

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

RAM KARAN



**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY DELHI
HAUZ KHAS, NEW DELHI-110016
DECEMBER, 2010**

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

by

RAM KARAN

DEPARTMENT OF CHEMISTRY

Submitted

in fulfillment of the requirements of the Degree of
DOCTORAL OF PHILOSOPHY

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

DECEMBER 2010

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.

December, 2010

Dr. S. K. Khare
Associate Professor of Biochemistry
Department of Chemistry
Indian Institute of Technology, Delhi

Acknowledgements

Pursuing a PhD project is both painful and enjoyable experience. It's just climbing a high peak, step by step, accompanied with bitterness, hardships, frustration, encouragement, and trust and with so many people's kind support and help. It's a pleasure to thank all of them. Those, whose names are not mentioned, kindly forgive me being rest assured that it, not at all lessens my gratefulness towards them.

First and foremost, I would like to express the deepest gratefulness to my PhD supervisor, **Dr. S. K. Khare**, for kindly accepting me as his PhD student to discover the 'halophiles' world in saline environment. I would like to thank you for your competent guidance, untiring efforts, constant support and care during the course of study and also for your enthusiasm and interest in research. Your wide knowledge and logical way of thinking have been of great value for me. Your understanding, encouraging and personal guidance have provided a good basis for the present thesis. I would have been lost without you. I have no words to express my gratitude for everything you have contributed in my PhD and without your blessings it was surely impossible for me to finish my work. For me, you are not only a teacher, but also someone I trust and admire. I thank you from the bottom of my heart.

Prof. M. N. Gupta: for constant encouragement, support and for being kind enough to allow us to use your lab facilities throughout my research span.

Prof. A. K. Singh, Head, Department of Chemistry: for providing me all the necessary facilities.

Prof. P. S. Pandey and Dr. Prashant Mishra: for being my SRC members and your kind support, guidance and helpful discussions.

Dr. Siddharth Pandey and Dr. Shashank Deep: for letting me use your laboratory facilities for fluorescence spectroscopy.

Prof. Santosh Satya: for your encouragement, support and good wishes.

Dr. Sanjay Kapoor, UDSC, New Delhi: for allowing me to carry out an important part of my thesis works in your lab. I am deeply indebted for your extensive help and teaching to initiate molecular biology experiments.

Prof. S. P. Singh, SU, Rajkot: for giving me the opportunity to work with you in your lab and for your encouragement.

Dr. Dinakar Salunke, NII, New Delhi: for allowing me to carry out N-terminal sequencing at NII, New Delhi. Also, I thank Ms. Sushma Nagpal for her kind help regarding the same.

Dr. A. K. Panda, NII, New Delhi: for your help during the N-terminal sequencing of my protein. That short span of time I spent in your lab proved to be quite helpful for me.

Dr. S. P. S. Khanuja, CIMAP, Lucknow: for providing me an opportunity to participate in the summer school organized by CIMAP, Lucknow. The training there, introduced me to molecular biological techniques which later helped a lot. I also extend my thanks to all GRB members at CIMAP.

I thank **Mr. Virender Sharma** for fabricating my glass columns in time and all staff members of the Instrument Lab for their cooperation.

My sincere thanks to both of my seniors **Dr. Anshu Gupta** and **Dr. Ruchi Gaur**, who have played a vital role in starting this PhD. I really admire you and your work and I feel very grateful in having the opportunity to exchange of ideas and useful conversations with you. Thanks for being cooperative, responsible and caring senior and for all you have done for me so far.

... Again, without following any specific order, thanks to my present research group **Mr. Arvind Sinha**, **Ms. Chetna Joshi**, **Mr. Sumit Kumar**, **Ms. Rajeshwari Sinha**, **Ms. Hemamalini**, **Mr. Rakib** and **Dr. Shivani Chaturvedi**. I am glad to have colleagues and friends like you. I thank you all from bottom of my heart for everything you have given to me. I cannot imagine my journey of research without you all. Thank you for creating a nice working atmosphere in the lab, for all the company and friendship, and for all the fun! I thank you for your help and suggestions with this thesis. I also thank all pass out M. Tech and M. Sc students for their cooperation.

A special thanks to **Arvind**, the most peaceful and prefect person I have ever met. People like you are hard to find. **Chetna** and **Rajeshwari**, you are the best company that I could ever find. **Sumit** for you, words will never be enough. I will never forget what you have done for me, a big sincere thank you!

