Molecular characterization of α-amylase from *Staphylococcus aureus*

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Abstract:
Staphylococcus aureus is one of the prominent Gram positive human pathogen secretes many surface and secretary proteins including various enzymes and pathogenic factors that favour the successful colonization and infection of host tissue. α-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 in any anatomical locales. In the present study we have cloned, sequenced, expressed and characterized α-amylase gene from *S. aureus* ATCC12600. The recombinant enzyme has a molecular weight of 58kDa and the kinetics showed V_{max} 0.0208±0.033 (mg/ml)/mg/min and K_m 10.633±0.737mg/ml. The multiple sequence analysis showed α-amylase of *S. aureus* exhibited large differences with *Bacillus subtilis* and *Streptococcus bovis*. As the crystal structure of *S. aureus* α-amylase was unavailable, we used homology modelling method to build the structure. The built structure was validated by Ramachandran plot which showed 90% of the residues in the allowed region while no residue was found in the disallowed region and the built structure was close to the crystal structure with Z-Score: -6.85. The structural superimposition studies with α-amylases of *Bacillus subtilis* and *Streptococcus bovis* showed distinct differences with RMSD values of 18.158Å and 7.091Å respectively which correlated with enzyme kinetics, indicating α-amylase is different among these bacteria.

Keywords: α-amylase, *Km*, *Vmax*, Z-score, RMSD.

Background:

Staphylococcus aureus can colonize and survive in a wide variety of environmental niches. It is capable of causing a range of mild to life-threatening diseases, including septicaemia, meningitis, toxic shock syndrome, food poisoning and skin abscesses [1]. It colonizes tissues using a repertoire of virulence determinants that includes cell surface-associated protein, as well as extracellular proteins. One of the extracellular proteins is α-amylase secreted by the bacteria. α-amylase is randomly cleaves the 1,4-α-D-glycosidic linkages between adjacent glucose units to yield smaller starches and ultimately Maltrotriose, maltose, amylose, glucose and limit dextrin from amylopectin. These sugars are further converted to glucose-6-phosphate; this very step is the basis for the synthesis of exopolysaccharide matrix,

Polysaccharide intracellular adhesion (PIA), and thus finally leading to Biofilm formation. Biofilms are defined as communities of microorganisms that are encased in a self-synthesized extracellular polymeric matrix (EPS) and grow attached to a biotic or abiotic surface [2]. *Staphylococcal* biofilms have a significant impact on human health [3] as they frequently exhibit an enhanced pathogenic capability relative to bacteria in solution by virtue of their sessile behaviour, increased resistance to antimicrobial agents, and the potential for detachment and distal embolization of large biofilm fragments [4].

α-amylases are classified as family 13 of the glycosyl hydrolases which have similar structures and catalytic sites and the same

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catalytic mechanisms [5]. In general they are composed of three domains. Domain A central barrel containing the active site residues and chloride ion binding site, domain B a long loop region inserted between third β strand and the α-helix of domain A, that contains calcium binding site and domain C a terminal β-sheet domain consisting of a motif which appears to show some variability in sequence and length between amylases according to the organism [6].

