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Gene Identification and Molecular Characterization of Solvent Stable Protease from A Moderately Haloalkaliphilic Bacterium, *Geomicrobium* sp. EMB2

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Cloning and characterization of the gene encoding a solvent-tolerant protease from the haloalkaliphilic bacterium *Geomicrobium* sp. EMB2 are described. Primers designed based on the N-terminal amino acid sequence of the purified EMB2 protease helped in the amplification of a 1,505-bp open reading frame that had a coding potential of a 42.7-kDa polypeptide. The deduced EMB2 protein contained a 35.4-kDa mature protein of 311 residues, with a high proportion of acidic amino acid residues. Phylogenetic analysis placed the EMB2 gene close to a known serine protease from *Bacillus clausii* KSM-K16. Primary sequence analysis indicated a hydrophobic inclination of the protein; and the 3D structure modeling elucidated a relatively higher percentage of small (glycine, alanine, and valine) and borderline (serine and threonine) hydrophobic residues on its surface. The structure analysis also highlighted enrichment of acidic residues at the cost of basic residues. The study indicated that solvent and salt stabilities in *Geomicrobium* sp. protease may be accorded to different structural features; that is, the presence of a number of small hydrophobic amino acid residues on the surface and a higher content of acidic amino acid residues, respectively.

Keywords: Halophiles, protease gene, *Bacillus clausii*, *Geomicrobium* sp., solvent-tolerant protease

Proteases are the earliest-known enzymes and extensively characterized from a variety of sources. The ability to withstand detergents and stability in solvent medium are new attributes pointed out in some of these enzymes [4, 16, 24]. The stability in salt/solvents and the structural features that are responsible for these enzymatic properties are yet to be fully understood.

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A few proteases from moderately halophilic bacteria have been purified and studied; namely, those from *Bacillus* sp. no. 21-1 [13], haloalkaliphilic *Bacillus* sp. Vel [7], *Filobacillus* sp. RF2-5 [8], *Halobacillus* sp. SR5-3 [22], *Salinivibrio* sp. strain AF-2004 [16], haloalkaliphilic bacterium sp. AH-6 [4], and *Halobacillus karajensis* [15]. Reports on the protease gene are still less documented; for example, halolysin 172P1 from *Natrialba asiatica* [12], halolysin R4 from *Haloferax mediterranei* [11], Spt A from *Natrinema* sp. J7 [30], SVP2 from *Salinivibrio* sp. strain AF-2004 [17], Nep from *Natrialba magadii* [3], CPI from *Pseudoalteromonas ruthenica* [28], and PCP-03 from *Pseudoalteromonas* sp. SM9913 [32]. Only limited knowledge is available on their three-dimensional structure [20]. Characterization of the biochemical properties in combination with the gene information would be helpful to improve the understanding of halophilic proteases.

We have previously reported the isolation of a moderately haloalkaliphilic *Geomicrobium* sp. EMB2 strain from Sambhar Salt Lake, India. This strain was polyextremic, and thus able to grow in the presence of high salt concentrations as well as alkaline conditions. Furthermore, it secreted a novel protease, which was catalytically active and stable at high concentrations of a wide range of organic solvents. *Geomicrobium* sp. EMB2 protease was purified to homogeneity by hydrophobic interaction chromatography and found to be a 38-kDa serine protease [14].

While comparing the properties of *Geomicrobium* sp. EMB2 protease with other known haloalkaliphilic proteases, it became apparent that EMB2 protease differs from other halophiles with respect to higher pH optima, high pH stability, stabilities in surfactant and detergent, and tolerance to organic solvents. The basis of these differences and unique attributes needs to be elucidated. Understanding of such structural principles will provide necessary tools for protein designing.

The present study was undertaken to (i) characterize its gene by cloning and sequencing, (ii) carry out an *in silico*

analysis to predict the three-dimensional structure of EMB2 protease, and (iii) understand the underlying structural features of this protein responsible for its unique enzymatic properties, specifically salt and solvent stabilities.

Materials and Methods

Microbial Strains

Geomicrobium sp. EMB2, a haloalkaliphilic microorganism that was isolated from saline water (Sambhar Salt Lake) and producing a proteolytic enzyme, was used [14]. The culture has been deposited in the public culture collection, Microbial Type Culture Collection (MTCC), Chandigarh, India with accession number MTCC 10310. *E. coli* strain XL-1 Blue MRF' (Stratagene, USA) was used as a host for amplification of the recombinant plasmids.