I am grateful to **Mr. Sohel**, **Mr. Nagraj**, **Mr. Ganeshan**, **Mr. Sandeep**, **Mr. Kamal**, **Ms. Shruti** and **Ms. Unnati** for their help whenever required.

I thank **Mr. Mohan** and **Ms. Manupriya** for helping me to initiate the molecular biology experiments, **Ms. Priyanka** and **Mr. Vinay** for their help during my stay at UDSC.

I would like to thank the CSIR and DBT for their financial support.

I am thankful to AIF, JNU for extending CD facility.

I wish to thank my friends **Somnath, Yogesh, Jeeva, Tufan, Rana, Anuj, Sunil, Hari, Sachin, Bijendra, Sanjay, Deepak, Ravindra, Amit, Ashwini** and **Surjeet** for helping me get through the difficult times, and for all the emotional support, camaraderie, entertainment, and caring you provided.

My deepest gratitude goes to my **family** for their unflagging love and support throughout my life; this thesis is simply impossible without them. I am indebted to my **parents**, for their care and love. As typical parents in an Indian family, they worked industriously to support the family and spare no effort to provide the best possible environment for me to grow up and attend school. They had never complained in spite of all the hardships in their life. Although they are no longer with us, they are forever remembered. I am sure they share our joy and happiness in the heaven. I dedicate this thesis to my parents.

I do not have enough words to express my gratitude to my **brothers, sister** and lovely **nephews** and **nieces** for their love and support in this endeavour. It was only because of your support, constant encouragement, and prayers.

Everyone needs some special persons in life whose endless support smoothen the path of life. I am grateful to God for giving me wonderful friend **Anupama**, my wife. Without you it was not possible for me to complete my research work. It was your emotional support, deep love, confidence, encouragement and endless help that enabled me to reach such a beautiful destination. Thank you for always being with me.

I am forever thankful to Almighty God.

Ram Karan

New Delhi

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.

Table of Contents

Certificate	i
Acknowledgements	ii
Abstract	v
Table of Contents	viii
List of Figures	xiii
List of Tables	xvi

CHAPTER 1 INTRODUCTION

1.1	Rationale of the work	1
1.2	Objectives	4
1.3	Scope of the thesis	5
1.4	Review of literature	7
1.4.1	Halophiles: Microbial Life in saline environment	7
1.4.2	Commonly studied saline habitats for halophiles	9
1.4.3	Halophilic diversity	14
1.4.4	Halophilic bacteria	18
1.4.5	Adaptive features of halophilic microorganisms	18
1.4.6	Adaptive features of halophilic proteins	25
1.4.7	Applications of halophiles and their proteins	30
1.4.8	Enzymes from halophiles	37
1.4.9	Proteolytic enzymes	40
1.4.10	Proteases from halophiles	45
1.4.11	Protease production from halophilic microorganisms	47
1.4.12	Purification of halophilic proteases	50
1.4.13	Properties of halophilic proteases	56
1.4.14	Molecular characterization	71
1.4.15	Proteases from <i>Geomicrobium</i> sp.	73

CHAPTER 2 ISOLATION AND CHARACTERIZATION OF HALOPHILIC MICROORGANISMS FOR SCREENING OF POTENTIAL PROTEASE PRODUCERS

2.1	Introduction	76
2.2	Materials and Methods	77
2.2.1	Materials	77
2.2.2	Isolation of halophilic microorganism	77
2.2.3	Characterization of the organisms	78
2.2.4	Screening of strains for protease activities	78
2.2.5	Identification of strains by 16S rDNA sequencing	79
2.2.6	Scanning and Transmission Electron Microscopy	79
2.2.7	Protease production from halophilic strains	80
2.2.8	Protease assay	80
2.2.9	Preliminary characterization of halophilic crude proteases	81
2.3	Results and Discussion	81
2.3.1	Sites for sample collection	81
2.3.2	Isolation of halophilic microbes	82
2.3.3	Protease producer halophiles	85
2.3.4	Biochemical and physiological characterization	86
2.3.5	Identification by 16S rDNA sequencing	88
2.3.6	<i>Geomicrobium</i> sp. EMB2	90
2.3.7	Scanning and transmission electron microscopy	90
2.3.8	Protease from <i>Geomicrobium</i> sp. isolate	91
2.3.9	Growth kinetics and protease production	92
2.3.10	Preliminary characterization of <i>Geomicrobium</i> sp. EMB2 protease	93
2.4	Conclusions	95

