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Research Article

SCREENING FOR STAPHYLOKINASE PRODUCING *STAPHYLOCOCCUS* SPP. FROM BOVINE MILK SAMPLE

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ABSTRACT

The present research work is designed to extract the Staphylokinase from *Staphylococcus* sp. isolated from milk samples. Mannitol salt agar and Blood agar was used to isolate the Staphylococcal isolates.

Casein hydrolytic assay and heated plasma agar assay was performed to screen the Staphylokinase producing *Staphylococcus* sp. Satoh's media was used as the production media for the enzyme. Partial purification and molecular weight of the enzyme was determined by ammonium sulphate precipitation and SDS PAGE analysis. Enzyme activity was confirmed using Holmstrom method.

Performing the modified Holmstrom method it was observed that staphylokinase extracted from *Staphylococcus* sp. showed thrombolytic activity at concentrations of 80-100 µl. After precipitation of the enzyme, maximum activity was determined at concentrations as low as 10 µl. Molecular weight of the extracted staphylokinase was determined as 14.5 kDa.

Thus this research was successfully carried out to find an economical and safer clot buster agent compared to chemical agents such as EDTA or heparin.

Keywords: Staphylokinase, *Staphylococcus* sp., Fibrin, Thrombolytic therapy, Holmstrom method.

INTRODUCTION

Thrombolytic therapy with intravenous infusion of plasminogen activators has become an established treatment for patients with acute myocardial infarction. The most frequently used thrombolytic agents are streptokinase and recombinant tissue-type plasminogen activator. Streptokinase is inexpensive but immunogenic and relatively inefficient for early coronary artery recanalization. Staphylokinase is a 136 amino acid protein produced by certain strains of Staphylococcus aureus1. It belongs to fibrin-specific plasminogen activator. *Staphylococcus* sp has a potential contribution in the field of medicine. Its unique nature to produce an enzyme Staphylokinase has a role in anti-clotting functioning. Staphylokinase, a Staphylococcal extracellular protein aids in converting plasminogen to plasmin, a major factor in blood clotting [2, 3] (Fig. 1). Thrombolyis can lead to life threatening disease like myocardial infarction or commonly known as heart attack due to blood clot. Staphylokinase (SAK) is considered as a thrombolytic agent that helps in dissolving plasminogen to inactive proenzyme plasmin [4] thus acting as a clot busters. Staphylokinase also help in clot lysis by its proteolytic action on fibrin, a major constituent of thrombus [5]. The main purpose for selecting the enzyme is because Staphylokinase could be relatively inexpensive when compared to other thrombolytic agents and an alternative cure against cardial blood clot.

Therefore, the objectives of this study were to isolate and screen Staphylokinase (SAK) producing staphylococcal sp from milk sample, to produce and purify SAK and to determine its clot buster ability.

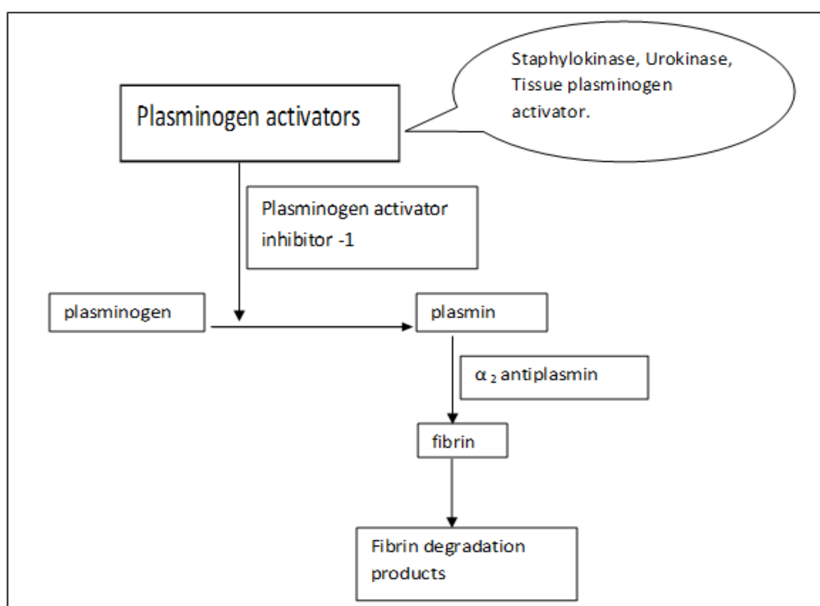

Fig. 1: Schematic representation of fibrinolysis [6].

MATERIALS AND METHODS

Chemicals

All analytical reagents and media components were purchased from Hi-Media (Mumbai, India)

Sample collection

Bovine milk samples were collected from in and around Vellore district and brought aseptically to the lab and cultured on Nutrient agar medium.