The kinetics of α-amylases varies distinctly different among bacteria for example in case of *Psuedoalteromonas arctica* and *Bacillus subtilis* the kinetics of α-amylase showed K_m of 7.28 mg/ml; 2.68 mg/ml and V_{max} 13.07 (mg/ml)mg/min; 1.773U/ml/min respectively [7, 8]. The α-amylase gene from *Bacillus stearothermophilus* was expressed in *S. aureus* and secreted in to the culture filtrate. All *staphylococcal* species were able to secrete α-amylase, since more than 80% of the enzyme activity was found in the culture supernatant [9] making very important role in this human pathogen, large amounts of monosaccharides which may have profound role in the pathogenicity of *S. aureus* therefore; the present study is focussed on characterization of α-amylase from *Staphylococcus aureus* ATCC 12600 and its comparison with *Bacillus subtilis* and *Streptococcus bovis* α-amylases.
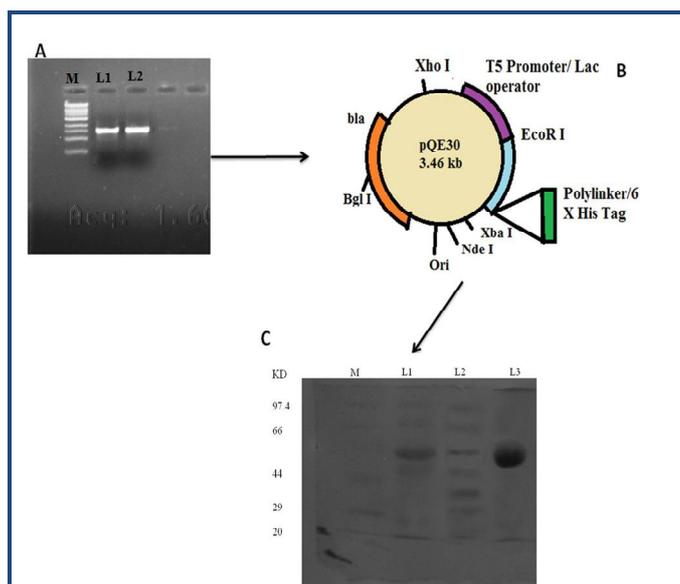

Figure 1: Cloning of α-amylase gene from *S.aureus* ATCC 12600. **A**. PCR amplification of α-amylase gene using α-amylase primers from the *S. aureus* ATCC12600 following the method described. Lane *M* molecular size marker obtained from Bangalore genei pvt ltd, lane *L1* and *L2* PCR amplified products. **B**. Schematic representation of pQE 30 plasmid vector, **C**. Electrophoretogram showing the expression of recombinant α-amylase from PLAM1clone in SDS-PAGE (10%). *Lane M*: Molecular weight markers obtained from Bangalore Genei Pvt ltd. Lane 1: induced cell lysate of PLAM1 clone *Lanes* 2 : uninduced cell lysate of PLAM1 clone. *Lane* 3: pure recombinant α-amylase eluted from nickel metal chelate chromatographic column.

Methodology:

Staphylococcus aureus ATCC12600 was grown on modified Baird Parkar Agar media at 37ºC. After overnight incubation single black shiny colony with distinct zone was picked and cultured in Brain Heart Infusion (BHI) broth at 37ºC. Thus, grown *S. aureus* ATCC12600 culture was used to characterize α-amylase enzyme and extraction of chromosomal DNA [10].

α-amylase enzyme assay

The α-amylase activity was measured with the Dinitro salycylic acid (DNS) according to the method described protocol by [11] using 10mg/ml starch dissolved in a 50 mM phosphate buffer pH 6.9 and 42º C. One unit of amylase activity was defined as the amount of enzyme that released 1mg of reducing end groups per minute at 42ºC. Maltose was used as standard of reducing end sugar.

α-amylase gene was amplified from *S. aureus* chromosomal DNA using the primers α-amylase -F: 5'-CATGAATAAGCAATGG-3' and α-amylase -R: 5'-TTAATTTAGTTCGAT 3' which were designed from the α-amylase gene sequence of *S. aureus* Mu50 strain [12]. The reaction mixture contained in a final volume of 50µl which consisted of 100ρmoles of each primers, 100µmol of dNTPS mix, 10 mM Tris- HCl (pH 8.8), 1.5 mM $MgCl_2$, 1U of hot start Taq DNA Polymerase (Bangalore Genei pvt ltd) and 0.25µg of chromosomal DNA. Amplification parameters included an initial denaturation step for 10 min at 94°C; 35 cycles of 94°C for 60 seconds of denaturation, 60 seconds of annealing at 33ºC and 100 seconds of amplification at 72°C which was followed by a final extension step at 72°C for 5 min in a Mastercycler gradient Thermocycler (Eppendorf). Amplified products were purified with NP-PCR Purification kit, Taurus Scientific, USA and were sequenced by dye terminating method at MWG Biotech India Ltd. Thus, obtained α-amylase gene sequence was deposited at Gen Bank(http://www.ncbi.nlm.nih.gov/nuccore/HM067708).