CHAPTER 3 OPTIMIZATION OF CULTURE CONDITIONS FOR MAXIMUM PROTEASE PRODUCTION BY *GEOMICROBIUM* SP. EMB2

3.1	Introduction	96
3.2	Materials and Methods	97
3.2.1	Materials	97
3.2.2	Microorganism	97
3.2.3	Growth and protease production by <i>Geomicrobium</i> sp. EMB2	97
3.2.4	Protease assay	98
3.2.5	Optimization of protease production media	98
3.2.6	Growth and protease production under optimized conditions	99
3.3	Results and Discussion	100
3.3.1	Protease from <i>Geomicrobium</i> sp. EMB2	100
3.3.2	Production of <i>Geomicrobium</i> sp. EMB2 protease	100
3.4	Conclusions	113

CHAPTER 4 PURIFICATION AND CHARACTERIZATION OF *GEOMICROBIUM* SP. EMB2 PROTEASE

4.1	Introduction	114
4.2	Materials and Methods	115
4.2.1	Materials	116
4.2.2	Estimation of enzyme activity and protein concentration	116
4.2.3	Bacterial strain	116
4.2.4	Culture conditions for protease production	117
4.2.5	Purification of EMB2 protease	117
4.2.6	Polyacrylamide gel electrophoresis	118
4.2.7	Activity staining of purified protease	118
4.2.8	Characterization	118

4.3	Results and Discussion	123
4.3.1	Purification of <i>Geomicrobium</i> sp. EMB2 protease	123
4.3.2	Gel electrophoresis	125
4.3.3	Characterization of EMB2 protease	126
4.4	Conclusions	141

CHAPTER 5 IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF PROTEASE GENE

5.1	Introduction	142
5.2	Materials and Methods	144
5.2.1	Bacterial strains	144
5.2.2	N-terminal amino acid sequencing	144
5.2.3	Designing of PCR primers	144
5.2.4	Isolation of genomic DNA of <i>Geomicrobium</i> sp. EMB2	145
5.2.5	Polymerase chain reaction for amplification of the protease gene	145
5.2.6	Cloning of PCR product	145
5.2.7	Plasmid DNA isolation and restriction analysis	146
5.2.8	DNA sequencing, protein sequence comparison and phylogenetic analysis	146
5.2.9	Modelling of the 3D structure	147
5.2.10	Nucleotide sequence accession number	147
5.3	Results and Discussion	148
5.3.1	Amplification and cloning of the protease gene	148
5.3.2	Sequence analysis of the protease gene	150
5.3.3	Phylogenetic analysis of the EMB2 proteas	156
5.3.4	Structural features of EMB2 protease contributing to salt and solvent stability	157
5.4	Conclusions	162

CHAPTER 6 PROTEIN FOLDING AND STRUCTURAL STUDIES ON *GEOMICROBIUM* SP. EMB2 PROTEASE

6.1	Introduction	163
6.2	Material and Methods	165
6.2.1	Materials	165
6.2.2	Bacterial strain	165
6.2.3	Culture conditions for protease production	166
6.2.4	Purification of EMB2 protease	166
6.2.5	Protease assay	166
6.2.6	CD spectroscopy	166
6.2.7	Fluorescence measurements	167
6.2.8	Effect of NaCl on the structure of protease	167
6.2.9	Effect of NaCl on organic solvent stability of protease	167
6.2.10	Effect of NaCl on thermal denaturation	168
6.2.11	Protective effect of NaCl during guanidine hydrochloride unfolding	168
6.3	Results and Discussion	169
6.3.1	Purification and characterization of haloalkaliphilic <i>Geomicrobium</i> sp. protease	169
6.3.2	Effect of NaCl on protease activity and stability	169
6.3.3	Effect of NaCl on protease activity and stability in organic solvents	173
6.3.4	Thermal denaturation	178
6.3.5	Guanidine hydrochloride (GdnHCl)-induced denaturation	180
6.4	Conclusions	184
	Overall Conclusions	185
	Future Perspectives	187
	References	188
	List of Publications	241
	Brief Bio-Data	245