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Production and purification of staphylokinase from *Staphylococcus hominis* MSD1isolated from Kadi: A traditional Indian fermented food

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Abstract: The present study focuses on the production ofstaphylokinase (SaK) from *Staphylococcus hominis* MSD1 which is isolated from a new source, kadi (a traditional food). Out of 12 isolates from fermented sample, single potent strain was found to be an efficient producer of SaK which was screened by heated plasma and skim milk agar assay. The purified enzyme showed a maximum specific activity of 2994 U mg^{-1} and zone of hydrolysis on radial caseinolytic and heated plasma assay.The blood clot lysis activity was compared with the standard, concluded that minimum 0.18 U (10µL) of purified SaK was required to dissolve the blood clot. The purity and molecular weight of SaK was analysed by SDS-PAGE and found to be approximately around 15 KDa.The present study is the first to report SaK from *Staphylococcus hominis*isolated from kadi, an traditional food.
Keywords: Staphylokinase, *Staphylococcus hominis* MSD1,clot buster, Kadi.

Introduction

Thrombolytic disorders have emerged to be one of the main causes of human mortality worldwide[1]. A blood clot (thrombus) developed in the circulatory system can cause vascular blockade leading to life threatening consequences. Various fibrinolytic enzymes derived from microbial source which are useful for thrombolytic therapy, currently used in managing and preventing cardiovascular diseases from fermented foods[2]. Many fibrinolytic enzymes have been identified from snakes, earthworms, and bacteria: *Streptococcus pyogenes, Aeromonashydrophila,Serratia*E15, *B. natto*, *Bacillus amyloliquefacens*, *Staphylococcus aureus,* actinomycetes and fungi: *Fusariumssp*; *Mucor*sp, *Armillariamellea*[3]. Currently, routine thrombolyticagents in clinical applications are recombinant human tissue plasminogen activator (tPA), urokinase (UK) and streptokinase (SK). SaK is a bacterial plasminogen activator protein which is currently in clinical testing for the treatment of myocardial infarction and peripheral thrombosis. SaK forms a bimolecular complex with human plasmin (ogen) and changes its substrate specificity by exposing new exo-sites that enhances accession of substrate plasminogen (PG) to the plasmin (Pm) active site. SaK, a 16kDaprofibrinolytic protein from the *Staphylococcus aureu*s, has been demonstrated to induce highly fibrin-specific thrombolysis in both human plasma and in limited clinical trials [4,5,6,7].The present study focuses on isolation and screening ofSaK producing *Staphylococcus* sp. from an Indian traditional food, kadi. The study progresses extensively with the production and purification of Sak from *Staphylococcus hominis* MSD1 as well as fibrinolytic activity of the purified Sak.

Materials and methods

Isolation and screening for SaK producing *Staphylococcus* sp.

The food sample (kadi) was serially diluted and spread plated on nutrient agar medium, incubated at 37°C for 24 h. The yellow pigmented colonies were selected on mannitol salt agar and hemolysis pattern was observed on blood agar medium. The isolated colonies were screened for SaK production by heated plasma agar[8]. Depending upon the zone of clearance, the strain MSD1 was selected for further experimental studies. The isolated strain was characterized by cultural, morphological and biochemical characters according to Bergey's Manual of Systematic Bacteriology[9].

Molecular characterization of the potent strain

The genomic DNA of the selected strain was isolated by phenol chloroform method[10]. The amplification of the 16S rDNA was carried out using the primers FC27 (5' to 3' AGAGTTTGATCCTGGCTCAG) and RC1492 (5' to 3' TACGGCTACCTTGTTACGACTT) [11].The PCR product was detected by agarose gel electrophoresis. Sequencing was performed using big dye terminator cycle sequencing kit (Applied Bio Systems, USA).The acquired sequences were used for a gene homology search, with the 16S rDNA sequences available in the public databases from BLAST and were identified to the generic level. Using the CLUSTAL-W Multiple sequence alignment program (Strasburg, France), the 16SrDNA sequences of the strains were aligned with sequences of related organisms obtained from Gen Bank and a phylogenetic tree was constructed by neighbor - joining method using the Evol-View program[12].

Production of Sak

The production of SaK was carried out in a Satoh's medium containing 1% nutrient broth, 0.3%yeast extract, 0.5% sodium chloride, 1% glycerol with pH 7.4 adjusted with 1N NaOH or 1N HCl[13]. 1% of the seed culture was inoculated in the medium and maintained at 37°C for 24 h in a shaker incubator (200 rpm). After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further purification.