Cloning, expression and purification of α-amylase gene

α-amylase gene was cloned in the Sma I site of pQE 30 and transformed into *E.coli* DH5α. The resultant clone was named as PLAM1. The insert in the clone was sequenced and after ascertaining the sequence, the α-amylase was over expressed with 1mM IPTG. The r α-amylase was purified from the cytosolic fraction of PLAM 1 clone by passing through nickel metal chelate agarose column (by following QIA express expression system protocol) and protein was eluted using 300mM immidazole hydrochloride the product was analysed on 10% SDS-PAGE [10, 13]. The enzyme kinetics of purified α-amylase was performed as described earlier in section.

In silico structure prediction

As the α-amylase structure of *S. aureus* and *Streptococcus bovis* were not available so far in the PDB we have constructed 3D structure using homology modelling method. The three dimensional models of *S. aureus* and *Streptococcus bovis* α-amylase was constructed by using Modeller 9v8 tool. The *S. aureus* α-amylase protein sequence was subjected to BLASTp [14] against PDB and the crystal structure of a putative α-amylase from *Geobacillus* sp (PDB ID: 2ZE0A) showing the maximum identity of 52% was chosen as template. Similarly the α-amylase of *Streptococcus bovis* was subjected to BLASTp against PDB and the crystal structure of putative α-amylase from (PDB ID:1WP6) showed 48% identity with maltohexose producing α-amylase from alkalophilic *Bacillus sp*. Alignment

files were generated in PIR format for Query and template sequences using ClustalX tool **[15]**. Python scripts were written and 20 best models for each organism were generated. The model with the lowest discrete optimized protein energy (DOPE) score was selected for further analysis.

Validation of *S. aureus* and *Streptococcus bovis* α-amylases Model

The stereo chemical quality of the predicted model was validated by PROCHECK and ProSA web servers. Both can read the atomic co-ordinates of the 3D model and judge the quality of the structure. Ramachandran plot generated from PROCHECK **[16]** validation server was used to access the quality of the model by looking into the allowed and disallowed regions of the plot. A Z-score value was generated from ProSA web server that can determine the overall quality of the model and its identity nearest to crystal structure.

