## Original Article:

# Isolation, purification and characterization of pyruvate kinase from *Staphylococcus aureus* : a potential drug target

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## ABSTRACT

**Background:** With emergence of multidrug-resistant strains of *Staphylococcus aureus,* there is an urgent need for the development of new antimicrobials which are narrow and pathogen specific. In this context, pyruvate kinase (PK) an important enzyme in the glycolysis, which catalyses the formation of pyruvate which is the key intersection in the network of metabolic pathways was isolated and purified from *Staphylococcus aureus* ATCC12600.

**Methods:** Purification steps included 10%-20% ammonium sulphate fractionation, diethyl aminoethyl cellulose ion exchange chromatography followed by gel filtration on Sephadex G-100. The pure PK molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and $K_m$ and $V_{max}$ for the PK was demonstrated.

**Results:** The pure PK obtained from Sephadex G-100 gel filtration column exhibited $K_m$ of $0.78 \pm 0.18$ µM and Vmax $76.47 \pm 0.82$ µM NADH/mg/min and molecular weight of 250 kDa in solution. However, in SDS-PAGE showed single band with a molecular weight of 63 kDa confirming the homotetramer nature. In all steps of purification the $K_m$ remained constant indicating presence of only one kind of enzyme. The PK gene searched in the genomic sequences of *Staphylococcus aureus* also confirmed the same.

**Interpretation and conclusions**: In *Staphylococcus aureus* presence of only one kind of PK unlike in other Gram positive bacteria exhibiting distinct differences in enzyme kinetics. This enzyme also showed the functionality of PK is found to be different from its human host. Therefore, PK probably is regarded as an ideal drug target in the development of new potent antimicrobials.

**Key words:** *Pyruvate Kinase, Diethylaminoethyl cellulose, Pyruvate, Km, Vmax*

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## INTRODUCTION

*Staphylococcus aureus* is ubiquitous organism and it can cause variety of life threatening infections ranging from skin abscess to severe illness like endocarditis, toxic shock syndrome, pneumonia, mastitis.[1] *Staphylococcus aureus* can infect any anatomical region of human body of bloodstream, cardiovascular, eye, ear, nose, and throat infections. An Indian working group Global Antibiotic Resistance Partnership (GARP) research estimates that of the approximately 190,000 neonatal deaths each year due to sepsis.[2] An increasing percentage of *Staphylococcus aureus* infections are caused due to the occurrence of "multidrug resistant (MDR) strains". Even after the entry of antibiotics it was difficult to control *Staphylococcus aureus* infections, because of its adaptability and persistence in various environmental conditions. Though, newer classes of antibiotics were introduced to treat *Staphylococcus aureus* infections the organism acquired resistance to all those antibiotics. *Staphylococcus aureus* is even resistant to vancomycin, a glycopeptide antibiotic which is supposed as last resort for prescription in the treatment and management of *Staphylococcus aureus* infections.[3] These drug resistant bacteria showed increased pathogenecity and it has been observed that drug resistance regulates various metabolic pathways in the organism.

*Staphylococcus aureus* derives its energy by the catabolism of glucose through Embden-Meyerhoff-Parnas pathway.[4] The final product

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pyruvate is the most important step of glycolysis because pyruvate further enters into tricarboxylic acid (TCA) cycle where, it further catabolise and it controls the carbon flux of glycolytic intermediates and regulates the level of adenosine triphosphate (ATP) in the cell and TCA cycle is linked to pathogenecity of the organism. Pyruvate is a key intersection point the activation of TCA cycle and formation of pyruvate is catalysed by pyruvate kinase (PK) enzyme.[5]

The enzyme PK (ATP: pyruvate 2-O-phosphotransferase E.C.2.7.1.40) catalyzes irreversibly by the conversion of phosphoenolpyruvate (PEP) to pyruvate, coupled to the synthesis of one molecule of ATP. The PK belongs to group of transferases which couples the free energy of phosphoenol pyruvate (PEP) hydrolysis to the synthesis of ATP to form pyruvate and this process requires participation of both monovalent ($K^+$) and divalent ($Mg^{2+}$) cations as co-factors.[6,7] The presence of bound substrate and metal ions also increases the affinity of fructose-1,6-bisphosphate (FBP) for the allosteric site.[8] ATP, alanine, phenylalanine becomes negative allosteric inhibitors of PK and serves as a switch between the glycolytic and gluconeogenic pathways.[9] This regulation flux by PK turns directs the concentrations levels of glycolytic intermediates, biosynthetic precursors, and nucleoside tri-phosphates in the cell which inturns regulates the cell proliferation and survival of *Staphylococcus aureus*.[10,11]

