Original Article:

Characterization of succinate dehydrogenase flavoprotein from *Staphylococcus aureus* ATCC 12600

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ABSTRACT

Background: *Staphylococcus aureus* possesses complete tricarboxylic acid (TCA) cycle. Succinate dehydrogenase (SDH) links TCA cycle with electron transport chain and could therefore be an ideal target in the development of new antimicrobials. Hence, present study is aimed at characterization of SDH flavoprotein from *Staphylococcus aureus* ATCC 12600.

Methods: *Staphylococcus aureus* ATCC 12600 was grown in brain heart infusion (BHI) broth and from the membrane fraction SDH was isolated and purified using diethyl aminoethyl (DEAE) cellulose. The kinetic parameters Michaelis constant (K_M), maximal velocity (V_{max}) and rate constant (k_{cat}) for both native (SDH) and recombinant succinate dehydrogenase (rSDH) enzyme flavoproteins were determined through Hanes-Woolf plot. The SDH flavoprotein gene was amplified using polymerase chain reaction (PCR) and was cloned in pQE30 vector and expressed in DH5α strain of *Escherichia coli*. The molecular weights of native and rSDH flavoproteins were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Results: SDH flavoprotein was purified from the membrane fraction of *S.aureus* and the present methodology gave 40 folds purification with molecular weight of 66kDa. The V_{max}, K_M and k_{cat} of SDH flavoprotein are 199±1 µM/mg/min, 143.5±0.1 µM and 995±4/min. The pure recombinant SDH flavoprotein exhibited similar properties with that of native SDH flavoprotein.

Conclusions: In multidrug-resistant strains of *Staphylococcus aureus* up regulation of SDH was observed favouring increased biofilm formation which is one of the key pathogenic factor and in view of the differences observed in the kinetics of *Staphylococcus aureus* SDH flavoprotein with other bacteria this enzyme is probably regarded as an ideal drug target in the development of new antimicrobials.

Key words: *Succinate dehydrogenase flavoprotein, K_M, V_{max}, Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus is a catalase positive, coagulase positive, facultative anaerobe that causes severe nosocomial, community acquired and chronic infections by the secretion of several virulence factors resulting significant morbidity, mortality and economic loss.[1-3] Virulence factors such as toxins, adhesions and superantigens regulate tricarboxylic acid cycle (TCA) of the pathogen, through availability of macromolecules.[4] *Staphylococcus aureus* uses several strategies to resist antibiotic therapy[5] resulting in up regulation of several metabolic enzymes. One of such metabolic enzyme up regulated in central metabolism is succinate dehydrogenase (SDH) which links TCA cycle and electron transport chain.[6]

TCA cycle is the central cyclic metabolic pathway that plays an integral part in metabolisms.[7] The intermediates from different metabolic pathways enters into TCA cycle through succinyl coenzyme A (succinyl CoA), which is further phosphorylated to form succinyl phosphate. The phosphoryl group is then transferred from succinyl phosphate to histidine residue of the succinyl coA synthatase releasing succinate.

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Krebs cycle releases free energy in the form of reducing equilivants reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$) during oxidation of carbohydrates, lipids and aminoacids[8] thus the growth of organism and respiration can be optimally controlled through this cycle.[6]

Among the enzymes of Krebs cycle SDH differs from other dehydrogenases due to its redox properties and is the only membrane bound dehydrogenase.[9] It consists of three subunits: FAD containing subunit SdhA; an SdhB subunit containing iron-sulfur units of the 4Fe-4S; and membrane-bound cytochrome b558 (SdhC and SdhD).[6,10-11] This membrane bound dehydrogenase links the TCA cycle with respiratory chain and its activity is modulated by several activators and inhibitors. It catalyzes the oxidation of succinate to fumerate by SdhA subunit which contains FAD as cofactor linked to histidine residue.[9] Then the iron-sulfur centers of succinate dehydrogenase relay the electrons from SdhA to membrane domains. It releases free energy by the oxidation of succinate to fumerate, while FAD is reduced to $FADH_2$ in a two electron process. ATP is generated in respiratory chain from $FADH_2$.[6] SDH also plays a major role in oxygen sensing and in combination with ubiquinone SDH acts as an antioxidant controlling the superoxides scavenging activity of respiratory chain.[9] Hence, the present study was designed to study the characterization of SDH flavoprotein from *Staphylococcus aureus* ATCC 12600.