Isolation and Morphological characterization

The screening for *Staphylococcus* sp was done using selective media - Mannitol salt agar (MSA) and differential media - Blood agar, to check the haemolytic and acid producing properties. The selected strains were sub cultured and preserved at 4° C for further testing. Gram staining was performed to characterize the Gram's nature and external morphology of the isolates.

Production of Staphylokinase (SAK)

Production of Staphylokinase by *Staphylococcus* sp was carried out by growing them in a medium containing (Satoh's medium) 10g/L nutrient broth, 3g/L yeast extract, 5g/L NaCl, and 10ml/L glycerol, at 30°C, at 100 rpm for 24 h. The pH of the medium was adjusted to pH 6.8 before sterilization. The isolated culture was then inoculated into the production medium. The cells were then harvested by centrifugation at 10000 rpm, at 4 ◦ C for 10min.

Screening for staphylokinase activity

The enzyme produced from the isolated Staphylococcus sp was screened by Casein hydrolysis assay and heated plasma agar assay to check the proteolytic and the plasmolytic activity of the enzymes respectively.

Casein hydrolysis assay [5]

Casein hydrolysis assay agar was prepared by mixing non fat dry milk (casein), serum and nutrient agar. The serum was prepared by collecting 5ml of blood and the blood was allowed to clot for 5 hrs. The yellow colour fraction was used as serum. Well diffusion plate technique was used to check the caseinolytic activity of the enzyme.

Heated plasma agar assay

Heated plasma agar assay method is one of the important methods to see the activity of the enzyme. Crude enzyme was taken and centrifuged at 10000 rpm for 25 mins. 15ml of nutrient agar medium was prepared. Plasma was prepared by collecting 10ml of blood. Anticoagulant (EDTA) was added and blood was centrifuged at 13000 rpm for 10 mins. The supernatant served as plasma. The plasma was then heated at 56 °C for 20 mins. Then it was mixed with nutrient agar and plated. Well diffusion technique was used to check the plasmolytic activity of the enzyme.

Partial purification and molecular weight determination of Staphylokinase

Ammonium salt precipitation method was used for the partial purification of the enzyme. 50 ml of the supernatant was taken and ammonium sulphate was added slowly under (30%, 50%, 70% saturation) magnetic stirrer at 4° C. After 1 h the samples were centrifuged at 8000 rpm for 30 mins, pellets collected were resuspended with 1X PBS buffer. SDS-PAGE was done to confirm the presence of the Staphylokinase (14.5 kDa) enzyme by using molecular weight marker 97 kDa to 14.5 kDa.

Determination of enzyme activity by Modified Holmstrom method [7]

The thrombolytic activity of the enzyme was checked using Modified Holmstrorm 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 eppendroff tubes and allowed to clot. After the blood clotted completely, enzyme was added at a concentration of 10-100 µl. The minimum concentration of the enzyme which completely liquefies 1ml of clotted blood is considered as 1 enzyme unit.

RESULTS

Isolation and Morphological characterization

A total of 8 Staphylococcal strains were isolated from various Milk samples collected from in and around Vellore. The isolates exhibited yellow round colonies on nutrient agar plates. All the isolates were found to be Gram positive in nature with grape like appearance when viewed under oil immersion objective.

Fig. 2: Growth of *Staphylococcus* sp on Mannitol salt agar plate

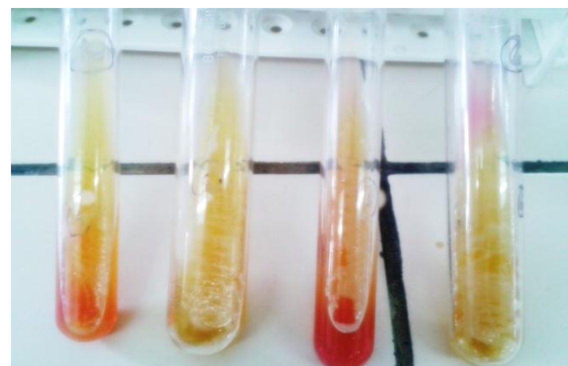

Fig. 3: Growth of *Staphylococcus* sp on Mannitol salt agar slant

The organisms were on MSA and BA, which showed its acid producing and haemolytic properties. The enzyme produced from the selected strains after incubation showed distinct halo zones indicating proteolytic and plasmolytic activity. The 8 strains isolated were narrowed down to 6 strains on the basis of the growth characteristics on MSA and BA plate. These 6 strains were used for enzyme production. The Staphylokinase (SAK) extracted from the selected strains was named as SAK 1, 4, 5, 6, 7, 8 for further analysis.