N-Terminal Amino Acid Sequencing of EMB2 Protease

For Edman degradation sequence analysis, purified protease was separated on 12% SDS–PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (SVF, MDI, India), and then stained with 0.2% Ponceau S dye (Sigma Mo., USA) and the protein bands excised for N-terminal sequence analysis. Protein sequencing was performed by the Procise 494 protein-sequencing system (Perkin-Elmer, Applied Biosystems, Weiterstadt, Germany), as described by Zhang *et al*. [35].

Primers for PCR

The N-terminal amino acid sequence of the purified protease was used to search the NCBI database (http://ncbi.nlm.nih.gov/BLAST/) for its homologs, using BLASTP (Protein–Protein Basic Local Alignment Search Tool) program. A match with a protease gene having a GenBank accession number YP_177585 was found in the database. To amplify the complete ORF of the protease gene, forward and reverse primers were designed from 5' and 3' regions of the cDNA sequence using Gene Runner software (Hastings Software Inc., USA) and synthesized commercially (Sigma). The primers used were forward 5'-GATTTGTTTATACGTCGCTTTGTTC-3' [Tm= 67.0°C] and reverse 5'-CTGTTTAGAGGGAAGGGGTATAATC-3' [Tm=67.0°C].

Isolation of Genomic DNA

Geomicrobium sp. EMB2 was grown in CMB (complete medium broth) (pH 8.0) containing (g/l): glucose, 10.0; peptone, 5.0; yeast extract, 5.0; KH_2PO_4, 5.0; and NaCl, 100. Genomic DNA was isolated from 10.0 ml of the culture using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's specifications. Electrophoresis was carried out as described by Sambrook and Russell [26], with 0.8% agarose in Tris-acetic acid-EDTA buffer.

Polymerase Chain Reaction for Amplification of the Protease Gene

Polymerase chain reaction was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Switzerland) and JumpStart *Taq* DNA polymerase (Sigma-Aldrich Corp., St. Louis, MO, USA) as described by Thummler *et al*. [30]. Thermal cycling conditions were as follows: hot start cycle at 94°C for 1 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1.30 min, and a final extension step at 72°C for 5 min. The amplified PCR products were analyzed by gel electrophoresis with 0.8% agarose.

Cloning of PCR Product

The 1,505-bp PCR-amplified product was resolved on 0.8% agarose and gel extracted using the QIAquick Gel Extraction Kit (Qiagen, Germany). The purified fragment was ligated with the pGEM-T Easy plasmid vector (Promega, USA). This ligation mixture was used to transform *E. coli* strain XL1-Blue MRF′ competent cells as described by Sambrook and Russell [27]. The white bacterial colonies containing recombinant plasmids were selected on LB agar medium containing 0.1 mM X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside), 50 µg/ml ampicillin, and 12.5 µg/ml tetracycline.

Plasmid DNA Isolation and Restriction Analysis

Five ml of overnight grown cultures was prepared from putative recombinant colonies, at 37°C and 150 rpm in LB medium containing ampicillin. Plasmids were isolated using the alkaline lysis protocol described by Birnboim and Doly [1]. These plasmid DNA samples (about 200 ng) were digested in a 20-µl reaction mixture with *Eco*RI and *Hin*cII (Roche, Germany) for 3.5 h at 37°C. The digested samples were resolved on 0.8% agarose gel along with standard size markers (1-kb plus ladder) to analyze the restriction patterns.

DNA Sequencing, Protein Sequence Comparison, and Phylogenetic Analysis

The nucleotide sequences were determined by using a Big Dye Terminator sequencing kit (ABI Prism) on an automated sequencer (ABI Prism Biolab, Perkin Elmer, Switzerland) by the primer-walking technique with M13 universal primers as initial primers. The sequences thus obtained were assembled using Sequencher DNA software (version 4.0.5; Gene Codes, USA).

The amino acid sequence was deduced using ExPasy (http://expasy.org/tools). Sequence homologies to genes in the GenBank database were identified using the BLAST algorithm of the NCBI at the National Library of Medicine. The acquired sequences were aligned using Clustal X (version 2.0) [9]. Based on the sequences of the family of serine proteases, a phylogenetic tree was constructed according to the neighbor-joining method clustering strategy [25] in Clustal X and analyzed using the TreeView 1.6.5 program. Other DNA and protein sequence analyses (*viz*., restriction analysis and hydropathy plots) were performed with the Lasergene sequence analysis software (DNASTAR, Madison, WI, USA).

