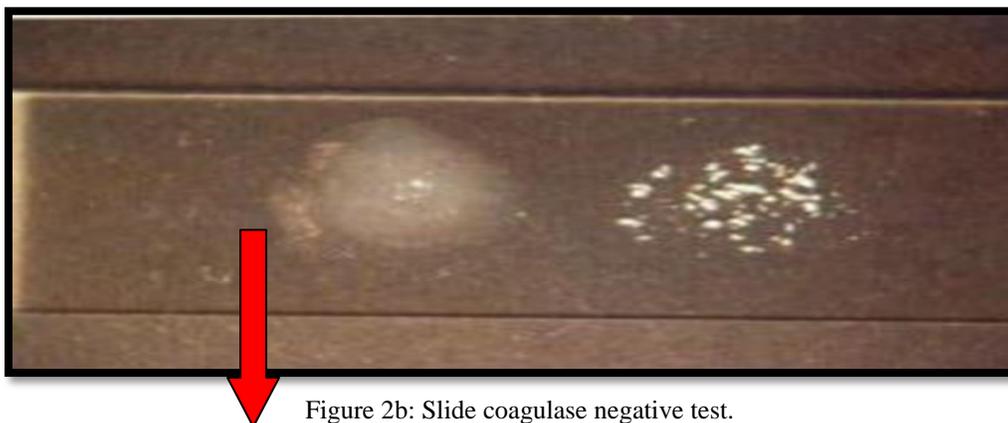


Figure 2b: Slide coagulase negative test.

C. DNase Test:

Another Specific test for identification of lysogenic strain of *S. aureus*. Lysogenic strain exhibit DNase negative, this distinguished lysogenic strain from the other non-lysogenic *S. aureus* strains. Deoxyribonuclease (DNase) is an enzyme that breaks down DNA. Presence of clear halos surrounding colonies is positive for their ability to digest the DNA and thus indicates presence of DNase [3] as shown in Table 5 and Figure 3.

Table 5: DNase test results

Sample	10^{-1} bovine milk	10^{-2} bovine milk	Clinical sample
Dnase	-ve (nohalo zone)	-ve (nohalo zone)	+ve (presence of halo zone)

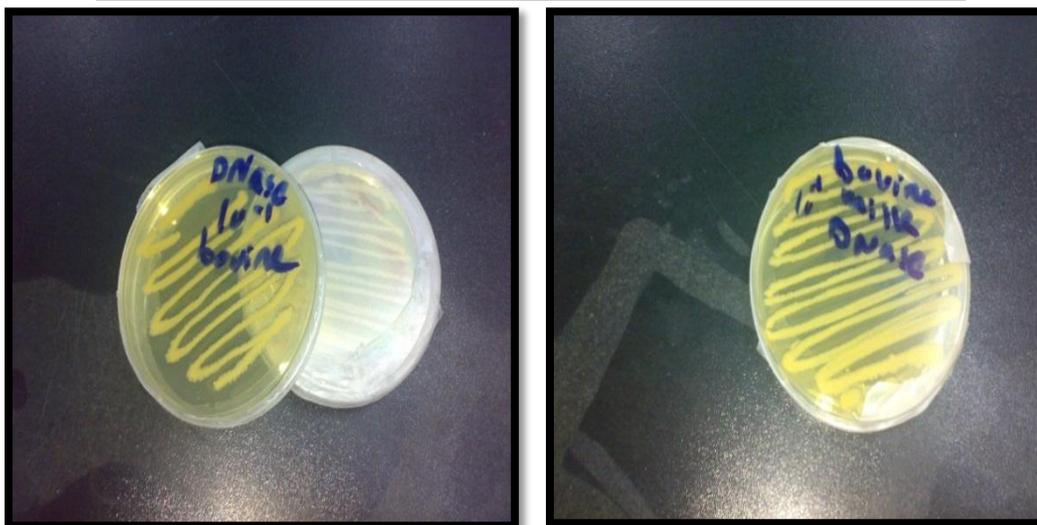


Figure 3: Negative DNase test

Screening for Staphylokinase Activity

1. Casein hydrolysis assay:

Casein hydrolysis assay after incubation at 37 °C onto the casein agar plate, there was a formation of clear caseinolytic halos.

This test involves cleavage of casein analogues to fibrin in the clot when activated by the added plasma Figure 4.



