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ISOLATION AND PURIFICATION OF STAPHYLOKINASE (SAK) FROM STAPHYLOCOCCUS AUREUS LOCAL ISOLATES

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ABSTRACT

The *Staphylococcus aureus* local isolated no 10. was carrying *luk* gene, which choose to produced staphylokinase and purification by different methods like ion Exchange-Chromatography and Gel Filtration Chromathography were used. The molecular weight of the native staphylokinase was determined by chromatography through Sephorase 6B column and by SDS polyacrylamide gel and estimated as 15,848 KD, isoelectric focusing in polyacrylamide gel was used to determine the iso electric point (PI) value and estimated as 6.3.

Key words: Staphylokinase, *Staphylococcus aureus*, Purification of Staphylokinase, Isoelectric point.

INTRODUCTION

Staphylococcus aureus is an opportunistic human pathogen that causes life-threating infections, it is regarded as the most frequently isolated bacterial pathogen in hospital-acquired infections including burns, wounds and post operative wound infections, despite its ability to cause other dangerous infections including bacteraemia and endocarditis, it may cause several skin infections including boils, pimples, abscesses, and impetigo. In addition gastroenteritis, Osteomyelitis, toxic shock syndrome and scalded skin syndrome are of Staphylococcus origin. It's responsible for food poisoning due to its ability to produce different types of enterotoxins (Jin *et al*.,2004). Staphylokinase, a protein produced by certain strains of Staphylococcus aureus, was shown to have profibrinolytic properties more than six decades ago (Lack, 1948). Natural staphylokinase has been purified from S. aureus strains that were transformed with bacteriophages containing the staphylokinase gene, which had undergone lysogenic conversion to staphylokinase production (Rita *et al*.,2005).

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Natural staphylokinase has been purified by precipitation with ammonium sulphate and chromatography on CM-cellulose, or by affinity chromatography on plasmin- Sepharose' or on plasminogen-Sepharose (Kondo *et al*., 1981).

Growth of S. aureus for SAK production

Staphylococcus aureus isolates were cultured on horse blood agar overnight and the bacteria within a single c.f.u. were used for the preparation of a stock culture. A stock culture suspension was prepared by incubating a separated colony in (5ml) brain heart infusion broth and incubated at 37°C to (0.D= 0.25). Total viable count was estimated by making a serial dilution of bacterial growth (10-1-10-6 cells), a (100µl) of each dilution was spread on the surface of brain heart infusion agar and incubated at 37oC for 24 hrs. Bacterial colonies were calculated for each dilution. A (2.5 ml) of this stock culture was inoculated into (250 ml) Todd-Hewitt broth medium and incubated for 18 hrs at 37oC.

Detection of SAK in cell free filtrate (Erechati *et al*., 1984)

SAK production in bacterial cell free filtrates was determined by incubation of 0.2ml of bacterial supernatant

with 0.2 ml of fresh human plasma, and 0.1 ml rabbit thromboplastin at 37C for 24 hr. After the incubation time, liquid production indicates production of SAK by the bacteria.

Determination of SAK activity in cell free filtrate

SAK activity was determined by incubation of different volumes (100,150,200µl) of cell free filtrates with 0.2 ml of fresh human plasma, and 0.1 ml rabbit thromboplastin at 37C for 24 hr. After the incubation time the amount of liquid produced was measured, the more liquid produced indicates the more SAK activity .The two highest SAK activity bacteria were selected for further extraction of staphylokinase.

Extraction of Staphylokinase

The Todd-Hewitt broth culture was centrifuged at 4000 rpm for 10 min (4 C), and the medium filtered by mille pore filter 0.45µm. Two volumes of cooled ethanol 5Cº were added to one volume of cooled supernatant 5°C, then the mixture was left in refrigerator at 4C for 24 hr. Centrifugation was also performed at 6000 rpm for 10 min, the resulted precipitate was dissolved in 0.05ml phosphate buffer pH 7.4, and then preserved in 4°C until use.

Purification of SAK

Purification of SAK by Ion Exchange Chromatography [10] . The DEAE-cellulose was suspended in (0.05 M) phosphate buffer pH (7) until the pH of the exchanger reached (7). The DEAE- cellulose was packed into the column (7.5x3.5 cm), then the column was equilibrated with the same buffer overnight.

Partially purified concentrated SAK (12 ml) was separately passed after loaded onto the column carefully. Then (100 ml) of (0.05M) phosphate buffer pH (7) was added. Proteins were eluted by using (200 ml) of a gradient from (0.05-0.3 M) phosphate buffer (pH 7). Fractions of (5 ml) were collected and absorbance was monitored at (280 nm). The presences of the SAK were estimated from each fraction of the major peaks, then activities were determined for the collected active fractions.