MATERIAL AND METHODS

Bacterial strains and conditions

Staphylococcus aureus ATCC 12600 was grown on modified Baird parker media at 37 ^{o}C.[12] After overnight incubation a single black shiny colony with distinct zone was picked and grown in brain heart infusion (BHI) broth for the extraction of genomic deoxyribonucleic acid (DNA) and characterization of SDH flavoprotein.

Purification of Succinate dehydrogenase flavoprotein from Staphylococcus aureus

Baird-Parker Agar media, Brain heart-infusion broth, Tris compound, Ampicillin, EDTA and X-gal were procured from Hi-media company. Ammonium sulphate, sodium chloride, sodium dodecyl sulphate, di-chlorophenal Indophenal (DCIP) FAD, Triton-X-100 and sodium succinate were obtained from Sisco Research Laboratories (SRL) Company. Taq DNA polymerase, dNTP's, T4 DNA polymerase, smaI restriction enzyme, calf intestinal alkaline phosphatase and T4 DNA Ligase were procured from Merck company

Staphylococcus aureus ATCC 12600 culture was grown upto late log phase and membrane fraction was collected to study the kinetics of SDH flavoprotein. The membrane fraction was initially purified using different concentrations of ammonium sulphate; 20%-40% of ammonium sulphate fraction showed maximum activity and this fraction was used for further purification on diethyl aminoethyl (DEAE) cellulose column using 20 mM sodium chloride and absorbance was taken at 280 nm. The molecular weight of purified enzyme was determined by running 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).[13]

Study of kinetics of succinate dehydrogenase flavoprotein

For the enzyme assay DCIP was used as proton (H^+) acceptor, which reduces FAD to $FADH_2$ that can be read spectrophotometrically at 600 nm. The reaction mixture contains 0.01 mg/mL of sodium succinate, 0.01% (v/v) tritonX100, 10 mM Tris HCl and 50 µL of enzyme. The absorbance was measured at 600 nm

against blank. Enzyme activity was expressed as concentration of product formed per minute per mL. Michaelis constant (K_M)and maximal velocity (V_{max}) for SDH were determined using Hanes-Woolf plot ([S] vs [S] / V).[14]

Polymerase chain reaction (PCR) for amplification of SDH flavoprotein

Staphylococcus aureus ATCC 12600 genomic DNA was isolated and used as template for amplification of SDH flavoprotein using the forward primer: 5'-CATGGCAGAGAAA CATCT-3' reverse primer: 5'-TAGACT TACTTGTGTAAT-3' in Master cycler gradient machine (Eppendorf) place of manufacture. The cocktail mixture containing 30 µL PCR water, 5µL of 10x polymerase buffer, 5 µL of dNTP's mix, 2 µL forward primer, 2µL reverse primer, 1µL (2 U/µL) TaqDNA polymerase, 5 µL of Template was run upto 40 cycles under 94 °C of initial denaturation for 10 min, 94 °C of denaturation for 1 min, 37.35 °C of annealing temperature for 90 sec, 72 °C of amplification for 2 min and final extension temperature of 72 °C for 5min.[15,16]

