STUDIES ON A NOVEL PROTEASE FROM HALOALKALIPHILIC *GEOMICROBIUM* SP. EMB2 ISOLATED FROM SAMBHAR SALT LAKE

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by

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Submitted

in fulfillment of the requirements of the Degree of

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Dedicated to my Parents

CERTIFICATE

This is to certify that the thesis entitled "Studies on a novel protease from haloalkaliphilic *Geomicrobium* sp. EMB2 isolated from Sambhar Salt Lake" being submitted by **Mr. RAM KARAN** to the Indian Institute of Technology, Delhi for the award of the degree of *Doctor of Philosophy* in Chemistry is a record of bonafide research work carried out by him. Mr. Ram Karan has worked under my guidance and supervision, and has fulfilled the requirements for the submission of the thesis which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

Dr. S. K. Khare
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December, 2010

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Ram Karan

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Abstract

Halophiles are the group of microorganisms that thrive in the presence of moderate to high salt concentration. There is an increasing interest in their enzymes for catalysis in high salt and non-aqueous conditions. The thesis concerns the studies of proteolytic enzymes from halophilic microorganisms. The main part of the work deals with the isolation of halophilic microorganisms, screening novel activities among them, purification and characterization of chosen novel protease and understanding the molecular basis of its salt and solvent adaptations.

Thirty eight halophilic bacterial strains were isolated from the soil and water samples collected from Sambhar Salt Lake, Rajasthan, India. The isolates were related to genus *Geomicrobium*, *Staphylococcus* and *Bulleidia* by biochemical tests and 16S rDNA sequencing. 16S rDNA sequences of all the isolates have been submitted to GenBank, USA and accession numbers assigned. Amongst these, one of the potential strains, *Geomicrobium* sp. EMB2 was selected for further investigation.

Geomicrobium sp. EMB2 was a Gram-positive cocci and moderately halophilic in nature, requiring 5-20% NaCl for growth. The isolate is deposited in Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India with Accession no. MTCC 10310.

Geomicrobium sp. EMB2 secreted a novel protease for which the production was optimized by Plackett-Burman and Response Surface Methodology (RSM). Under un-optimized conditions the isolate secreted 37 U/ml. The optimization lead to about 20 fold

increase in production. Optimized culture conditions were casamino acid (0.6%), yeast extract (0.6%), peptone (0.2%), $MgCl_2$ (0.1%), NaCl (12.0%), pH 8.5, 30 °C, and constant shaking at 150 rpm, at which production reached to about 721 units protease per ml in 72 h.

The protease was purified to homogeneity by hydrophobic interaction chromatography and found to be a monomeric protein of 38 kDa molecular mass. It was stable in the pH range 7.0-12.0. The pH and temperature optimum were pH 10.0 and 50 °C respectively. The K_m and V_{max} towards casein were found to be 2.31 mg/ml and 526 U/min respectively. It was a serine type protease with broad substrate specificity. The activity towards Acetyl-L-tyrosine ethyl ester (ATEE) and Benzoyl-L-tyrosine ethyl ester (BTEE) indicated it to be chymotrypsin like protease.

The *Geomicrobium* sp. protease can be considered novel, as it withstood range of detergents, surfactants, alkaline pH, salt and solvents. Stability was observed in all the solvents having log *P* (logarithm of the partition coefficient of a particular solvent between n-octanol and water) above 2.0.

In order to understand the structural features responsible for imparting salt and solvent stability, the primary structure, conserved motif and hydropathy profile of the protease were investigated and compared with other known serine-proteases. N-terminal amino acid sequence of the purified protease was determined experimentally. Based on this sequence, NCBI database was searched for its homologues using the BLASTP program. A match with a serine protease gene from *Bacillus clausii* KSM-K16 was found in the database. A pair of specific primers was designed from the 5' and 3' UTR regions

and used for amplification of open reading frame (ORF) by polymerase chain reaction (PCR). The PCR amplified product (~1.50 kb) was cloned in pGEM-T Easy vector and was used to transform *E. coli* strain XL-1 Blue MRF'. Of more than 50 transformants, 16 white colonies were selected for plasmid isolation. Isolated plasmids were checked for their restriction digestion pattern to confirm the identity of the clones. The nucleotide sequence of the cloned PCR product was determined and analyzed.

The deduced 375 amino acid sequence (molecular mass of 43.2 kDa) based on nucleotide sequence was quite similar to the protease of *Bacillus clausii* KSM-K16 [AP006627.1], *Bacillus psuedofirmius* OF4 [CP001878.1] and Proteobacterium HTCC2207 [NZ_AAPI01000012]. The first 40 nucleotide residues constituted a signal peptide (4.2 kDa), followed by a 2.5 kDa propeptide (23 residues) and 35.4 kDa mature enzyme (311 residues). The three dimensional structure was predicted by I-TASSER server. It showed that the surface of the protein was covered by large number of acidic residues which may be responsible for its stability in salt.

Intrinsic fluorescence and circular dichroism spectroscopy measurements revealed predominantly α-helical structure of protease in presence of 0.85-1.70 M NaCl. It unfolded in salt-free medium, but addition of 0.85 M NaCl, led to refolding and regain of secondary and tertiary structure. This confirmed the necessity of salt for maintaining the native protein structure. Presence of NaCl also exerted the protective effect against thermal, organic solvent and guanidine hydrochloride denaturation by stabilizing the native structure.

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