To expand understanding the mechanism of acquiring MDR strains in the system we need to study the regulatory enzymes which, contribute for resistance and aids for pathogens survival. In the present study has been focused on the isolation, purification and biochemical characterization of PK from *Staphylococcus aureus* ATCC 12600.

## MATERIAL AND METHODS

For the present study, chemicals were obtained from Sisco Research Laboratories Pvt. Ltd., India, Hi-Media Laboratories Pvt.Ltd., India, Sigma-Aldrich, USA, New England Biolabs, USA and QIAGEN Inc., Valencia, CA.

### Bacterial strains and conditions

*Staphylococcus aureus* ATCC12600 was grown on modified Baird Parkar media[12] at 37 °C. After overnight incubation single black shiny coloured with distinct zone colony was picked and cultured in brain heart infusion (BHI) broth at 37 °C. Thus, grown *Staphylococcus aureus* ATCC12600 culture was used for the isolation, purification of PK enzyme and extraction of chromosomal deoxyribonucleic acid (DNA).

### Isolation and purification of Pyruvate kinase from *S. aureus* ATCC 12600

*Staphylococcus aureus* ATCC12600 was grown in Brain heart infusion broth (BHI) at 37 °C up to late log phase [optical density at 540nm ($OD_{540}$) = 0.9] from the culture the cytosolic fraction was isolated[13] and used for PK enzyme assay. PK from the cytosolic fraction was purified first by concentrating initially with 0%-10% $(NH_4)_2SO_4$. This fraction showed minimum activity therefore further $(NH4)_2SO_4$ fractionation was followed and at 10%-20% $(NH4)_2SO_4$, the fraction showed maximum activity which was used for further purification. Thus obtained pellet after centrifugation at 10,000 rpm for 10 min at 4 °C was suspended in 2 mL of 0.1M Tris-HCl pH 7.4, and dialyzed against the same buffer. PK was further fractionated on DEAE cellulose column. For this, 1mL of 10%-20% $(NH_4)_2SO_4$ concentrate was loaded on DEAE cellulose column and PK was eluted with stepwise gradient of NaCl concentration prepared in 0.1M Tris-HCl pH 7.4. The peak fraction in each gradient was assayed to check the presence of enzyme. Maximum PK activity was observed in 20 mM NaCl fraction which was dialyzed against 0.1M Tris-HCl pH 7.4. The dialyzed fraction was concentrated using Lyophilizer (Delvac) and fractionated on Sephadex G-100 (superfine grade, 30 cm x 1.5 cm) which was swollen in 0.1M Tris-HCl pH 7.4 and the PK was eluted with 0.1M Tris-HCl pH 7.4 containing 50mM NaCl. The calibration of the column was performed using catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa). Proteins were eluted with 0.1M Tris-HCl pH 7.4 containing

50mM NaCl at a flow rate of 0.5mL/min. Each peak fraction was assayed to identify the presence of enzyme. In all the steps of protein purification the concentration of the protein was determined[14] method and the molecular weight of the purified enzyme was determined by running SDS-PAGE (10%).[15]

**Kinetics study**

The cytosolic fraction was collected from the *S. aureus* to perform the enzyme assay and kinetics for PK. The reaction mixture contains 50mM TrisHCl, pH 7.5; 45mM adenosine diphosphate (ADP); 6.6mM nicotinamide adenine dinucleotide-reduced (NADH); 4.5mM PEP; pure and crude enzyme in cytosolic fraction and pure lactate dehydrogenase (LDH). The reaction mixture was incubated at 4-5 min at 37 $^{o}$C and absorbance was read at 340 nm by consumption of NADH against reference blank. The maximum velocity of the enzyme catalyzed reaction was calculated by taking varying concentrations of substrate PEP from 1mM to 10 mM and Km and Vmax for PK was determined using Hanes-Woolf plot ([S] vs [S]/V).