Skim milk agar assay

Skimmed milk agar supplemented with 2 ml of fresh human serum was prepared. The supernatant of the production medium was added in the wells and the plates were kept for overnight incubation at 37°C to observe the zone of hydrolysis [14].

Radial caseinolytic assay

Casein hydrolysis was performed by incorporating casein, fresh human serum in 1% nutrient agar. Well diffusion plate technique was used to check the caseinolytic activity of the enzyme present in the supernatant of the production medium and incubated overnight at 37°C[15].

Purification of SaK

The crude enzyme supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation. All subsequent steps were carried out at 4 °C. The protein was resuspended in 20mM phosphate buffer, pH 7.0, and dialyzed against the same buffer. Then the partially purified enzyme was then applied to anion exchange chromatography column packed with DEAE cellulose , bed volume of 25 mL equilibrated with 20 mM phosphate buffer. pH 7.0. The bound proteins were eluted with a linear gradient of 0.05–0.5 M NaCl in 20 mM phosphate buffer, pH 7.0. The eluted fractions at the flow rate of 2 mL min^{-1}were determined for SaK activity and protein concentration.

SDS-PAGE and HPLC

The molecular weight of the obtained enzyme was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions obtained from gel filtration column chromatography were analyzed for protein purity by High Performance Liquid Chromatography (HPLC) using

a Waters 2487 HPLC system consisting of a dual detector, a Waters 1525 binary pump, and equipped with a Waters Symmetry C18 column (5 mm, 4.6150 mm) and Waters Sentry TM universal guard column (5 mm, 4.620 mm) (Waters Corporation, Milford, MA, USA). The mobile phase was acetonitrile and water in the ratio of 1:1. The protein was compared with standard SK (10 KU, Sigma-Aldrich) and purity was authenticated at 250 nm.

Determination of Sak activity

The Sak activity was determined using casein digestion method, which is based on the determination of the liberated tyrosine from casein after plasminogen activation. The values obtained are converted to 1 µM of tyrosine released per minute [16].

Protein Assay

The total protein content was determined by Lowry's method with bovine serum albumin (BSA) as standard [17].

In vitro blood clot lysis method

Clot lysis activity of the purified enzyme was determined by modified Holmstrom method [18,19].

Results

All the strains isolated from fermented food sample were screened for staphylokinase production. Out of 12 isolates only one potent strain MSD1 showed maximum hydrolysis of diameter 18mm on heated plasma plate assay (Figure 1).

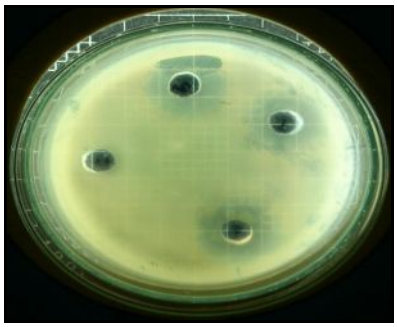

Fig. 1 Zone of hydrolysis on heated plasma plate assay by MSD1

The isolated strain appeared to be yellow pigmented colonies on mannitol salt agar and γ-hemolysis pattern on blood agar plate. The strain was microscopically identified as gram positive cocci with grape like clusters, thus, depicts the characteristic feature of genus *Staphylococcus*. The cultural, morphological and biochemical characteristics feature confirms the strain MSD1 belongs to the genus *Staphylococcus* (Table 1,Figure 2).

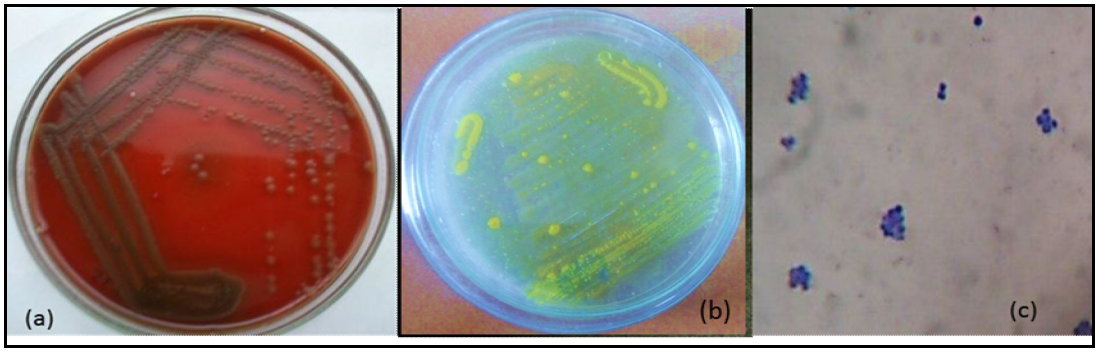

Fig. 2 *Staphylococcus hominis* MSD1 on (a) Blood agar (b)Mannitol salt agar (c) Gram positive cocci in clusters under 100X light microscopy

