



Original Research Article

Cloning and characterization of α -amylase from a clinical isolate of staphylococcus aureus resistant to vancomycinV L Asha Latha^{1,*}, B Sushma², P V G K Sharma²¹Dept. of Biochemistry, Mahavir Institute of Medical Sciences (MIMS), Vikarabad, Telangana, India²Dept. of Biotechnology, Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati, Andhra Pradesh, India

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ABSTRACT

Introduction: Staphylococcus aureus (*S. aureus*) is one of the prominent gram positive human pathogen secretes many surface and secretory proteins including various enzymes and pathogenic factors that favour the colonization and infection of host tissue. Alpha-amylase is one of the enzymes secreted by *S. aureus* which catalyses the breakdown of complex sugars to monosaccharides, which are required for colonization and survival of this pathogen.

Materials and Methods: In the present study we have cloned, sequenced, expressed and characterized α -amylase from the clinical isolate of *S. aureus* resistant to vancomycin (VRSA). The 25kb plasma DNA was isolated from the clinical isolate of *S. aureus* and a 600bp PCR amplified product was obtained.

Results: The SDS-PAGE analysis of induced and un-induced clone SB3 indicates that α -amylase has a M.W. of 22KD. The α -amylase was eluted from the gel and enzymes assay was performed. The Km was found to be close to Km of native α -amylase identified in the extracellular and in the cytosolic fraction of *S. aureus* resistant to vancomycin.

Conclusion: The current study clearly indicate that there is clear distinction between the α -amylase of human and different prokaryotes, particularly *S. aureus* which made this enzyme an ideal target in the development of drugs against infections of tooth and oral cavities.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is gram positive cocci about 1 μ m in diameter arranged in grape like irregular clusters. Infections associated primarily with *S. aureus* pathogenic strains are responsible for the formation of abscesses, localized pus-producing lesions; strains of staphylococci produce a variety of metabolic end products which may play role in the organism's pathogenicity.¹ They are coagulase causes clot formation, leukocidin which causes lysis of white blood cells and enterotoxin for gastro enteritis. *S. aureus* shows resistance to wide range of antibiotics such as ampicillin, gentamycin, kanamycin, chloromaphenical, methicillin and vancomycin.^{2,3} *S. aureus* produces a wide variety of exoproteins that contribute to its

ability to colonize and cause disease in mammalian hosts. All strains secrete a group of enzymes and cytotoxins which includes four hemolysins (α , β , γ , δ), nucleases, proteases, lipases, hyaluronidase and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Some strains produce one or more additional exoproteins which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB, SECN, SED, SEG, SEH & SEI) the exfoliative toxins (ETA & ETB) and leukocidin. Each of these toxins is known to have potent effect on cells of the immune system and biological effects as well.⁴ Alpha-amylase belongs to family of glycosyl hydrolases abundantly present in plants, animals and microorganisms. It is a major digestive enzyme present in the pancreatic juice and urine. In humans, α -amylases link to chromosome 1p21 randomly cleaves the 1,4- α -D-glycosidic linkages

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between adjacent glucose units to yield smaller starches and ultimately maltotriose, maltose, amylose, glucose and limit dextrin from amylopectin.⁵ Several workers⁶ characterize the α -amylase binding to streptococcus species to identify the salivary components which interact with oral bacteria and determine the mechanism responsible for their binding to bacterial surface strains. Alpha-amylase helps to degrade starch and colonize on food materials, and in the mouth, teeth which may have implications for dental plaque and caries formation. In view of important role of α -amylase in the pathogenesis of *S. aureus*, the current study was carried out on cloning and characterization of α -amylase gene on a clinical isolate of *S. aureus* resistant to vancomycin.

2. Materials and Methods

2.1. Bacterial isolation and Identification

The clinical isolate of *S. aureus* was obtained from the sputum sample of patients at Sri Venkateswara Institute of Medical Sciences (SVIMS) Tirupati, a tertiary care hospital which serves the population of Rayalseema (Chittoor, Kadapa, Anathapur, Ongole & Nellore). The study protocol was approved by Institutional ethical committee. Identification of clinical isolates of *S. aureus* was performed by traditional biochemical methods including gram staining, grown on agar media, catalase and coagulase testing. The culture was found to be resistant to vancomycin with minimum inhibitory concentration (MIC) of 2.5 μ g/mL.

2.2. Isolation of VRSA plasmid from clinical isolate *S. aureus*

Pure *S. aureus* culture showing resistance to vancomycin was grown in Lysogeny broth (LB) media that contains 50 μ g/ml of vancomycin. Single colony was picked and grown in LB broth that containing 25 μ g/ml of vancomycin and plasmid DNA from the organism was isolated by the method.⁷ The culture was centrifuged at 6000rpm for 2min. The pellet was suspended in 500 μ l of TEG buffer and re-centrifuged at 6000rpm for 2min and repeated for thrice. The obtained pellet was vortexed and suspended in 150 μ l of 10mg/ml ice cold lysozyme prepared in TEG buffer and incubated at 37°C for one and half hour. 30 μ l of 10mg/ml pancreatic RNAase was added and incubated for 30min at 37°C. Lysis buffer (300 μ l) was added and kept in ice for 30min. The tubes were centrifuged at 10,000rpm for 10min at 4°C in which supernatant was taken and plasmid DNA was precipitated by ethanol and the pellet, obtained was suspended in TEG buffer and the DNA was analyzed by running 1% agarose gel in 1X TAE system. Thus isolated plasmid was named as VRSA.