Modeling of the 3D Structure

Three-dimensional structures of the EMB2 protease were modeled using the online I-TASSER server for protein 3D structure prediction, the highest scoring server at the CASP7 structure-prediction competition [31, 33, 34]. The server predicts the folds and secondary structure by profile profile alignment (PPA) threading techniques. For the EMB2 protein, 5 models were obtained. The model figures were drawn using the Accelrys ViewerLite.

Nucleotide Sequence Accession Number

The DNA sequence of the EMB2 gene identified in the present study was submitted in the GenBank database under the GenBank accession number ADH93590.

RESULTS AND DISCUSSION

The *Geomicrobium* sp. EMB2 protease was characterized for its enzymatic properties. It was found to be a serine type of protease with 38 kDa molecular mass. It exhibited pH optimum at pH 10.0, with stability in the alkaline range. It was found that *Geomicrobium* sp. EMB2 protease was endowed some industrially useful properties. For instance, its alkaline nature and compatibility with detergents/ surfactants could be potentially useful for laundry applications. The stability in organic solvents was an unusual characteristic. Investigations were undertaken for its molecular characterization and to evaluate the distinguishing structural features.

Amplification and Cloning of the Protease Gene

The first 20 N-terminal amino acids sequence of the purified protease was as follows:

N-Thr-Gln-Ile-Pro-Asn-Asp-Leu-Asp-Cys-Gln-Asn-Ala-Glu-Asn-Arg-His-Ile-Asn-Pro-Ser-.

This sequence was used to search the NCBI database to identify the protein by using the Protein–Protein Basic Local Alignment Search Tool program (BLASTP). A match with a serine protease from *Bacillus clausii* KSM-K16 (GenBank Accession No. YP_177585) was found in the database. The protein had a 1,221-bp open reading frame (ORF). In order to clone the corresponding gene in *Geomicrobium* sp. strain EMB2, the cDNA sequence of *Bacillus clausii* KSM-K16 protease was used for designing the primers for PCR amplification.

The genomic DNA of *Geomicrobium* sp. EMB2 strain was isolated. The coding region of this gene was PCR amplified by using primers homologous to 5' and 3' untranslated regions (UTR). An approximately 1.5-kb amplified DNA fragment was obtained, which was gel purified (Fig. 1), cloned in pGEM-T Easy vector, and transformed in *Escherichia coli* strain XL-1 Blue MRF'. Amongst approximately 50 transformants of *E. coli* strain XL-1 Blue MRF', eight white colonies were selected for plasmid isolation. All the clones showed an insert of 1.5 kb along with a 3.0-kb vector band after digestion with *Eco*RI (Fig. 1). Since the protease gene was found to have internal *Hinc*II restriction sites, the plasmids were individually digested with this enzyme. The restriction pattern confirmed the cloning of the protease gene (Fig. 1).

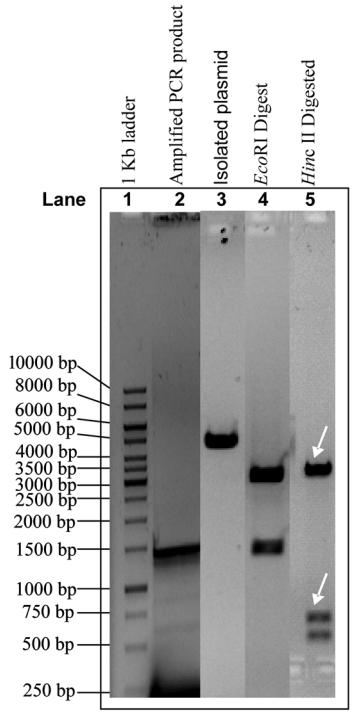

Fig. 1. Analysis of the PCR-amplified ORF (protease gene) fragment and confirmation of cloning of the protease gene-specific fragment (1.50 kb) from *Geomicrobium* sp. in the pGEM-T Easy vector.

Lane 1, 1-kb ladder; Lane 2, PCR-amplified sample; Lane 3, Isolated plasmid; Lane 4, Ethidium bromide-stained gel showing the presence of ~1.50 kb fragments in *Eco*RI-digested clones; Lane 5, Ethidium bromide-stained gel showing the fragments of *Hinc*II-digested clones. Arrows indicate the expected fragments of ~3,500 bp and ~744 bp in *Hinc*II-digested clones.