Cloning and expression of succinate dehydrogenase flavoprotein

PCR amplified product of SDH flavoprotein gene was electro-eluted and suspended in 10 mM Tris HCl pH 8.0 then it was blunt ended with 1U/µL of DNA polymerase purified from the bacteriophase T4. The plasmid DNA, pQE30 was used as expression vector containing T5 promoter site was digested with 1U/µL of restriction enzyme SmaI at 25 °C and it was dephosphorylated using 1U/µL of calf intestine alkaline phosphatase. Then insert and SmaI digested pQE30 (1:5) was ligated using 100U of Taq DNA ligase and transformed into *Escherichia coli* DH5α. The transforments were screened using X-gal and isopropyl-β-D-1 thiogalactopyranoside (IPTG) on lysogeny broth (LB) agar plate containing ampicillin. The resultant clone was named as SDH clone1 and it was over expressed optimally with 0.25mM IPTG. Then the recombinant SDH flavoprotein was eluted from the SDS-PAGE. The excised gel pieces were taken in sterile micro centrifuge tubes and 1 mL of elution buffer [50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5] was added so that the gel pieces are completely immersed. Then the gel pieces were crushed using a clean pestle and incubated in a rotary shaker at 30 °C overnight following centrifugation at 10,000 revolutions per minute (rpm) for 10 minutes and carefully pipette supernatant into a new micro centrifuge tube. An aliquot of the supernatant was tested for the presence of protein by subjecting it to 10% SDS-PAGE.[15,17] This purified protein activity was determined by subtracting the activity of uninduced enzyme from the purified recombinant enzyme and the kinetic parameters K_M, V_{max}, K_{cat} for recombinant succinate dehydrogenase (rSDH) flavoprotein were determined using Hanes-Woolf plot ([S] vs [S] / V).[14]

RESULTS

Kinetic parameters of SDH flavoprotein identified in the membrane fraction of *Staphylococcus aureus* ATCC 12600, V_{max}, K_M and k_{cat}, were found to be 199±1 µM/mg/min, 143.5±0.1 µM and 995±4/min (Table 1 and Figure 1). Increased enzyme activity was observed with purification and the methodology yielded 40 folds of purification from crude fraction (Table 1). The SDH flavoprotein was eluted from DEAE cellulose column at 120 mM NaCl concentration. In course of purification enzyme activity was not lost and K_M remained constant. This fraction gave a single band (molecular weight 66 kDa) in 10% SDS-PAGE (Figure 2). The genomic DNA was isolated from *Staphylococcus aureus* ATCC12600 (Figure 3). PCR amplification of this gene using the primers designed from the gene sequence reported in the

Table 1: Purification and characterization of Succinate dehydrogenase from *Staphylococcus aureus* ATCC 12600

Enzyme fraction	Protein concentration (mg/mL)	V_{max} (µM/mg/min)*	K_M (µM)*	k_{cat} (/min)*	Fold purification
Staphylococcus aureus ATCC12600 Crude	0.789	192.3±0.2	144.23±0.1	1282±1	0
20%-40% $(NH_4)_2SO_4$ fraction	0.3017	195±2	144±0.2	1300±4.5	4.5
DEAE cellulose column	0.2618	199±1	143.5±0.1	995±4	40

*Expressed as mean ± SD obtained from three determinations
$(NH_4)_2SO_4$=Ammonium sulphate; DEAE=diethyl aminoethyl; K_M=Michaelis constant; V_{max}=Maximal velocity; k_{cat}=rate constant; SD=standard deviation