## RESULTS

In the present study PK was identified in the cytosolic fraction of *Staphylococcus aureus* ATCC12600 with enzyme activity of 0.015 ± 0.001 µM NADH/mL/min, $V_{max}$ 44.1 ± 0.24 µM NADH/mg/min and Km 0.75 ± 0.2 µM. From this fraction PK was purified first by 10%-20% $(NH_4)_2SO_4$ concentration followed by DEAE cellulose chromatography (Figure 1) and gel filtration on Sephadex G-100 column. The PK in anion exchange column was fractionated using discontinuous gradient of NaCl, the PK activity was observed in the peak fraction of 20 mM NaCl gradient, the eluted protein was dialysed and lyophilized. This was further fractionated on Sephadex G-100 and the first elution peak showed maximum enzyme activity. The molecular weight determined from Sephadex G-100 indicated PK has molecular weight of 250 kDa and the pure PK exhibited $K_m$ 0.78 ± 0.18 µM and $V_{max}$ 76.47 ± 0.82 µM NADH/mg/min (Figures 2A and 2B). In all the steps of protein purification the enzyme activity increased with the increase in the purification. The $K_m$ in all steps of purification remained almost constant and indicated presence of only one kind of PK in the *Staphylococcus aureus* (Table 1). These results were also in accordance having only one putative gene products for PK found in the genomic sequences of all the strains of *Staphylococcus aureus*. The eluted protein was concentrated and was electrophoresed in 10% SDS-PAGE on staining with silver nitrate a single band with a molecular weight of 63 kDa was observed (Figure 3) and single band in SDS-PAGE indicating PK exists as homotetramer enzyme in solution.

## DISCUSSION

It is well known that prokaryotes derive their energy maximally from glucose catabolism[16,17] and PK is one of three regulatory enzymes in glycolysis which controls the overall activity of the pathway it catalyses the formation of pyruvate from phosphoenol pyruvate by producing one ATP molecule.[18,19] Pyruvate is a key intersection in the network of metabolic pathways[20], therefore; in the present study PK of *Staphylococcus aureus* ATCC12600 was biochemically characterized.

The purification protocol adopted yielded 32 folds purified PK from *Staphylococcus aureus* ATCC12600. In all steps of purification increased enzyme activity was observed (Table 1). The PK from the cytosolic fraction was concentrated successively by ammonium sulphate concentration initial concentration of 0%-10% ammonium sulphate showed no enzyme activity however; 10%-20% ammonium sulphate concentration gave maximum activity compared to 20%-30% which showed very low PK activity.[21] From this PK was successfully fractionated on DEAE cellulose column and the peak fraction of 20mM NaCl gradient showed maximum PK activity (Figure 1 and Table 1).This fraction was lyophilized and fractionated on Sephadex G-100 column,the first elution fraction contained maximum PK activity and molecular weight determined from gel filtration column indicated molecular weight more than 250 kDa. Further, the enzyme was electrophoresed in SDS-PAGE (10%) which gave single band with a molecular weight of 63 kDa confirming the PK exists as homotetramer[22-25] in solution (Figure 3).

**Table 1: Purification and characterization of pyruvate kinase from *Staphylococcus aures* ATCC 12600**

| Purification methods | Protein concentraton | Enzyme activity (µM NADH/mL/min) | Velocity (µM NADH/mg/min) (µg/µL) | $K_m$ (µM) | Fold purifi-cation |
|---|---|---|---|---|---|
| Crude | 0.56 | 0.015 ± 0.001 | 44.1 ± 0.24 | 0.75 ± 0.2 | 0 |
| $(NH_4)_2SO_4$ (10-20%) fractionation | 0.52 | 0.018 ± 0.002 | 52.3 ± 0.32 | 0.76 ± 0.25 | 8 |
| DEAE cellulose column | 0.44 | 0.024 ± 0.002 | 68.2 ± 0.64 | 0.78 ± 0.22 | 4 |
| Gel filtration G-100 | 0.4 | 0.027 ± 0.003 | 76.47± 0.82 | 0.78 ± 0.18 | 32 |

Values are the mean ± SD obtained from three determinations; DEAE = diethyl aminoethyl