Table 1 Cultural, microscopic and biochemical characteristics of *Staphylococcus hominis* MSD1

Characterization	Tests	Results
Cultural	Colony morphology Mannitol salt agar Blood agar medium	Small, round, circular, convex yellow pigmented colonies γ-hemolysis
Microscopic	Gram staining Shape Arrangement Motility	Gram positive Cocci Grape like clusters Non motile
Biochemical	Methyl Red Vogesproskauer Citrate Utilization Arginine utilization Glucose Lactose Arabinose Sucrose Sorbitol Mannitol Raffinose	+ + + + + + - + - + -

The 16S rDNA sequencing confirmed the strain MSD1 gene sequence were found 99% similarity with the members of the closely related genera *Staphylococcus hominis* . The strain MSD1 was coined as *Staphylococcus hominis* MSD1. The phylogenetic tree was constructed and the bootstrap analysis was performed to assess the confidence limits of the branching (Figure 3).

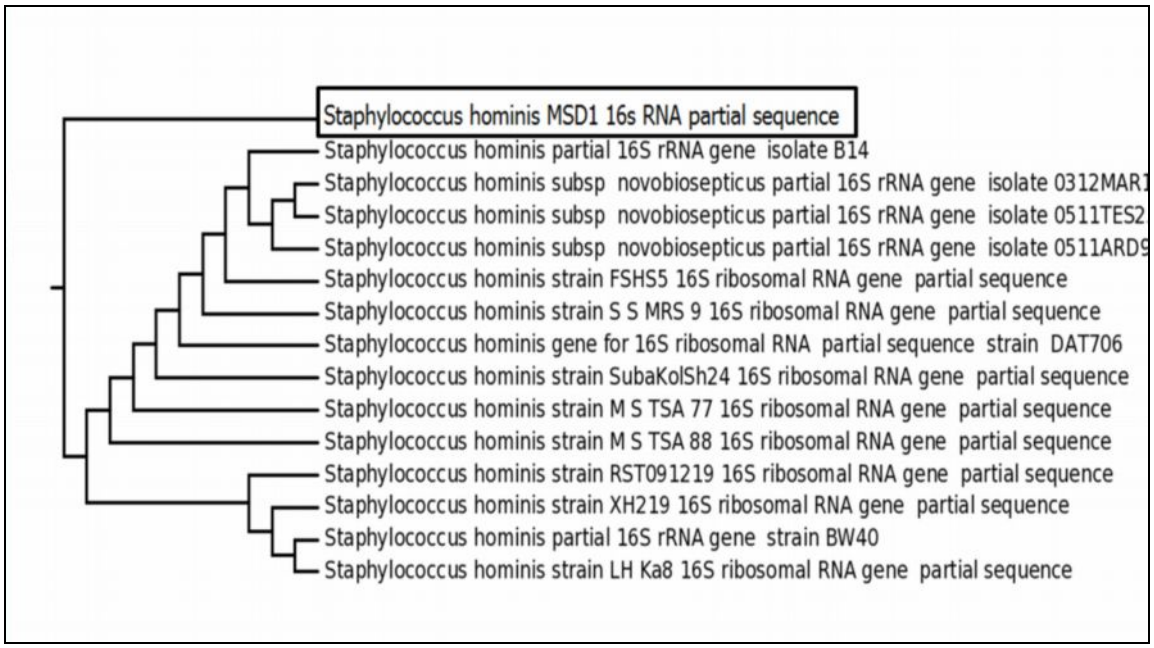

Fig. 3 Phylogenetic tree of *Staphylococcus hominis* MSD1

The crude enzyme from production medium of MSD1 strain was confirmed on skim milk agar and radial caseinolytic assay which maximum zone of hydrolysis of 8mm and 14mm respectively (Figure 4).