Sequence Analysis of the Protease Gene

DNA sequencing of the insert was performed using M13 forward and M13 reverse primers, and two internal primers, IPF and IPR (Fig. 2). The nucleotide sequence of *Geomicrobium* sp. EMB2 genomic DNA exhibited more than 70% homology with the serine protease gene of *Bacillus clausii* KSM-K16 (GenBank Accession No. AP006627.1) and *Bacillus pseudofirmus* OF4 (GenBank Accession No. CP001878.1). Thus, this protease can be concluded to be of the serine-type protease.

The 1,505-bp ORF of the EMB2 gene had a coding capacity of 375 amino acids (Fig. 2). Comparison of the N-terminal sequence with the predicted ORF revealed that the amino terminus of the mature extracellular purified protease matched with the 65th amino acid onwards of the predicted polypeptide sequence (Fig. 2). This suggested that the protease would probably be synthesized as a 42.7-kDa preproenzyme consisting of 375 amino acids, which would then be processed to produce a mature enzyme of 35.4 kDa (311 amino acids). Amino acid sequence homology analysis of the EMB2 protease with other serine-type proteases indicated that three amino acid residues (H77, H134, and S235) likely form the catalytic triad.

Phylogenetic Analysis of the EMB2 Protease

The full-length amino acid sequence deduced for EMB2 protease served as a template to screen structurally similar proteases by using BLASTP at the National Center for Biotechnology Information (NCBI). Resulting from this analysis, proteases exhibiting maximum sequence identity

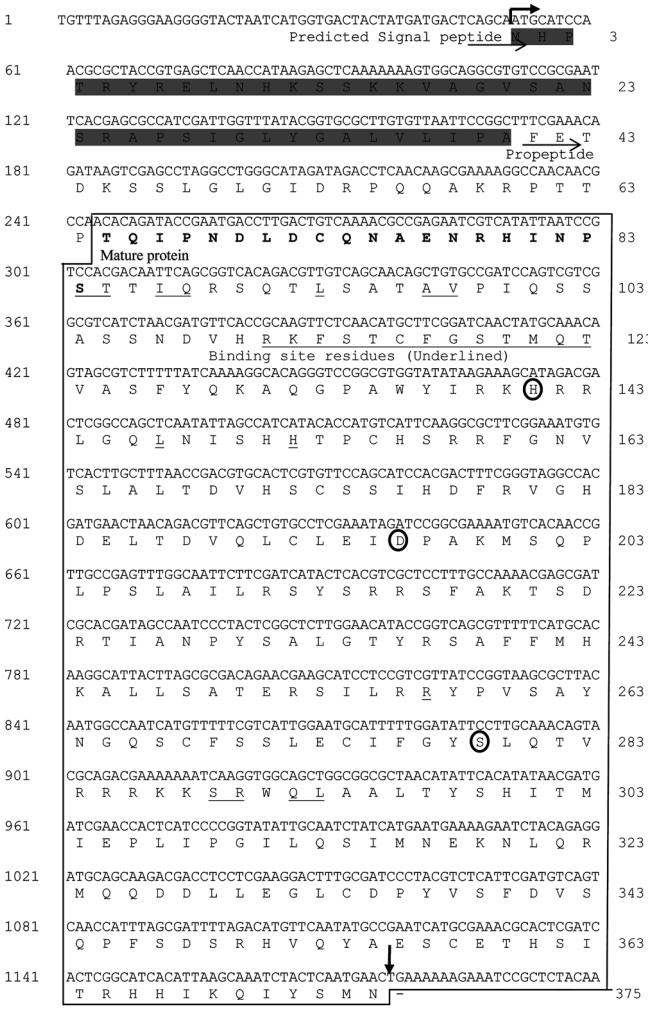

Fig. 2. Nucleotide and deduced amino acid sequence of the *Geomicrobium* sp. protease gene.
The nucleotide sequence was obtained after the assembly of all sequenced fragments and deduced amino acid sequence (in single letter codes) of the protease gene encoding a 42.6-kDa protein from *Geomicrobium* sp. EMB2. The start codon (ATG) and the stop codon (TGA) are marked by a rightward and a downward arrow, respectively. The shaded region represents the signal peptide. The boxed region represents the mature protein. The underlined region is the binding residues. The numbers on the left are nucleotide counts, whereas those on the right represent amino acid sequences. Bold-fonted amino acids are the N-terminal amino acid sequence of the purified protease determined by the Edman degradation method. Circles indicate essential amino acids constituting the active site.

