

**PURIFICATION AND BIOCHEMICAL
CHARACTERIZATION OF RECOMBINANT
STREPTOMYCIN ADENYLYLTRANSFERASE**

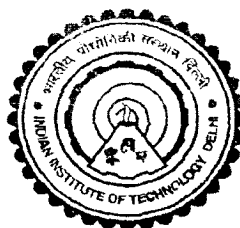
by

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Streptomyces Adenylyltransferase

Enzymes - Streptomyces

Inhibitors - Aminoglycosides

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CERTIFICATE

This is to certify that the thesis entitled "**Purification and Biochemical Characterization of Recombinant Streptomycin Adenylyltransferase**" being submitted by **Snehasis Jana** to the Indian Institute of Technology, Delhi, for the award of the degree of **Doctor of Philosophy**, is a record of the bonafide research work carried out by him under my supervision and guidance in conformity with the rules and regulations of the Indian Institute of Technology, Delhi. The research work and the results presented in the thesis have not been submitted to any other University or Institute for the award of any other degree or diploma.



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
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ABSTRACT

Resistance to the aminoglycosides is generally the result of the synthesis of modifying enzymes, which can use ATP to either phosphorylate or adenylate the drugs, or acetylCoA to acetylate them. These modified aminoglycosides are no longer effective antibiotics and thus the cell manifests a resistance phenotype. Aminoglycoside nucleotidyltransferase catalyzes the transfer of nucleotides to the hydroxyl group of aminoglycoside antibiotic, accompanied by the release of pyrophosphate and AMP-Aminoglycoside. One important mechanism of streptomycin modification is through ATP- dependent – O- adenylation, catalyzed by streptomycin adenylyltransferase (SMATase). The microbial resistance to streptomycin was reported about 35 years ago. So far, no detailed biochemical characterization of SMATase has been described to date. However, the exact biochemical functions of the catalytic motifs in this enzyme have yet to be confirmed by more detailed biochemical and structural studies. Therefore, the present study was undertaken to purify and characterize the recombinant SMATase. Fusion SMATase was purified by Ni²⁺ -IDA-His-Bind Resin column chromatography. Thioredoxin-His₆ tagged –SMATase fusion protein was produced in a bacterial intracellular expression system mainly in a soluble form. The purified fusion protein showed a single band on SDS–PAGE corresponding to 49 kDa. The recovery of fusion protein was 85% and fold purifications was 22. There was no significant difference in enzyme activity and K_m and V_{max} values of fusion and native SMATase. This suggests that fusion part does not play any role in the enzymatic activity of SMATase. The dissociation constant (K_d) value was $9.02 \pm 0.22 \mu\text{M}$ and the total binding sites (B_{max}) was $101.72 \pm 2.54 \mu\text{mol/mg}$ protein. Initial velocity and dead-end inhibition studies showed that SMATase followed an ordered sequential mechanism.

Native SMATase possesses secondary structural content with 65% α -helix, 9% β -sheet, 4% β -turn, and 22% random coil. The analysis of the CD features for the determination of secondary structural class reveals that it is predominantly α -type protein. Far-UV CD and fluorescence spectroscopy probed the structure-function relationship of SMATase. The denaturation data indicates that the protein undergoes complete denaturation in 6 M GdnHCl. The effect of GdnHCl on the native conformation of protein is attributed to its destabilization of hydrogen bond and ionic interactions. It was observed that there was a concomitant decrease of enzyme activity with the loss of secondary structure. This happens due to its conformational change by GdnHCl. Comparative fluorescence emission spectra of SMATase at four GdnHCl concentrations, viz.. 1, 2, 4, and 6 M were recorded. With progressive increase in GdnHCl concentration up to 6 M, the relative fluorescence intensity gradually decreases, indicating alteration of the protein conformation. Also, the emission maximum (λ_{\max}) is red shifted (343 to 348 nm) by 5 nm as the protein encounters high denaturant concentration, which indicates that the microenvironment of aromatic amino acids (Trp residues) is getting exposed to more polar region. This result indicated that at 6 M GdnHCl, SMATase was existed in a conformation that was different from the native conformation. Conformational stability experiments under native and GdnHCl-induced denatured state of SMATase, proved that α -helices were essential for the enzyme activity. Quenching studies of SMATase revealed that nearly all Trp residues were quenched by non-ionic quencher acrylamide, however, the ionic quencher potassium iodide could quench only 80% of intrinsic tryptophan fluorescence. This result may reflect the fact that, although most of the tryptophan residues in SMATase are in the polar environment, at least one out of four Trp residues is buried in the polar region, which is not accessible to the ionic quencher potassium iodide.

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