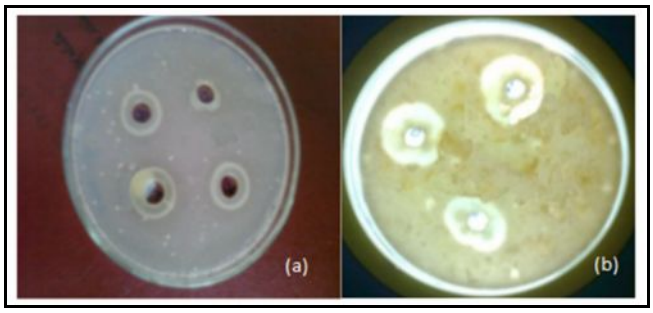

Fig. 4 Zone of hydrolysis of partially purified enzyme on Casein plasminogen agar plate and Skim milk agar assay

The casein digestion enzyme activity and the protein content of the crude supernatant was quantified calorimetrically and found to be 128±0.2 U $mL^{-1}$and 1.4 mg mL^{-1} respectively.The specific activity of the partially purified staphylokinase by dialysis was found to be 2830 U $mg^{-1}$and 2994 U mg^{-1}. Fractions 3 to 6 were collected from DEAE-cellulose column, the 5th fraction showed a maximum activity of 286 U mL^{-1} and protein content of 0.33 mg mL^{-1} (Figure. 5).

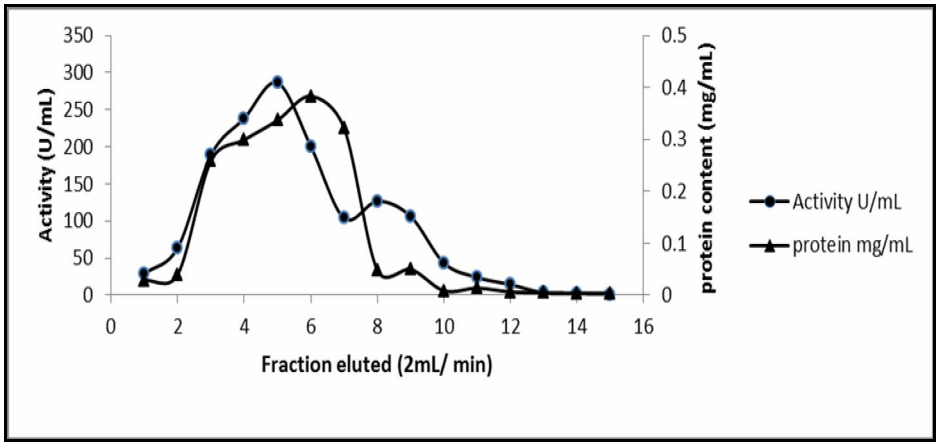

Fig. 5 Elution profile of SaK from ion exchange chromatography

The specific activity of the purified SaK was found to be 2994 U mg^{-1} andits corresponding purification fold of 3.3 with a total yield of 30.5% protein (Table 2). Blood clot lysis activity wasobserved visually at two different concentrations, 50 and 100µL of purified SaK. The enzyme volume of 100µL was able to liquefy the clot completely within 2 h of incubation at room temperature (Figure. 6). The band around 15 kDa in SDS-PAGE confirms the presence of purified SaK(Figure.7).The fractions from 3 to 6 from DEAE- cellulose column were pooled together and confirmed byHPLC. The retention time of purified SaK was 5.082 min (Figure.8).

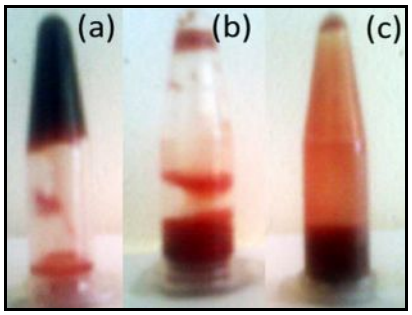

Fig. 6. Blood clot lysis (a) clot before adding enzyme (b) 50µL of purified enzyme (c) 100µL of purified enzyme