2.3. Blast search of SAVP 022 was performed from *S. aureus mu.50*

S. aureus Mu50 genome was sequenced and annotated.⁸ The genome contained vancomycin resistant plasmid which had 34 genes of them SAVP022 encoding a hypothetical protein was subjected BLAST search indicated that protein sequence has close homology with α -amylase. From the gene sequence primers were constructed for the amplification of the SAVP022 in our clinical isolate of *S. aureus*.

2.4. Amplification of SAVP022 encoding α -amylase from VRSA plasmid of clinical isolate *S. aureus*

The SAVP022 was amplified from VRSA plasmid isolated from a clinical isolate of *S. aureus*. For amplification the following primers were used

Forward Primer (BS 1): 5¹ CTA CCC CTT AAA TCT 3¹

Reverse Primer (BSS 11): 5¹ GAT TAA CTA TAA CTT 3¹

2.5. α -amylase enzyme assay

The α -amylase activity was measured with the Dinitrosalicylic acid (DNS) according to the method described protocol⁹ by using 10mg/ml starch dissolved in a 50mM phosphate buffer pH 6.9 and 42°C. One unit of amylase activity was defined as the amount of enzyme that released 1mg of reducing end groups per minute at 42°C. Maltose was used as standard of reducing end sugar.

2.6. Polymerase chain reaction for amplification of α -amylase

The VRSA plasmid isolated from the clinical isolate of *S. aureus* SAVP022 was amplified in Master cycler gradient thermocycler. The PCR product was analyzed on 1% agarose gel electrophoresis. The results were recorded in Vilber Lourmet gel documentation system.

3. Results

The 25kb plasma DNA was isolated from the clinical isolate of *S. aureus* resistant to vancomycin (Figure 1). The plasmid DNA of this strain having 34 hypothetical proteins, among this a hypothetical protein SAVP022, when subjected to BLAST showed homology to α -amylase with E-value; 1.3, 24% identities and gaps 5%. Thus primers were designed for SAVP022 and the α -amylase was amplified using plasmid DNA isolated for the clinical isolate of *S. aureus* resistant to vancomycin. A 600bp PCR amplified product was obtained. The SDS-PAGE analysis of induced and un-induced clone SB3 indicates that α -amylase has a M.W. of 22KD (Figure 2 A,B). The α -amylase was eluted from the gel and enzymes assay was performed. The Km was found to be close to Km

of native α -amylase identified in the extracellular and in the cytosolic fraction of *S.aureus* resistant to vancomycin (Table 1).

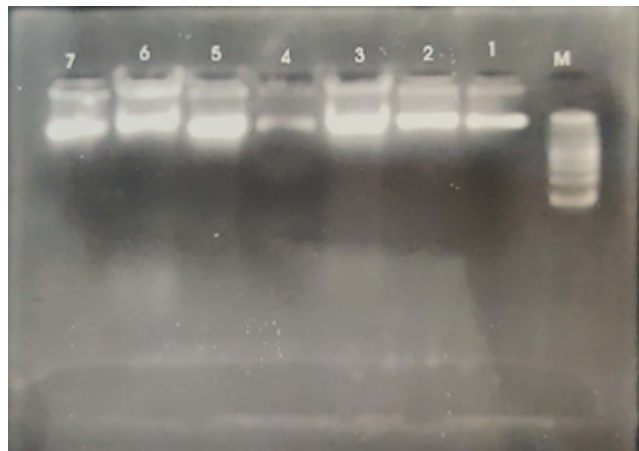


Fig. 1: Electrophoretogram showing the plasmid DNA isolated from a clinical isolate of *S.aureus* resistant to vancomycin

0.5 μ g of the plasmid DNA was loaded in 1% agarose gel and electrophoresis was performed in 1X TAE system and the gel was stained with 0.5 μ g/ml ethidium bromide.