Purification of SAK by Gel Filtration Chromatography

Sepharose -6B column (67x2.1cm) was prepared and packed according to the instruction of the manufacturing company (Pharmacia- Sweden). The column was equilibrated with (0.1 M) phosphate buffer (pH 7) at a flow rate of (50 ml/hr).

A (3 ml) sample of each concentrated partially purified SAKs was added to the column, carefully using pasture pipette. Elution of proteins was done with the application of (200 ml) of (0.1 M) phosphate buffer (pH 7). A (5 ml) fraction was collected for each SAK then protein contents were estimated by measuring the absorbance at (280 nm), the major peaks for each SAK were determined by plotting the absorbency of protein fractions versus the elution volumes. SAK activity was determined for each fraction of the major peaks. Protein concentrations and specific activities were also determined for the collected fractions of the major peaks of the different SAKs.

Determination of Molecular Weights of SAK by Gel Filtration Chromatography

Determination of the Void Volume of the Column Sepharose-6B column (67x2.1 cm) was prepared and packed according to the instructions of the manufacturing company (Pharmacia-Sweden). The column was equilibrated overnight with (0.1 M) phosphate buffer (pH 7) with a flow rate of (50 ml /hr).

A (2ml) of blue dextran 2000 solution was passed through the column, and (200 ml) of (0.3) phosphate buffer (pH 7) was added to the column. Fractions of (5 ml) were collected. The absorbency at (280 nm) for each fraction was measured. The column void volume (Vo) was determined by the estimation of total volume of fractions as characterized with start point movement of the blue dextrane to that of climax of absorbency of the blue dextran.

Determination of SAK Elution Volumes (Vo)

Sepharose -6B column (67x2.1cm) was prepared, packed and equilibrated for a second time.

A (3ml) of concentrated purified SAKs samples were passed separately through the column, carefully, and (200 ml) of (0.1 M) phosphate-buffer (pH 7), with a flow rate of (50 ml/hr) were passed through the column. Fractions of (5 ml) were collected. The elution volumes (Ve) were estimated separately for each separated and dissolved fractions of purified SAKs by following the absorbency at (280 nm).

Measuring of Standard Proteins Elution Volumes (Ve) (Stellwagen, 1990)

Different standard proteins were applied (Bovine serum albumin, ovalbumin, chymotrypsinogen, ribonuclease A) through Sepharose -6B column, and then eluted with (0.1M) phosphate buffer (pH 7) with a flow rate of (50 ml/hr).

The elution volume was estimated for each standard protein by following the absorbency for the separated fractions at wave length (280 nm). The (Ve/Vo) ratio was calculated for each standard protein and for the dissolved fractions and separated fractions of purified SAKs, then standardization was done, by plotting the elution volume (Ve) of each standard protein to the void volume (Vo) of the blue dextran 2000 (Ve/Vo) versus the log of each standard protein molecular weight;. The SAKs molecular weights were accordingly calculated.

Isoelectric focusing (IEF)
Gel mixed prepared

The final concentration of acrylamide in the separating gel used was 7.5%. 0.3 ml ampholyte solution was mixed with 3 ml from acrylamide-bisacryamide and 8 ml of distilled water was degassed under vacuum for 10 minutes. A 0.7 ml ammonium persulphate and 20 µl for TEMED were added, mixed well then used immediately,

Fill glass gaskets (100x95 mm) with gel , after gel polymerization put the glass gaskets in electrophoresis apparatus, use a pasteur pipette carefully fill the gel glass to the top with a protective solution consisting of 1% ampholyte, after that put the solution (Anode solution) on the bottom chamber and the cathode solution on the top chamber.

Staphylokinase sample

It was prepared by dissolving 1g from toxin and 0.1 gm sucrose in 1 ml of distilled water.

Isoelectric focusing (IEF) Conditions

The proteins (toxin) were focused at 50 mA / gel slab (constant current) by setting a maximum voltage of 240 volts. Under these conditions, focusing will take approximately 4 hours in gel slab. After focusing is completed, remove the gels from glass.

RESULTS AND DISCUSSION

Acute myocardial infarction is among the most prominent causes of death in the Western world. It is commonly caused by the formation of a pathologic clot that results in obstructing the blood flow to heart tissues. Staphylokinase (SAK), a 136-amino acid protein from certain lysogenic Staphylococcus aureus strains, is a plasminogen activator and a promising blood clot-dissolving agent with clinical potency that is at least as good as tPA (Collen *et al*., 1993). SAK does not bind directly to fibrin, it can bind indirectly through the fibrin binding of plasmin(ogen) by forming a 1:1 stoichiometric SAK-plasmin(ogen) complex. The resulting SAK-plasmin complex can then function as the plasminogen activator to convert plasminogen to plasmin for clot lysis (Armstrong *et al*., 2003).