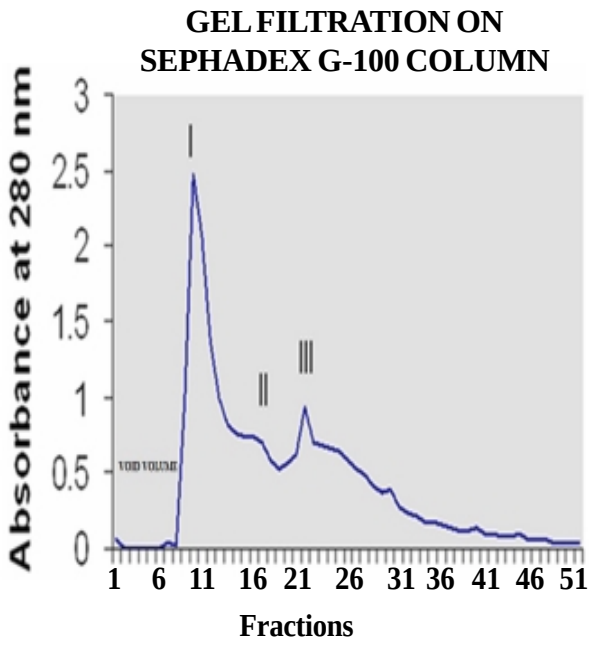


**Figure 1:** Gel filtration profile of *Staphylococcus aureus* pyruvate kinase
PK fractionation on Sephadex G-100 (superfine grade, 30cm x 1.5 cm). The PK was eluted using 0.1M Tris-HCl pH 7.4 containing 20mM NaCl and the enzyme appeared in first peak
PK= pyruvate kinase

The pure PK exhibited Km 0.78 ± 0.18 µM and Vmax 76.47 ± 0.82 ìM NADH/mg/min (Figures 2A and 2B); the Vmax was almost same obtained from both Hanes-Woolf plot and Line-Weaver Buck plot corroborating the kinetic results.[26-29] The enzyme kinetic data indicated Km for PK remained same in all the steps of purification indicating presence of only one kind of enzyme and these results are in congruence with the results obtained from the PK gene search in the genomic sequences of all *Staphyloccus aureus* strains which indicated as one enzyme. However; in *Escherichia coli*[30] and *Salmonella typhimurium*[31] contain two isoenzymes type I and type II with variable enzyme kinetics (Table 2).

*Staphyloccus aureus* possess complete TCA cycle unlike *Escherichia coli, Salmonella typhimurium*[32] and this makes this enzyme to adjust its redox conditions according to the host environmental conditions[33] and also *Staphyloccus aureus* can colonize in any anatomical locales in the host. The PK gene sequence of *Staphyloccus aureus* showed no homology with its human