Screening for staphylokinase activity

In Casein Hydrolytic Assay SAK 1, 4, 5, 7, 8 produced halo zones. SAK 2, 3, 6 did not give any halo zones. SAK 8 produced 2.9 cm halo zone which is found to be the largest zone whereas SAK 4 produced the least zone. SAK 1 and 5 produced zones around 2.5 cm each. This test involves cleavage of casein analogues to fibrin in the clot atmosphere when activated by the added plasma.

Table 1: Casein hydrolytic assay for extracted enzymes.

Samples	Halo formation	Diameter of the halo zone in cm
SAK 1	Yes	2.5
SAK 2	No	-
SAK 3	No	-
SAK 4	Yes	1.5
SAK 5	Yes	2.5
SAK 6	No	-
SAK 7	Yes	2.4
SAK 8	Yes	2.9

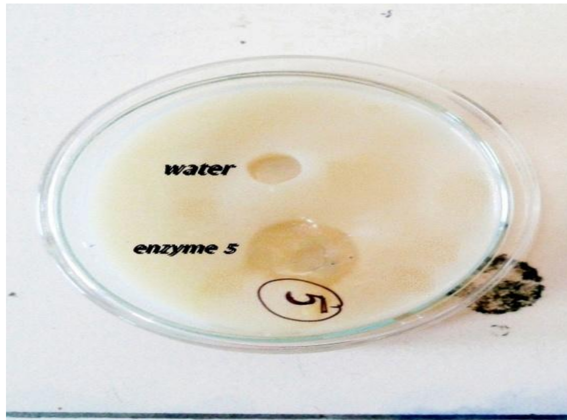

Fig. 4: Zone of inhibition on casein hydrolysis Assay agar using SAK 5.

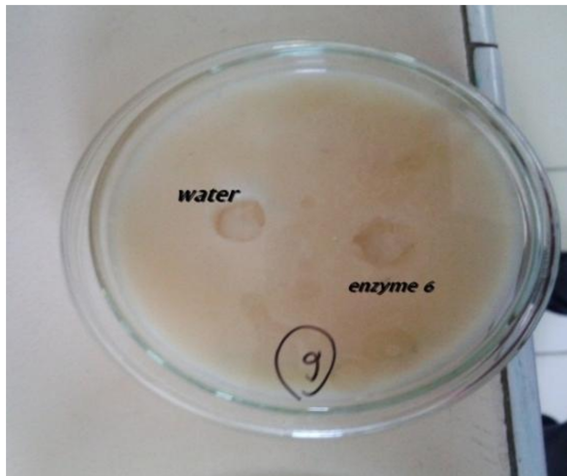

Fig. 5: No zone of inhibition on casein hydrolysis agar around well containing SAK 6.

In heated Plasma Assay after incubation at 37° C onto the heated plasma agar plate, there is a formation of clear fibrinolytic halos. SAK 6, 7, 8 produced halo zones whereas SAK 1, 4, 5 did not produce any zones. SAK 6 gave the best zone when compare to other SAK's.

Table 2: Heated plasma agar assay for the extracted enzymes.

Samples	Halo formation
SAK 1	No
SAK 2	No
SAK 3	No
SAK 4	No
SAK 5	No
SAK 6	Yes
SAK 7	Yes
SAK 8	Yes

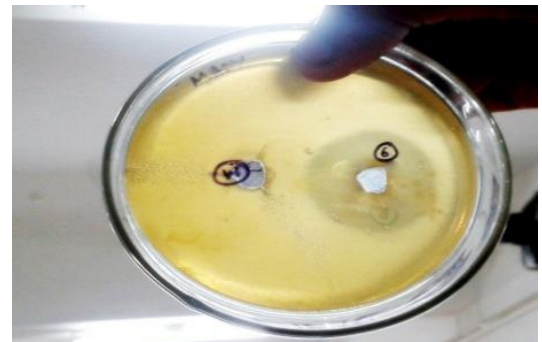

Fig. 6: Zone of inhibition on heated plasma agar around well containing SAK 6.

Partial purification and molecular weight determination of Staphylokinase

SDS-PAGE was done to separate out our protein of choice from other cell precipitates on the basis of molecular weight. Standard molecular weight markers were used to confirm the presence of Staphylokinase which has a molecular weight of 14.5 kDa.

Determination of enzyme activity by Modified Holmstrom method

Holmstrom method confirmed the thrombolytic property of the isolated enzymes. Crude SAK 1 showed thrombolytic property only at 100 µl concentration whereas precipitated enzyme showed the activity from concentrations of 20-100 µl. Precipitated SAK 4 and 5 showed thrombolytic activity 20 µl onwards. Whereas precipitated SAK 6, 7 and 8 showed 10 µl onwards. So, the least concentration that gives the complete lysis of 1 ml of clotted blood is only 10 enzyme units for SAK 6, 7, 8.