One of the most important aims of this study is to extract staphylokinase (SAK) enzyme in order to prepare it for further purification steps. Prior to a large scale production of this enzyme from Staphylococcus aureus, the selection of the highest producible isolate was made. All the α -, γ - and σ -hemolysin but not β-hemolysin producers and enterotoxin A positive isolates were chosen in order to test their abilities in the production of this enzyme.

Straphylokinase is carried by a triple-converting bacteriophage (Π 42) that encodes the staphylokinase gene (sak) and the enterotoxin A gene (entA). Lysogeny results in loss of expression of the chromosomal β-hemolysin gene and the expression of staphylokinase and enterotoxin A, thus, these isolates are BK+ (Rita et al., 2005).

The results showed that all the selected isolates (67 MSSA and MRSA isolates), carry sak gene. Nasir,(1999) reported that 100% of S. aureus isolated from burns produce staphylokinase, recently, it was reported that 95% of S. aureus produce staphylokinase and its production did not correlate with the profile of resistance to methicillin or formation under antibiotic pressure (Marzena *et al*., 2007).

Staphylokinase is one of the S. aureus virulence factors, Tao et al. (2003) suggested that SAK is part of the adaptive mechanism of S. aureus favorable for Bacterial symbiosis with the host.

SAK production was significantly less common in isolates from patients with lethal bactereamia than in the present non-lethal bactereamia or nasal carriage isolates, nasal isolates and strains of S. aureus giving rise to uncomplicated bactereamia were more often SAK producers (Tao *et al*., 2003).

Isolates with sak gene were tested for staphylokinase activity using thrombolysis method described by Tao *et al*.,(2003) . Higher amount of liquid produced indicates more secreted staphylokinase activity in fibrinolysis as well as thrombolysis (Table 1). Isolate no. 8 produce the highest amount of fluid (950 µl) by the action of plasminogene activation then conversion to plasmin which consequently degrades fibrin present in human blood causing lyses of thrombus.

Staphylokinase induces highly fibrin-specific thromboIyis in human plasma, dogs, rabbits and rats by the activation of plasminogen. However, it has no ability to activate cows' plasminogen (Dmitry *et al*., 1996).

Purification of staphylokinase from Staphylococcus aureus isolate

Purification of staphylokinase by ion-exchange chromatography (DEAE-cellulose column) was carried out by the concentration the sample which ppt by alcohol 12 ml was passed through the DEAE cellulose column. From the result, figure (1) indicated that washing with 100 ml of 0.05M phosphate buffer pH 7 allowed the presence of one peak of protein which represented to other proteins without any toxin activity. Then after elution of proteins with 200 ml of a gradient from 0.05M to 0.3M phosphate buffer pH 7, two peaks were obtained for which represented by fractions 28-32 and 34-44 and second peak was obtained high staphylokinase activity.

Accordingly, staphylokinase was obtained after purification with ion-exchange chromatography. Partially purified staphylokinase was passed through an Amicon-Filter P50 in (Ultrafiltration-Cell) to concentrate them to (5 ml). Purification of staphylokinase by gel-filtration chromatography (Sephorase-6B) column .A 5 ml of concentrated partially purified staphylokinase was added to the column. Figure (2) indicated the presence of three major peaks represented by fractions (25-33), (32-35) and (35-40) respectively. After the determination of staphylokinase

activity for all these peaks which reflected that fractions (32-36) was able to produce toxin activity. It has been suggested by several researchers that staphylokinase produced in the late exponential phase of growth (14-18 hrs after culturing) especially when S.aureus has been cultured on blood agar for 24hr previous to cultivation in Todd-Hewitt broth (Marzera *et al*., 2007; Tao *et al*., 2003).

Purification of staphylokinase had been used widely before the application of ion-exchange chromatography due to its high absorption capacity with proteins.

Collen *et al*.(1992) purified staphylokinase from 12 liter batches by chromatography on Sp-Sephadex with pH gradient elution, and sephacryl S-300 super fine gel filtration with a yield of 35%, contained a single band on SDS- poly acryl amide gel electrophoresis. High yield production about 10-15% of total cell protein and highly purified recombinant staphylokinase were obtained by chromatography on Sp-Sepharose and on phenyl- Sepharose columns with yield of 50-70% (Schlott *et al*., 1994; Sang *et al.*, 2000). Staphylokinase was purified by sequential chromatography through Sp-Sepharose, Sephadex G-50, and, Q- sepharose, high purity proteins was obtained over 98% (Hong *et al*., 2007).