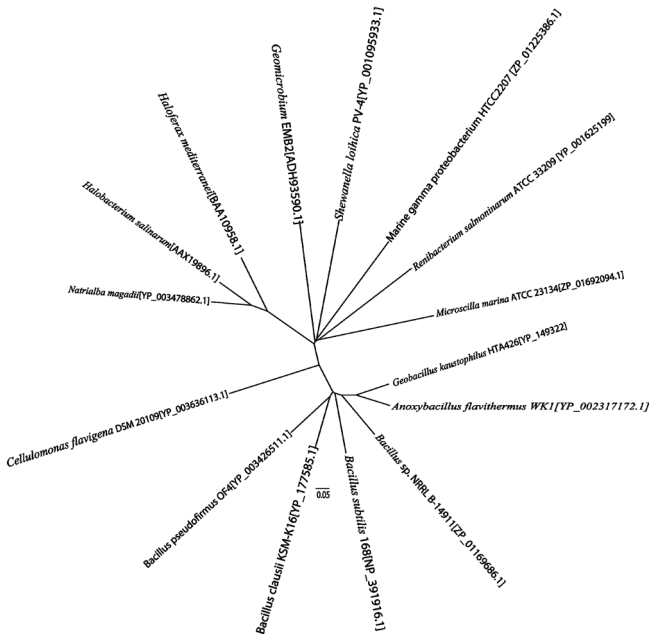

Fig. 3. Phylogenic relationship amongst the known proteases from various strains.
These data were generated by the CLUSTAL X software using the neighbor-joining method.

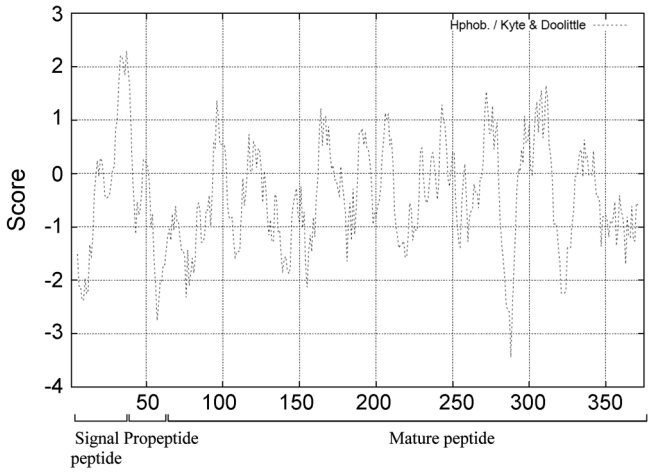

Fig. 4. Hydropathy analysis for EMB2 protease according to Kyte and Doolittle [18].
On the plot, a positive peak indicates a probability that the corresponding polypeptide fragment is hydrophobic (a negative peak indicates a probable hydrophilic segment).

to the EMB2 protease were selected for generating a phylogenetic tree. The EMB2 protease clustered with serine protease of marine gamma proteobacterium HTCC2207, *Shewanella loihica* PV-4, *Renibacterium salmoninarum* ATCC 33209, and other related *Bacillus* sp. (Fig. 3).

Structural Features of EMB2 Protease Contributing to Salt and Solvent Stabilities

The hydropathy profile of the deduced amino acid sequence of *Geomicrobium* sp. EMB2 protease, plotted according to the method of Kyte and Doolittle [18], showed hydrophobic inclination (Fig. 4). The increased presence of hydrophobic amino acids may possibly contribute to the solvent stability of *Geomicrobium* sp. protease.