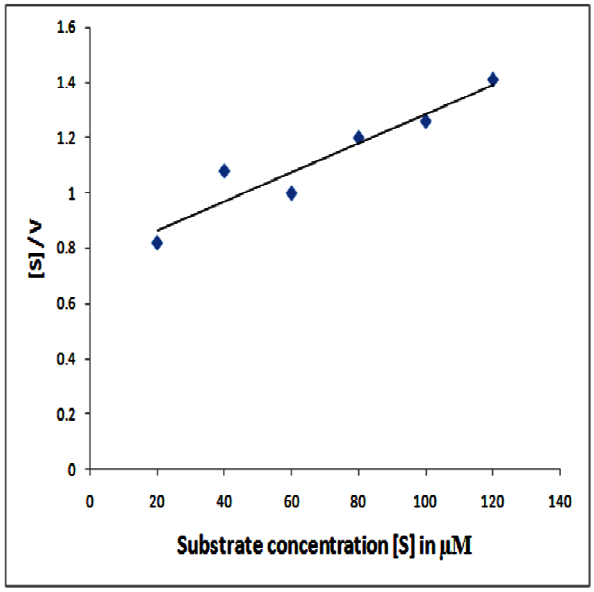

Figure 1: Kinetic plots of SDH flavoprotein for the determination of K_M and V_{max} using Hanes-Woolf Plot for crude SDH flavoprotein of *Staphylococcus aureus* ATCC 12600.

SDH=Succinate dehydrogenase; K_M=Michaelis constant; V_{max}=Maximal velocity

Staphylococcus aureus database gave 1.8 Kb PCR product (Figure 3B). This product was cloned in the SmaI site of pQE30 vector in -1 frame and expressed in Escerichia coli DH5α with 0.25mM IPTG. The expressed protein gave single band with 66kDa (Figure 4). The kinetic parameters V_{max}, K_M and k_{cat} for rSDH flavoprotein (Figure 5) were found to be 200±0.2 µM/mg/min, 138±0.5 µM and 1538±0.5/min respectively which were close to the native SDH flavoprotein of *Staphylococcus aureus* ATCC 12600 (Table 2). These results indicated presence of single SDH in *Staphylococcus aureus.*

Comparative analysis of SDH flavoprotein kinetics with other organisms is shown in Table 3.[18]

DISCUSSION

When glucose is completely consumed under anaerobic conditions, *Staphylococci* oxidize acetate through TCA cycle. The SDH flavoprotein is the central important component in TCA cycle. Its activity is associated with several effects like survival, virulence and production of biofilm slime substance, polysaccharide intracellular adhesion (PIA). The cells in a biofilm show various gradients of oxygen, nutrients and pH which affords a permanent adaptation to survive better in such complex environment.[6] So that SDH is up regulated in TCA cycle that leads to production of more reducing equivalents which can fuel the respiratory chain resulting in reductive conditions which was observed in most of the multidrug resistant and vancomycin resistant strains of *Staphylococcus aureus* thus SDH flavoprotein was characterized in the present study.[18-20] Enzyme kinetics of SDH flavoprotein indicates that the K_M of the enzyme remained constant throughout purification (Table 1).

In *Staphylococcus aureus* it has been observed that up regulation of SDH is due to the effects of survival under low nutrients and oxygen

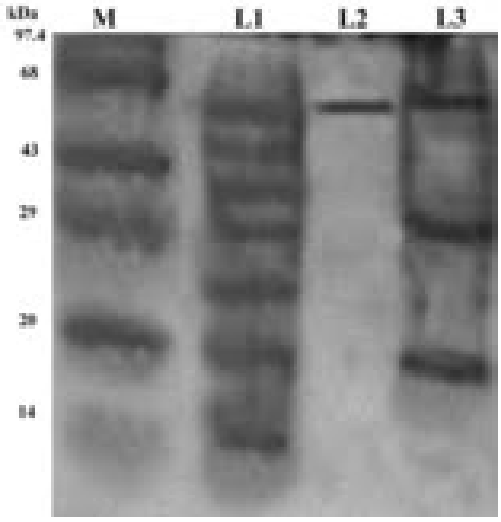

Figure 2: 10% SDS-PAGE protein profile analysis of *Staphylococcus aureus* SDH flavoprotein

M: Molecular size markers (obtained from Bangalore Genei Pvt ltd); L1: Crude membrane fraction of *Staphylococcus aureus* ATCC12600; L2: SDH flavoprotein obtained from DEAE cellulose column followed by the gel filtration; L3: 20-40% ammonium sulphate concentrate of *Staphyloccus aureus* membrane fraction.