Determination of molecular weight of staphylokinase

1. By gel-filtration through Sepharose 6B

The molecular weight of the native staphylokinase was determined by chromatography through Sephorase 6B column. The void volume (V^o) of the column was calculated by estimating the void volume of blue dextrin 2000 and the elution volume (Ve) for each standard protein and for dissolved fractions of purified staphylokinase.

The ratio of the elution volume of each standard protein as well as the dissolved fractions of purified staphylokinase to that of void volume of the blue dextrin 2000 was calculated.

Results in table (2) indicated that molecular weight of purified staphylokinase was estimated as (15848 daltons), which located between chymotrypsinogen A (25000 daltons) and Ribonuclease A (13700 daltons) (Figure 3). Several molecular forms of staphylokinase have been purified with slighlty different molecular weights (Mr: 16.500) on SDS-PAGE (Saikt *et al*., 1985; Gerlach *et al*., 1988).

Hong *et al* (2007), Characterized and purified recombinant staphylokinase with molecular weight of 15.4 Kda, which estimated by mass spectrometry and SDS-PAGE.

Colin *et al*.(1994) described staphylokinase of S. aureus as an enzyme with fibrinolytic properties to be used in human thrombosis therapy, therefore they determined the molecular weight of staphylokinase by SDS-PAGE about 18000 dalton.

Molecular weight determination by SDS polyacrylamide gel, the staphylokinase migrated as a single protein band (figure 4) with a mobility slightly high than that of trypsin (standard molecular weight of 24,000 daltons). When the mobility of the staphylokinase was compared to the mobility of the molecular weight standards on a logarithmic plot, a subunit molecular of 26,000 + 900 Daltons was obtained (figure 5).

The differences in both results may come from, the variability among strains studies, and the techniques applied for determination of the molecular weight of the staphylokinase gel-filtration chromatography and SDS-polyacrylamide gel electrophoresis for this purpose.

Isoelectric foucing (IEF) for staphylokinase from S. aureus:

The iso electric point for staphylokinase is around 6.2-6.5. Gerlach *et al*.(1988) purified SAK from Staphylococcus aureus bacteriophage 42D by ion exchange chromatography and gel filtration had a specific activity of 16,000 units/mg protein. Isoelectric focusing of the purified SAK revealed heterogeneity with respect to the isoelectric points. Four different SAK proteins were identified among which the majority fraction had an IEP of 6.3 (Figure 6).

Table 1. Staphylokinase activity of some selected *S. aureus* isolates

Isolate No.	SAK activity 200µl	150 µl	100 µ1
1	125	100	90
2	200	160	150
3	100	80	70
4	160	135	110
5	210	170	150
6	115	90	70
7	270	220	200
8	285	240	200
9	200	170	150
10	205	170	145

Table 2. Standardization of staphylokinase in accordance to the ratio of void volume and elution volume (Ve/ Vo) ratio

Standard protein and purified Sak enzymes	Molecular Weight (Dalton)	(Ve / Vo) ratio
Bovine Serum Albumin	67000	1.75
Ovalbumin	43000	1.9
Chymoytrypsinogen A	25000	2.5
Ribonuclease A	13700	2.9
Staphylokinase	15848	2.81

Figure 1. Purification of staphylokinase by ion exchange chromatography (DEAE- Cellulose) column (7.5x3.5cm). The column was washed by using (0.05M) phosphate buffer pH (7), and then eluted by using a gradient of (0.05M) to (0.3M) phosphate buffer pH (7)

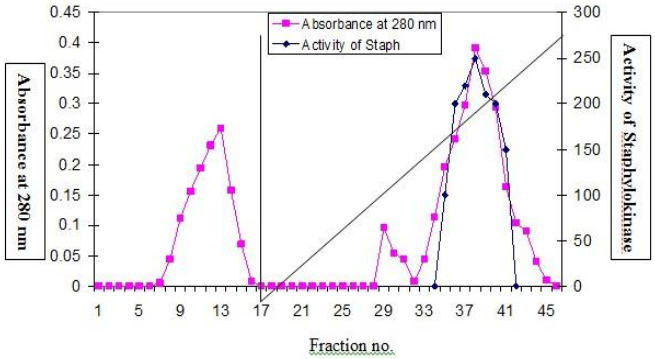


Figure 2. Purification of staphylokinase by gel-filtration chromatography (Sepharose 6B) column (67x2.1cm). Eluent: (0.1M) phosphate buffer pH (7) at a flow rate of (50ml/hour)