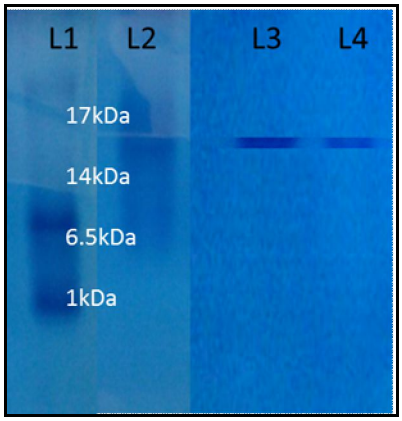

Fig.7 SDS-PAGE profile—Sak from *Staphylococcus hominis* MSD1 (Lane 1), Protein marker; L2 (Lane 2), 60 % ammonium sulfate precipitate; L3 (Lane 3) and L4 (Lane 4) purified Sak from DEAE-cellulose column chromatography

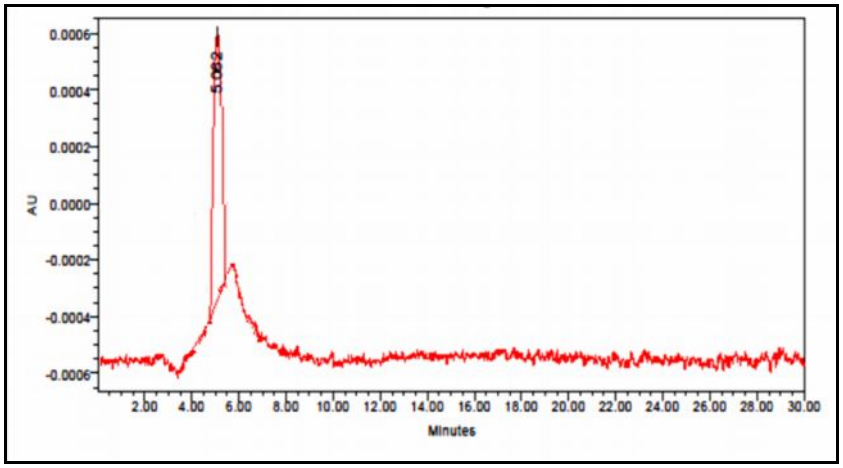

Fig.8 HPLC profile of purified staphylokinase from *Staphylococcus hominis* MSD1

Table 2 Purification table

Purification	Activity U mL^{-1}	Total activity U*mL	Total protein mg*mL	Specific activity U mg^{-1}	Fold purification	Yield %
Crude supernatant	128	12800	14.00	914	1.0	100.0
Ammonium sulfate precipitation	278	3336	1.30	2566	2.8	35.6
Dialysis	311	1868	0.66	2830	3.1	32.3
Ion exchange chromatography	356	713	0.24	2994	3.3	30.5

Discussion

Thrombosis is a major life-threatening disease and effective thrombolytic agents are of clinical value for alternative treatments[20]. SaK is a good clot buster as well as one among the fibrinolytic enzymes[21,22,23].SaK is reported to have a serine protease domain with no proteolytic activity unlike other plasminogen activators like tissue plasminogen activator (tPA) and urokinase[24].The thrombolytic properties of staphylokinase were indirectly investigated in the present study.The present study was performed to investigate the thrombolytic activities of potent isolate*S. aureus* MSD1from fermented food source,kadi. The conventional methods, to characterize the staphylokinase production based on heated Plasma Agar assay method were screened as previously reported by Naseer et al., [25].The zone of hydrolysis of MSD1 strain was 18mm which is lower than the SAK 8 isolate of 28mm by heated plasma agar assay was previously reported by Mohanasrinivasanet al., [26]. According to the Kloos et al.,*S.hominis* appears as yellow pigmented colonies on Mannitol salt agar and no

hemolysis (γ- hemolysis) on blood agar which depicts the similar identification in the present study[27]. The present study was first to report the staphylokinase production from *Staph.hominis.* The radial caseinolytic and skim milk agar assay incorporated with the serum for Sak activity was reportedby Subathra Devi et al., and Mohanasrinivasan et al., [26,28] .When whole cell proteins were analyzed, accumulation of the 14.5-kDa [26] and 15.5 kDa were detected [29]. The previous studies on clot lysis activity of about 0.01mL of the extracted Sak from the strain SAK 8 dissolved 50% of the clot when compared to the present study. Hence the present study proved the fibrinolytic potential of SaK produced from Staphylococcus*hominis* MSD1.Sak extracted from an Indian traditional food, Kadi, has proven and lead the pathway to screen more fibrinolyticenzymes and is comparable to the other plasminogen activators, such as streptokinase, urokinase, nattokinase and tissue plasminogen activator.

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