In order to correlate the structural features responsible for solvent-stable function, three-dimensional structures of EMB2 protease were modeled using the online I-TASSER server for protein 3D structure prediction. Fig. 5A shows

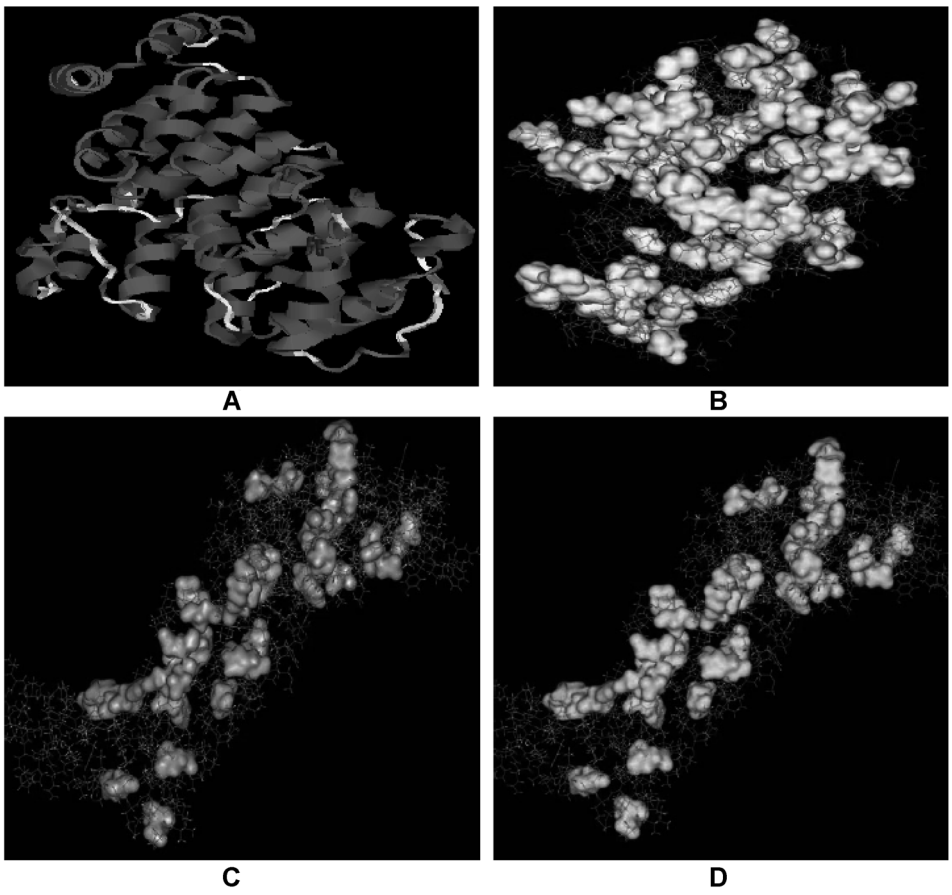

Fig. 5. 3D structure of the *Geomicrobium* sp. protease.
(**A**) Secondary structure. (**B**) Hydrophobic patches on the protein surface. (**C**) Acidic amino acids on the protein surface. (**D**) Negatively charged residues (aspartate and glutamate) on the protein surface.

the model with secondary structure topology. The molecule was found to have 51% α-helices, 7% β-strands, and 42% coils. In this protein, 40% of amino acid residues are hydrophobic, of which most were localized on the surface of this protein, as shown in Fig. 5B. The surface of the EMB2 protein has more number of small (glycine, alanine, and valine) and borderline (serine and threonine) hydrophobic amino acids as compared with the non-halophilic protein. The two solvent-labile proteases, namely thermolysin and *Aspergillus niger* protease (PDB code 1LNF and 1IZD, respectively) described by Gupta *et al*. [6], taken for comparison, contained 30.4% and 30% hydrophobic residues, respectively. Ogino *et al*. [23] and Gupta *et al*. [6] have reported that the presence of hydrophobic amino acids on the surface played an important role in the organic solvent stability of the PST-01 and Pse protease. Based on the above observations, we hypothesize that accumulation of hydrophobic amino acids on the surface of the polypeptide might impart solvent tolerance to EMB2 protease.

The amino acid composition of this polypeptide showed a high percentage (12.5%) of acidic residues as well, a feature in agreement with the acidic properties of other halophilic proteins; namely, *Pseudoalteromonas* sp. SM9913 (9.3%) [32], haloprotease CPI from *Pseudoalteromonas ruthenica* (11.2%) [28], SVP2 from *Salinivibrio* sp. strain AF-2004 (15.3%) [17], and halolysin R4 from *Haloferax mediterranei* (13.8%) [11]. The acidic and negatively charged (glutamic and aspartic) amino acids on the EMB2 protein surface are shown in Fig. 5C and 5D. Generally, proteins get precipitated owing to the salting-out effect in the presence of a high concentration of salts. The presence of negative charges on the surface of halophilic enzymes helps in binding of hydrated ions, thus reducing the chance to aggregate at high salinities [2, 19, 20]. Binding of water dipoles to a highly negative-charged surface of halophilic enzymes also helps in neutralization of the surface charge, rendering them more soluble and flexible at high salinities [21]. Similar mechanisms may be responsible for the salt stability of *Geomicrobium* sp. protease.