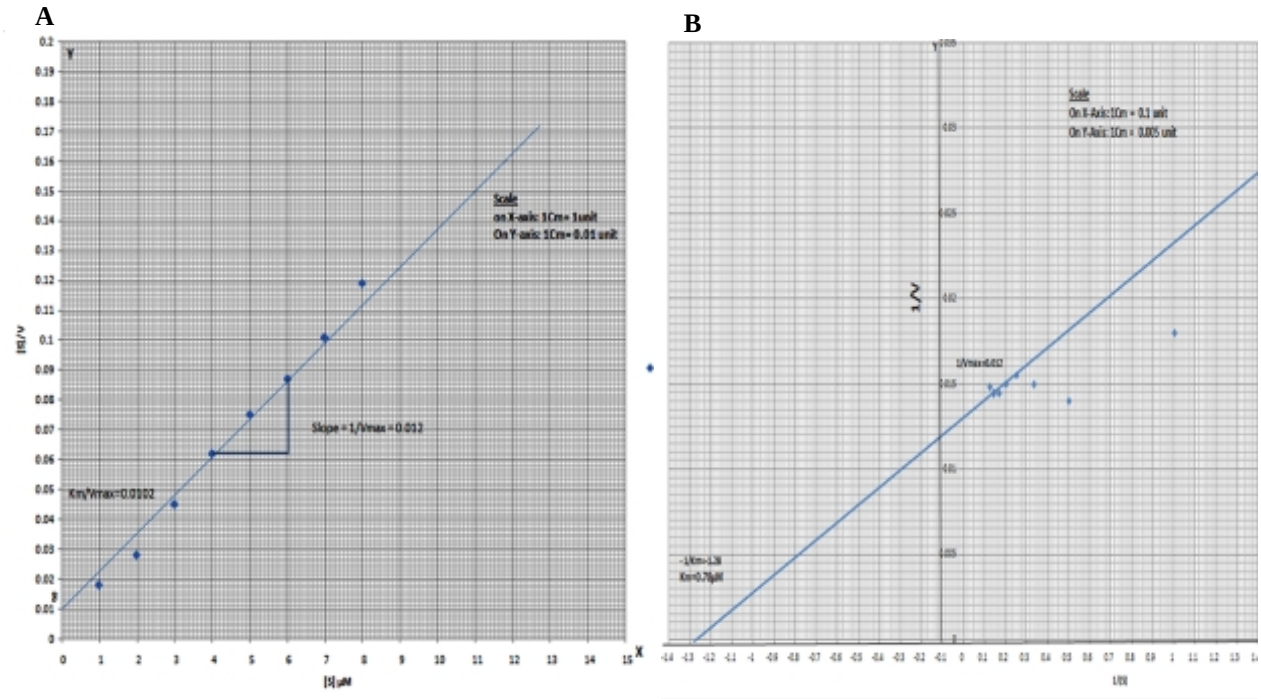


**Figure 2**: Kinetic plots of pyruvate kinase for the determination of $K_m$ and $V_{max}$ showing Hanes-Woolf Plot (**A**) and Line-Weaver Burk plot (**B**) (1/[S] vs 1/V)

**Table 2: Comparative analysis of pyruvate kinase kinetics with other Gram negative, Gram positive organisms and *Homo sapiens***

| Organism | $K_m$ (μM) | $V_{max}$ (Units/mg) | Reference |
|---|---|---|---|
| *Escherichia coli* | 0.08 | 0.714 | 27 |
| *Bacillus licheniformis* | 1.1 | 93 | 28 |
| *Salmonella typhimurium* | 1.5 | 53 | 31 |
| *Homo sapiens R/L isoform* | 1.2 | 11.9 | 9 & 19 |
| *Staphyloccus aureus* | 0.78 | 83.33 | present study |

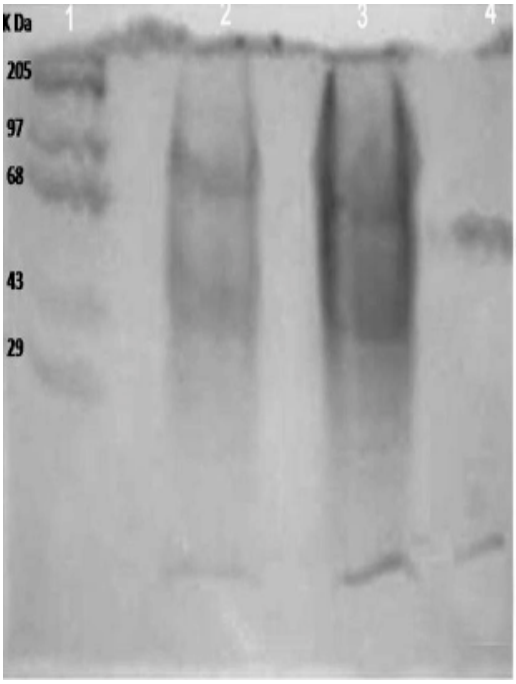


**Figure 3:** SDS- PAGE Protein profile analysis of pyruvate kinase

SDS–PAGE (10%) analysis of *Staphyloccus aureus* pyruvate kinase Lane 1: Molecular size markers obtained from Bangalore Genei Pvt ltd, lane 2: 10-20% ammonium sulphate concentrate of *Staphyloccus aureus* cytosolic fraction, lane 3: Crude cytosolic fraction of *Staphyloccus aureus* ATCC12600, lane 4: pyruvate kinase obtained from the gel filtration on Sephadex G-100

counterpart therefore; such enzymes which play key role in the metabolic flux in the *Staphyloccus aureus* can be potential drug targets in the development of potent antimicrobials which are pathogen specific.

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