Superimposition of *S. aureus* α-amylase

The comparative structural prediction studies to ensure the identity and variability of *S. aureus* α-amylase structure with other α-amylase structures were carried out using MATRAS (MArkovian TRAnsition of Structure) program. This program has unique features where it can define the structural similarity score as the log-odds of two probabilities using a scheme similar to Dayhoff's amino acid substitution score. In the program we have assigned structures by inputting the PDB code and by uploading the PDB format files in the user's computer. An alignment, superimposed structures and various kinds of structural similarities, such as raw score, RMSD values were predicted for the following structures. The validated 3D model of *S. aureus* α-amylase was super imposed with available and built structures of α-amylases from other bacteria such as *Bacillus subtilis* (1BAG), *Streptococcus bovis* (built structure).
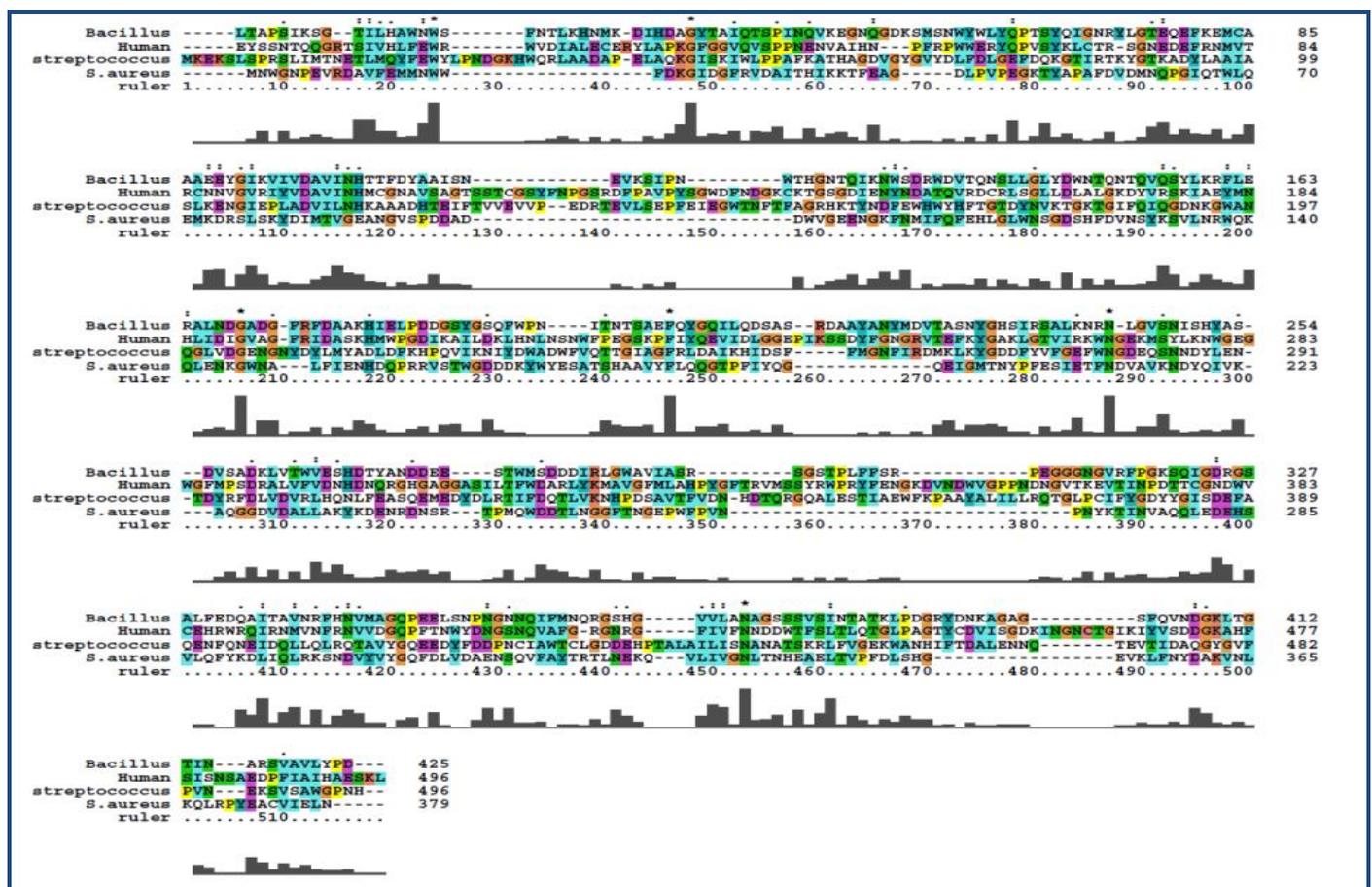

Figure 2: Multiple Sequence alignment of *S.aureus* α-amylase with *Bacillus subtilis* and *Streptococcus bovis*.

Result & Discussion:

In *S. aureus* cell wall biosynthesis is a crucial factor for its survival in the host and in the formation of small colony variants, which is one of the key factors observed in the host with increased relapsed episodes of *S. aureus* infections **[13, 17]** this is probably augmented in *S. aureus* due to the high activity of α-amylase which is secreted in the culture filtrate. This increased production of monosaccharides elevates the glucose levels which is phosphorylated by glk A **[18]** in this pathogen and is primarily utilized in the synthesis of PIA, exopolysaccharides and in cell wall biosynthesis **[2, 19, 20]**. This

in turn suppresses the TCA cycle resulting in reductive conditions **[20, 21]** and in this situation rate of biofilm formation is very high. This type of scenario is seen in all multi drug resistant strains (MDR) and Vancomycin resistant strains (VRSA) *S. aureus* **[19, 20, 21]**. Therefore in the present study α-amylase gene of *S. aureus* ATCC 12600 was cloned, sequenced and expressed in *E.coli* DH5α, the resultant clone was named as PLAM 1.