SDS-PAGE=sodium dodecyl sulphate polyacrylamide gel electrophoresis; ATCC= American type culture collection; DEAE= diethyl amino ethyl

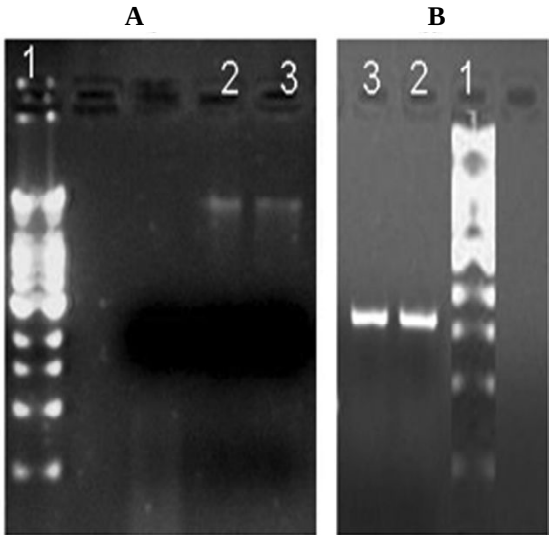

Figure 3: PCR amplication of SDH flavoprotein

A: Electrophoretogram showing the genomic DNA isolated from *Staphylococcus aureus* ATCC12600: Lane -super mix molecular size marker (33.5, 24.5, 15, 8.99, 7, 6,5,4,3,2,1,0.5Kb); Lanes 2 and 3-isolated genomic DNA.

B: PCR amplification of SDH flavoprotein gene: Lane 1- super mix molecular size marker; Lanes 2 and 3-and PCR products of SDH flavoprotein gene.

SDH=Succinate dehydrogenase; PCR=Polymerase chain reaction; SmaI=restriction enzyme; PQE30=plasmid DNA used as expression vector

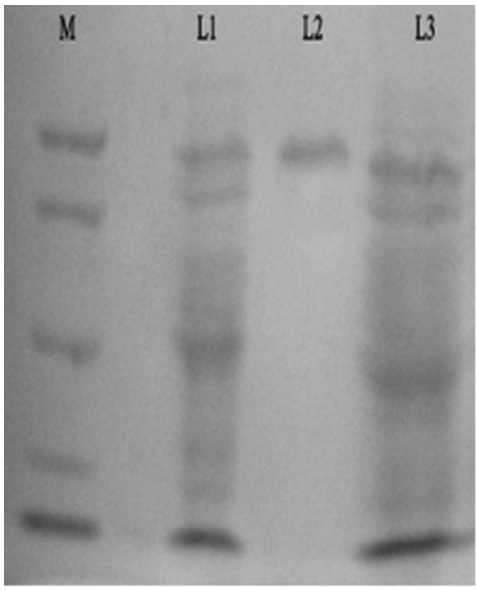

Figure 4: Expression of SDH flavoprotein gene from SDH clone 1

M=Molecular size markers (obtained from Bangalore Genei Pvt Ltd); L1=induced cell lysate of SDH clone1; L2=eluted fraction of SDH clone1; L3=uninduced cell lysate of SDH clone1; SDH=succinate dehydrogenase