Figure 4: Clear caseinolytic zone



2. Heated plasma agar assay:

In heated Plasma Assay, after incubation at 37 °C onto the heated plasma agar plate, there was a formation of clear fibrinolytic halos. The heated plasma agar plate test showed a very good clearance zones after overnight incubation through induction and prolonged incubation for 48 h increased the clearance zone as shown in Figure 5.

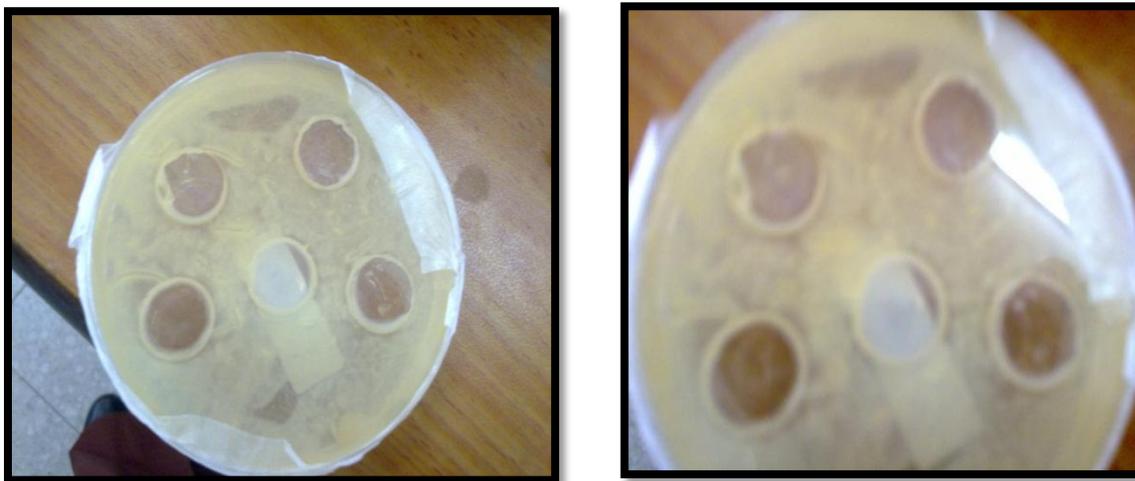


Figure 5: Clear fibrinolytic halos

Determination of Enzyme Activity:

The thrombolytic property of the isolated enzyme was confirmed by the modified Holmstrom method as the clot was degraded as shown in figure 6.



Figure 6: Clot degradation after addition of the enzyme

Discussion

The present study succeeded in the isolation of lysogenic *S. aureus* from bovine milk and used for Staphylokinase (SLK) production. This is in accordance with the earlier observation which shows that the isolates from a local wound formed due to lysogenic *S. aureus* was used for commercial production of SLK [10]. Our work provided low cost, easier and comparatively safer method for production of SLK as we used non-pathogenic environmental samples where as in all other previous research pathogenic samples were practiced which increases the chances of infection and requires safer handling techniques which also leads increase in processing cost. Our study is more rapid than previous study that screen for SLK producing *Staphylococcus spp.* from bovine milk samples [9] as we



used lysogenic strain characters that distinguished it from other *S. aureus* strain that cannot produce staphylokinase such as the absence of coagulase and deoxyribonuclease activities.

SLK has attracted attentions of researchers to use it as a therapeutic thrombolytic agent through special mechanism, also cause less allergic reaction compared with other thrombolytic agents [13].

Native SLK is useful for cost-effective thrombolytic therapeutic purposes in clinical areas. The SLK is a better thrombolytic agent than any other chemical agents like Heparin and EDTA [14].

Furthermore, cloning of the Staphylokinase gene in non-pathogenic microorganisms such as *E. coli* helps in production of the recombinant enzyme. Various biophysical and chemical modifications are being used to extend its half-life in the circulatory system of human. The SLK that was extracted has good clot bursting ability and is comparable to the other plasminogen activators, such as streptokinase, urokinase, nattokinase and tissue plasminogen activator [9].

Conclusion

SAK is one of the bacterial proteins having relatively good clot specificity than t-PA, but production from native *S. aureus* poses a great risk in the protein production, as it is pathogenic.

Recombinant protein production in the non-pathogenic host would be useful for cost effective therapeutic protein production in the clinical practice and safer.

The SLK is similar to the streptokinase produced by *Streptococcus pyogenes* except that it is slower acting and takes several hours to destroy a clot. It is postulated that in the *in vivo* situation the presence of SLK allows the *S. aureus* to break out of a clot once it has formed and then migrate to other parts of the body.

References

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