The α-amylase gene expression in PLAM 1 clone was obtained by adding extra **" C "** residue in the 5' end of the forward primer

such that the gene is cloned in −1 frame in the Sma I site of pQE 30, and thereby, correct expression of enzyme could be achieved **[10, 13]**. The insert in the PLAM 1 clone was confirmed by sequencing and ascertaining the sequence which is the same as the one deposited in the GenBank (accession number HM067708). The α-amylase gene expression was induced with 1mM IPTG. The product of α-amylase was purified with nickel metal chelate chromatography column and the pure recombinant enzyme showed similar properties with that of native α-amylase **[10, 13]**. The α-amylase identified in the extracellular fraction of *S. aureus* ATCC 12600 showed an enzyme activity of 0.0193±0.0015 mg/ml/min, V_{max} 0.032±0.0036 (mg/ml)/mg/min and K_m 11.57±0.409 mg/ml. The pure recombinant α-amylase showed single band in SDS–PAGE (10%) with a molecular weight of 58kDa, which is corresponding to the insert cloned and monomeric size of the protein **(Figure 1)**. The kinetics of pure recombinant α-amylase was close to the secreted native α-amylase of *S. aureus* ATCC12600 **Table 1 (see supplementary material)** which are in accordance with the results reported earlier **[9]**.
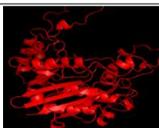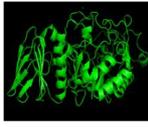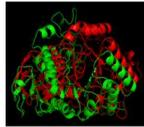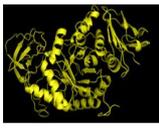
Organism	Super imposition	RMSD Value in Å
S.aureus		
Bacillus subtilis	*S.aureus- Bacillus subtilis*	18.158
Streptococcus bovis	*S.aureus- Streptococcus bovis*	7.091

Figure 3: Structural comparison of *S. aureus* α-amylase with *Bacillus subtilis* and *Streptococcus bovis*.

The sequence of *S. aureus* α- amylase (GenBank accession number for amylase gene is HM067708) showed complete homology with amylase gene of all the *S. aureus* strains reported in the database. The multiple sequence alignment of α-amylase protein sequence of *S. aureus* ATCC12600 showed highly conserved regions with *Bacillus subtilis* and *Streptococcus bovis* while very low identity in the sequence was observed with both human salivary α- amylase **(Figure 2)**. As the crystal structure of *S. aureus* α- amylase was not available hence in the present study we have built the 3D structure using homology

modeling method using X-ray crystal structure of *Geobacillus* sp as template which showed 52% identity. Similarly, the 3D α-amylase for *Streptococcus bovis* was also constructed using the X-ray crystal structure of Alkalophilic *Bacillus* Sp as template however, the comparative structural analysis showed extensive variations as indicated from the RMSD values which also correlated with the enzyme kinetic results of α-amylase **Table 2 (see supplementary material) & Figure 3)**. These results also suggest that in various MDR and VRSA strains of *S. aureus* α-amylase expression may be critical in the colonization and rate of biofilm formation.

Conclusion:
S. aureus can colonize and adopt on any anatomical locales in the human host for which the organism requires large amount of monosaccharides which can be easily generated by α-amylase. In this context this enzyme may be playing pivotal role in the colonization and biofilm formation which are one of the key factors in the pathogenesis of *S. aureus*.

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Supplementary material:

Table 1: Enzyme Kinetics of α- amylase from *S.aureus*

Source of α- amylase	Enzyme activity mg/ml/min	Specific activity (mg/ml)/mg/min	K_M mg/ml
S.aureus ATCC 12600 extracellular fraction	0.0193±0.0015	0.032±0.0036	11.57±0.409
Pure recombinant α- amylase from PLAM -1	0.052±0.0036	0.0208±0.033	10.633±0.737

*Values are the mean± SD from three determinations

Table 2: Enzyme Kinetics comparison of α- amylase from *S.aureus* with other Bacterial α- amylases

Organism	K_m	V_{max}	K_{cat}	Reference
Bacillus subtilis	2.68 (mg/ml)	1.773(U/ml)	0.601 mg/ml	[8]
Streptococcus bovis	0.88(mg/ml)	2.650 (U/mg)	2.510 mg/ml	[22]
Staphylococcus aureus	11.57±0.409 (mg/ml)	0.032±0.0036(mg/ml)/mg/min	1.6517±0.055 mg/ml	In the present study

*Values are the mean± SD from three determinations