The *Geomicrobium* sp. EMB2 protease gene was amplified by polymerase chain reaction and cloned for the determination of its nucleotide sequence. EMB2 contained

an ORF of 1,505 bp that showed a homology (~74%) to the serine protease gene of *B. clausii* strains. EMB2 is a 35.4-kDa single polypeptide of 311 amino acid residues. It has a strong hydrophobic bias, which may contribute to its solvent-tolerant nature. Furthermore, the bioinformatics analysis revealed that its primary structure that contained 12.5% acidic residues was folded in a conformation that favored its stability in salt and organic solvents.

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References

1. Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7:** 1513–1523.
2. Danson, M. J. and D. W. Hough. 1997. The structural basis of protein halophilicity. *Comp. Biochem. Physiol.* **117A:** 307–312.
3. De Castro, R. E., D. M. Ruiz, M. I. Giménez, R. A. Paggi, M. X. Silveyra, and J. A. Maupin-Furlow. 2008. Gene cloning and heterologous synthesis of a haloalkaliphilic extracellular protease of *Natrialba magadii* (Nep). *Extremophiles* **12:** 677–687.
4. Dodia, M. S., C. M Rawal, H. G. Bhimani, R. H. Joshi, S. K. Khare, and S. P. Singh. 2008. Purification and stability characteristics of an alkaline serine protease from a newly isolated haloalkaliphilic bacterium sp. AH-6. *J. Ind. Microbiol. Biotechnol.* **35:** 121–131.
5. Gimenez, M. I., C. A. Studdert, J. J. Sanchez, and R. E. De Castro. 2000. Extracellular protease of *Natrialba magadii*: Purification and biochemical characterization. *Extremophiles* **4:** 181–188.
6. Gupta, A., S. Ray, S. Kapoor, and S. K. Khare. 2008. Solvent-stable *Pseudomonas aeruginosa* PseA protease gene: Identification, molecular characterization, phylogenetic and bioinformatic analysis to study reasons for solvent stability. *J. Mol. Biol. Biotechnol.* **15:** 234–243.
7. Gupta, A., I. Roy, R. K. Patel, S. P. Singh, S. K. Khare, and M. N. Gupta. 2005. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. *J. Chromatogr. A* **1075:** 103–108.
8. Hiraga, K., Y. Nishikata, S. Namwong, S. Tanasupawat, K. Takada, and K. Oda. 2005. Purification and characterization of serine proteinase from a halophilic bacterium, *Filobacillus* sp. RF2-5. *Biosci. Biotechnol. Biochem.* **69:** 38–44.
9. Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgin, and T. J. Gibson. 1998. Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* **23:** 403–405.
10. Kamekura, M. and Y. Seno. 1990. A halophilic extracellular protease from a halophilic archaebacterium strain 172 P1. *Biochem. Cell Biol.* **68:** 352–359.
11. Kamekura, M., Y. Seno, and M. L. Dyall-Smith. 1996. Halolysin R4, a serine proteinase from the halophilic archaeon *Haloferax mediterranei*; gene cloning, expression and structural studies. *Biochim. Biophys. Acta* **1294:** 159–167.
12. Kamekura, M., Y. Seno, M. L. Holmes, and M. L. Dyall-Smith. 1992. Molecular cloning and sequencing of the gene for a halophilic alkaline serine protease (halolysin) from an unidentified halophilic archaea strain (172P1) and expression of the gene in *Haloferax volcanii*. *J. Bacteriol.* **174:** 736–742.
13. Kamekura, M. and H. Onishi. 1974. Protease formation by a moderately halophilic *Bacillus* strain. *Appl. Microbiol.* **27:** 809–810.
14. Karan, R. and S. K. Khare. 2010. Purification and characterization of a solvent-stable protease from *Geomicrobium* sp. EMB2. *Environ. Technol.* **10:** 1061–1072.
15. Karbalaei-Heidari, H. R., M. A. Amoozegar, M. Hajighasemi, A. A. Ziaee, and A. Ventosa. 2009. Production, optimization and purification of a novel extracellular protease from the moderately halophilic bacterium *Halobacillus karajensis. J. Ind. Microbiol. Biotechnol.* **36:** 21–27.
16. Karbalaei-Heidari, H. R., A. A. Ziaee, and M. A. Amoozegar. 2007. Purification and biochemical characterization of a protease secreted by the *Salinivibrio* sp. strain AF-2004 and its behavior in organic solvents. *Extremophiles* **11:** 237–243.
17. Karbalaei-Heidari, H. R., A. A. Ziaee, M. A. Amoozegar, Y. Cheburkin, and N. Budisa. 2008. Molecular cloning and sequence analysis of a novel zinc metalloprotease gene from the *Salinivibrio* sp. strain AF-2004 and its extracellular expression in *E. coli*. *Gene* **408:** 196–203.
18. Kyte, J. and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157:** 105–132.
19. Lanyi, J. K. 1974. Salt dependent properties of proteins from extremely halophilic bacteria. *Bacteriol. Rev.* **38:** 272–290.
20. Madern, D., C. Ebel, and G. Zaccai. 2000. Halophilic adaptation of enzymes. *Extremophiles* **4:** 91–98.
21. Mevarech, M., F. Frolow, and L. M. Gloss. 2000. Halophilic enzymes: Proteins with a grain of salt. *Biophys. Chem.* **86:** 155–164.
22. Namwong, S., T. Hiroga, K. Takada, M. Tsunemi, S. Tanasupawat, and K. Oda. 2006. Halophilic serine protease from *Halobacillus* sp. SR5-3 isolated from fish sauce: Purification and characterization. *Biosci. Biotechnol. Biochem.* **70:** 1395–1401.
23. Ogino, H., T. Uchiho, J. Yokoo, R. Kobayashi, R. Ichise, and H. Ishikawa. 2001. Role of intermolecular disulfide bonds of the organic solvent-stable PST-01 protease in its organic solvent stability. *Appl. Environ. Microbiol.* **67:** 942–947.
24. Ruiz, D. M. and R. E. De Castro. 2007. Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon *Natrialba magadii*. *J. Ind. Microbiol. Biotechnol.* **34:** 111–115.
25. Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.
26. Sambrook, J. and D. W. Russell. 2000. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