Table 3: The least concentration that gives the complete lysis of 1 ml of clotted blood after incubation at 37⁰ C.

Samples	SAK1	SAK4	SAK5	SAK6	SAK7	SAK8
Enzyme (units/ml)	80	30	20	10	10	10

Table 4: Modified Holmstrom Method results showing the clot busting ability of the extracted enzymes as crude samples and precipitated samples

Enzyme	10µl	20µl	30µl	40µl	50µl	60µl	70µl	80µl	90µl	100µl	Control
SAK 1 crude	-	-	-	-	-	-	-	-	-	+	-
SAK 1 precipitate	-	-	-	-	-	-	-	+	+	+	-
SAK 4 crude	-	-	-	-	-	+	+	+	+	+	-
SAK 4 precipitate	-	-	+	+	+	+	+	+	+	+	-
SAK 5 crude	-	-	-	-	-	-	-	+	+	+	-
SAK 5 precipitate	-	+	+	+	+	+	+	+	+	+	-
SAK 6 crude	-	-	-	-	+	+	+	+	+	+	-
SAK 6 precipitate	+	+	+	+	+	+	+	+	+	+	-
SAK 7 precipitate	+	+	+	+	+	+	+	+	+	+	-
SAK 8 precipitate	+	+	+	+	+	+	+	+	+	+	-

DISCUSSION

SAK can be used as a good clot buster than other commercially available chemical anti clotting agents such as heparin, EDTA. Isolation of native *Staphylococcus* sp from clinical samples has ethical issues because it is highly pathogenic. But the strains that were extracted from environmental samples are safe and economical [5].

The diameter of the halo zones for caseinolytic assay around the well was measured to check the functional activity of SAK proteins. Among all the strains, maximum zone of clearance of 2.9 cm was

observed for SAK 8, whereas work done by [5] on Staphylococcus sp from wound pus showed maximum halo zone of 3cm. Thus results are almost comparable with our work with a lesser risk of pathogenicity.

The enzyme was then purified using Ammonium sulphate precipitation method. Ammonium sulphate contains sulphate which has cosmotropic and protein molecule exclusionary power. This property helps in effective precipitation of protein. In SDS PAGE analysis protein bands are at around 14.5 kDa which indicates our target protein was extracted successfully.

Most of the in vitro methods that were usually or at present applied to study thrombolysis have many limitations. Some involve tedious calculations and mathematical skills that too give only theoretical prediction of the outcome and most are expensive to be performed in a laboratory. In context to that Holmstrom method is easy to perform and is cost effective too. After performing the modified Holmstrom method it was seen that all enzymes showed thrombolytic activity at concentrations of 80-100 µl. On the other hand it was seen that crude SAK from most of the isolated staphylococcus strains did not showed clot lysis activity at lower concentrations. Most of the precipitated enzymes showed clot lysis activity at fairly lower concentrations. SAK 1 and SAK 4 precipitated samples showed the clot lysis activity at a minimum concentration of 20 µl whereas the crude sample showed the same activity at a concentration of 100 µl for SAK 1 and at 60 µl and above concentrations for SAK 4. SAK 5 on the other hand showed the least clot lysis activity and was active only at the concentration 80µl and above in case of both crude and precipitated sample. SAK 6 showed clot lysis at all concentrations (10-100 µl) in case of precipitated sample whereas it showed clot lysis at the concentration of 50 µl and above in case of crude sample as seen in (Table 4). Based on our results it can be inferred that SAK 6 showed the best thrombolytic activity as compared to all the other.

Madhuri Doss H *et al,* 2011 reported that 0.12 ml of Streptokinase [8] liquefied 1ml of clotted blood in 18 h, whereas in our work only 0.01 ml of the extracted staphylokinase from (SAK 6, SAK 7, and SAK 8) produced gave the same results.

Thrombolytic therapy demands a more clot specific third generation molecule which will work efficiently in a shorter duration with minimum side effects. Native Staphylokinase is useful for cost-effective thrombolytic therapeutic purposes in clinical areas. Large quantities of Staphylokinase can be produced inexpensively by bacteria [9]. The Staphylokinase is a better thrombolytic agent than any other chemical agents like Heparin and EDTA. Our work showed that this enzyme can show maximum activity at concentrations as low as 10 µl. Thrombolytic agents with such low active concentrations can be very useful for preparation commercial formulation. Furthermore, cloning of the Staphylokinase gene in non-pathogenic microorganisms such as *E.coli* helps in production of the recombinant enzyme [10, 11]. Various biophysical and chemical modifications are being used to extend the half- life when in the circulatory system of human. The Staphylokinase 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.

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