Lane M: Super mix molecular size markers (33.5Kb, 24.5Kb, 8.99Kb, 7Kb, 6Kb, 4Kb, 3Kb, 2Kb, 1Kb, 0.5Kb)

Lanes 1-7: VRSA plasmid DNA isolated from *S.aureus*

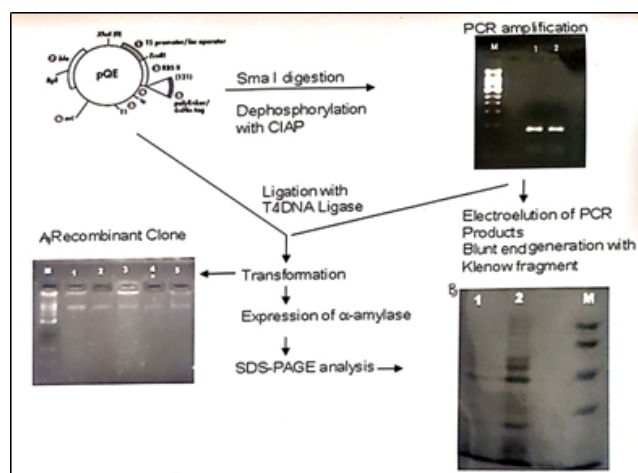


Fig. 2: Cloning Strategy of α -amylase

A) Electrophoretogram showing recombinant plasmid DNA isolated from the SB-3, 0.5 μ g recombinant plasmid was loaded in 1% agarose gel and the electrophoresis was performed in 1xTAE system and the gel was stained with 0.5 μ g/ml ethidium bromide.

Lane M: super mix molecular size markers (33.5Kb, 24.5Kb, 8.99Kb, 7Kb, 6Kb, 4Kb, 3Kb, 2Kb, 1Kb, 0.5Kb).

Lane 1: pQE30 plasmid DNA.

Lane 2 to 5: recombinant plasmid isolated from clones SB-1, SB-2, SB-3 and SB-4.

B) Electrophoretogram showing the expression of recombinant protein from MK-4 clone in 10% SDS-PAGE gel and the gel was stained with 0.125% coomassie brilliant blue R250.

Lane M: molecular size markers (97.4Kb, 68Kb, 43Kb, 29Kb, 20Kb, 14.3Kb)

Lane 1: uninduced cell lysate of SB-3

Lane 2: induced cell lysate of SB-3

4. Discussion

It is very well known that *S.aureus* can grow in several different niches, and produces several toxins which help the organism to spread its pathogenesis but also causes life threatening diseases. *S.aureus* enters into human body through wounds, surgical blades, forceps and scissors in the hospital environments. It can enter as food contaminant and spread its pathogenesis.^{10,11} In this case, the first step is to degrade large amount of starch which inhibits bacterial growth. In order to establish itself in this high carbohydrate content *S.aureus* produces several key enzymes which degrade starch one of them is α -amylase.¹²

The organism used in the present study i.e. a clinical isolate of *S.aureus* resistant to vancomycin showed high α -amylase activity in the cytosol fraction. The presence of enzymes with high enzymatic activity prompted us to search genes encoding such enzymes in the gene sequence of *S.aureus* Mu50. The SAVP022 hypothetical protein present on the plasmid DNA of *S.aureus* Mu50 showed strong homology to α -amylase, with E=1.3 and 24% identities with very less gaps.⁸ Accordingly, plasmid DNA from the clinical isolate of *S.aureus* resistant to vancomycin was isolated and found to be 25Kb. From this plasmid using primers designed from SAVP022, a 0.6kb DNA fragment was amplified and this was cloned into the sma 1 site of PQE=30 and expressed in *E.coli* DH5- α . The size of the expressed protein was found to be 22KD corresponding to the insert cloned into the pQE-30 (Figure 2 B). In prokaryotes, the α -amylase is about 48KD in size however, in *S.aureus* MU 50 and in this strain of *S. aureus* the protein with similar function has M.W. of 22KD.¹³

The enzyme was eluted from the gel and the enzyme activity was determined (Table 1) and the Km of α -amylase was found to be very close to that of native α -amylase indicates that enzyme would successfully be expressed in the clone SB-3. This enzyme plays a key role in infections pertaining to tooth and buccal cavities, high expression of this enzyme in the *S. aureus* indicate that this enzyme may affect the tooth demineralization.

Table 1: Enzyme activity, specific activity and km of α -amylase in cytosolic fraction

S.No		Enzyme activity (μ M/ml/min)	Specific activity (μ M/mg/min)	K_M (μ M starch)
1	Cytosolic fraction of VRSA	4.9	1.87	50
2	Extra cellular fraction of VRSA	–	–	–
3	Induced SB-3	8.1	284.2	55.55
4	Un-induced SB-3	4.6	167.2	83.5

VRSA- Vancomycin resistant staphylococcus aureus

5. Conclusion

It is very well known that salivary α -amylase play an important role in dental plaque and caries formation. Alpha-amylase bound to bacteria in plaque facilitates dietary starch hydrolysis to provide additional glucose for metabolism by plaque micro organisms in close proximity to tooth surface. The resulting lactic acid produced may be added to the pool of acid in plaque to contribute to tooth demineralization. In the current study α -amylase was identified on the plasmid DNA of a S. aureus resistant to vancomycin which makes this organism can infect tooth and other parts of oral cavities. The results from the present study clearly indicate that S. aureus infections in tooth can be very serious and can spread from tooth to various parts of the body.

The current study clearly indicate that there is clear distinction between the α -amylase of human and different prokaryotes, particularly S.aureus which made this enzyme an ideal target in the development of drugs against infections of tooth and oral cavities.

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7. Source of Funding

None.

8. Conflict of Interest

Authors state no conflict of interest.

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