27. Sanchez-Porro, C., E. Mellado, C. Bertoldo, G. Antranikian, and A. Ventosa. 2003. Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76. *Extremophiles* **7:** 221–228.
28. Sánchez-Porro, C., E. Mellado, A. P. Pugsley, O. Francetic, and A. Ventosa. 2009. The haloprotease CPI produced by the moderately halophilic bacterium *Pseudoalteromonas ruthenica* is secreted by the type II secretion pathway. *Appl. Environ. Microbiol.* **75:** 4197–4201.
29. Shi, W., X. F. Tang, Y. Huang, F. Gan, B. Tang, and P. Shen. 2006. An extracellular halophilic protease SptA from a halophilic archaeon *Natrinema* sp J7: Gene cloning, expression and characterization. *Extremophiles* **10:** 599–606.
30. Thummler, F., A. Beetz, and W. Rudiger. 1990. Phytochrome in lower plants: Detection and partial sequence of a phytochrome gene in the moss *Ceratodon purpureus* using the polymerase chain reaction. *FEBS Lett.* **275:** 125–129.
31. Wu, S., J. Skolnick, and Y. Zhang. 2007. *Ab initio* modeling of small proteins by iterative TASSER simulations. *BMC Biol.* **5:** 17–26.
32. Yan, B. Q., X. L. Chen, X. Y. Hou, H. He, B. C. Zhou, and Y. Z. Zhang. 2009. Molecular analysis of the gene encoding a cold-adapted halophilic subtilase from deep-sea psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913: Cloning, expression, characterization and function analysis of the C-terminal PPC domains. *Extremophiles* **4:** 725–733.
33. Zhang, Y. 2007. Template-based modeling and free modeling by I-TASSER in CASP7. *Proteins* **8:** 108–117.
34. Zhang, Y. 2008. I-TASSER server for protein 3D structure prediction BMC. *Bioinformatics* **9:** 40.
35. Zhang, Y., D. D. Bak, H. Heid, and K. Geider. 1999. Molecular characterization of a protease secreted by *Erwinia amylovora. J. Mol. Biol.* **289:** 1239–1251.