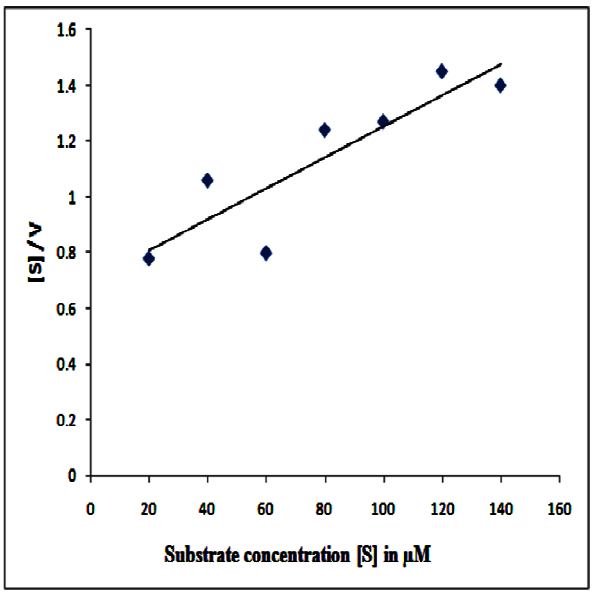

Figure 5: Hanes-Wolf plot for recombinant SDH flavoprotein for the determination of K_M and V_{max} of *Staphylococcus aureus* ATCC 12600

SDH=Succinate dehydrogenase; K_M=Michaelis constant; V_{max}=Maximal velocity

Table 2: Comparison of kinetics of SDH with rSDH flavoprotein

Strain (µM/mg/min)*	V_{max}	K_M (µM)*	k_{cat} (/min)*
Native SDH from *Staphylococcus aureus* ATCC 12600	192.3±0.2	144.23±0.1	961.2±1
rSDH	200±0.2	138±0.5	1538±0.5

*expressed as mean ± SD obtained from three determinations
K_M=Michaelis constant; V_{max}=maximal velocity; k_{cat}=rate constant; SD=standard deviation; SDH=succinate dehydrogenase; rSDH=recombinant succinate dehydrogenase

Table 3: Comparative analysis of SDH flavoprotein kinetics with other organisms

Organism	V_{max} (µM/mg/min)	K_M (µM)	Reference
Escherishia coli	264	20	18
Mycobacterium pheli	19	120	18
Staphylococcus aureus	199	143.5	Current study

K_M=Michaelis constant; V_{max}=Maximal velocity; SDH=succinate dehydrogenase

limitation leads to more reductive conditions that are advantageous for biofilm formation. *Staphylococcus aureus* adapt to rapid changes in the environment for growth and survival under biofilm formation which is one of the key pathogenic factor of this organism.[6] The results of native SDH purification and SDS-PAGE (Figure 2) encouraged us to clone the large subunit of SDH which is a flavoprotein that primarily functions in the TCA cycle while other two subunits are directly linked to the electron transport system. Thus only the large subunit of SDH i.e., SdhA was amplified and cloned in SmaI site of pQE30 vector and expressed in *Escherishia coli* DH5α and the clone was named as SDH clone 1. In order to achieve correct expression in SDH clone 1 the gene was cloned in the –1 frame in pQE30 vector for which "C" residue was added to the 5' end of forward primer. The expressed protein gave single band in 10% SDS-PAGE with a molecular weight of 66kDa (Figure 4).[17] The eluted protein from the gel showed similar properties with that of native enzyme indicating no isoforms of SDH are present in *Staphylococcus aureus*. Different bacteria have variation in their TCA cycle according to their needs.[22-23] as evidenced by different kinetic parameters of different bacteria(Table 3).[18, 24]

It has been observed that impaired acetate catabolism is the key feature in vancomycin resistant *Staphylococcus aureus* strains. In *Staphylococci* acetate catabolism is implicated in growth yield, antibiotic tolerance, regulation of cell death and biofilm forming ability.[21] In human tissues its infection is more pronounced in the abscess where, low oxygen condition prevails. From the previous studies it is evident that TCA cycle plays central role in metabolism and SDH flavoprotein which is an intermediate of TCA cycle is up regulated in biofilm cells of Staphylococci and deletion of SDH genes leads to small colony variant phenotype that have altered metabolisms.[20] Therefore, considering the linking of SDH flavoprotein of TCA cycle, with electron transport chain as well as large variation in the functional properties when compared to other bacteria and human host, it appears that this enzyme is probably a suitable drug target for infections caused by multidrug resistant *Staphylococcus aureus*.[25]

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