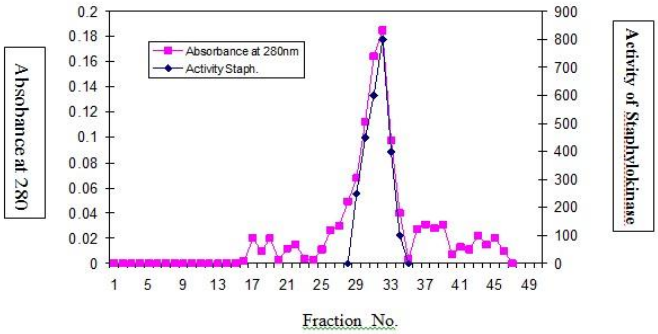


Figure 3. Determination of the molecular weight of Staphylokinase by using standard proteins of different molecular weights. The column was Sephorase 6B (67x2.1cm), eluted with 0.1M phosphate buffer pH 7.0 of a flow rate of 60 ml/ hour. 2. By SDS polyacryamide gel electrophoresis

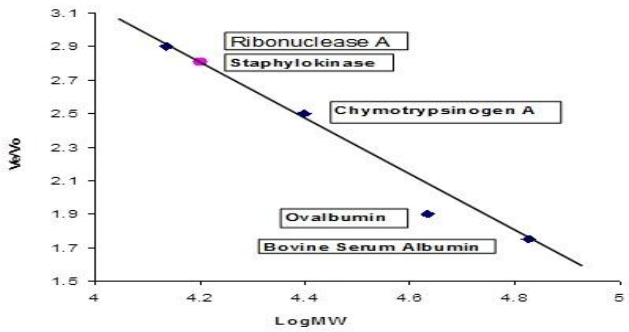


Figure 4. The electrophoresis of staphylokinase taking from S. aureus in acrylamide gel in presence of SDS and mercaptoethanol

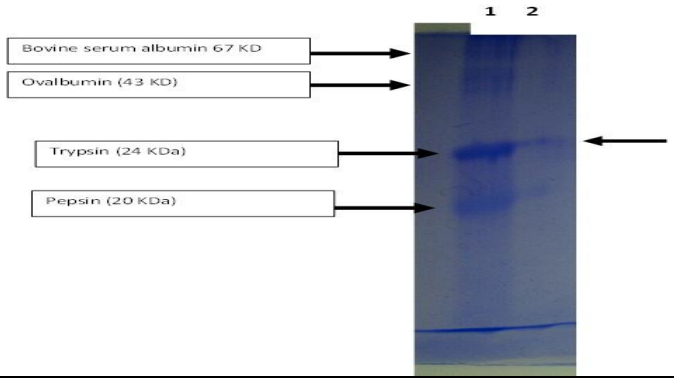


Figure 5. Molecular weight determination of Staphylokinase using sodium dedocyl sulphate polyacrylamide gel electrophoresis. Mobility of Staphylokinase compared to the protein standards

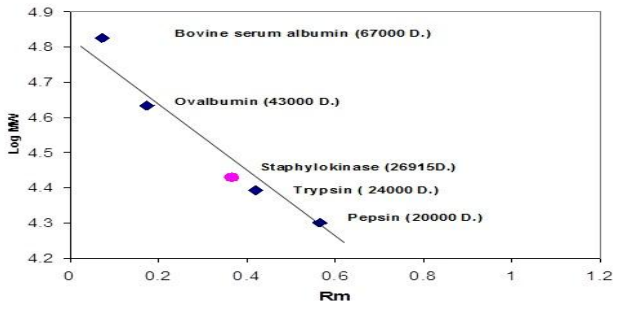
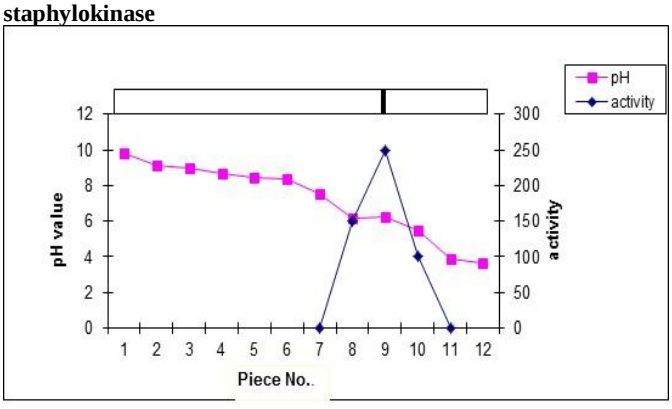


Figure 6. Determination of the iso electric point (IEP) for staphylokinase



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