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Expression and characterization of alkaline protease from the metagenomic library of tannery activated sludge

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Metagenomics has the potential to facilitate the discovery of novel enzymes; however, to date, only a few alkaline proteases have been characterized from environmentally-sourced DNA. We report the identification and characterization of an alkaline serine protease designated as Prt1A from the metagenomic library of tannery activated sludge. Sequence analysis revealed that Prt1A is closely related to S8A family subtilisins with a catalytic triad of Asp_{143}, His_{173}, and Ser_{326}. The putative protease gene (*prt*-1A) was subcloned in pET 28a (+) vector and overexpressed in *Escherichia coli* BL21(DE3)pLysS cells. This 38.8 KDa recombinant protease was purified to homogeneity by nickel affinity chromatography and exhibited optimal enzyme activity at elevated pH (11.0) and temperature (55°C). The enzyme activity was enhanced by the addition of 5 mM Ca^{2+} ions, and was stable in the presence of anionic detergent, oxidizing agent and various organic solvents. The enzyme displayed high affinity and catalytic efficiency for casein under standard assay conditions (V_{max} = 279 U/mg/min, K_m = 1.70 mg/mL) and was also compatible with commercial detergents. These results suggest that Prt1A protease could act as an efficient enzyme in various industrial applications.

[**Key words:** Metagenomics; Alkaline protease; Activated sludge; Enzyme kinetics; Thermostability]

Soil microorganisms are valuable sources of novel enzymes, antibiotics and other biomolecules. Microbial enzymes are potentially useful in a broad range of industrial and agricultural applications. While a large number of enzymes are obtained from culturable microbes using standard microbiological techniques, these enzymes are derived from less than 1% of the total microorganisms present in the environment (1). Hence, the rate of discovery of novel enzymes has decreased significantly and vast microbial resources remain untapped. To circumvent this, metagenomics has emerged as an alternative approach to bioprospecting that involves direct cloning and screening of environmental DNA (2). As a result, functional metagenomics has led to the discovery of novel enzymes from unculturable organisms.

Proteases hold a major share of the global enzyme market due to their wide range of applications (3). Although proteases are produced by plants, animals, bacteria, fungi and archaea, microbial proteases have the greatest industrial importance because they offer ease of genetic manipulations and high yields. Alkaline serine proteases play significant role in detergents, leather and food processing, peptide and pharmaceutical synthesis, brewing, and wastewater treatment (4). Serine proteases are endopeptidases that utilize serine (S) within the active site as a nucleophile to cleave peptide bonds. Aspartate (D) and histidine (H) are also involved in the formation of the catalytic triad at the enzyme active site. Although many serine proteases have been previously characterized, the search for additional enzymes is still in progress to obtain more efficient enzymes for different industrial purposes. Further, the metagenomic approach has only recently contributed serine proteases from goat skin, forest and desert soil (5–7).

The activated sludge wastewater treatment process is a conventional biological treatment of tannery effluent which employs microbes for the degradation of proteins, lipids, and other pollutants. Activated sludge from tannery effluent is therefore a rich source of diverse microorganisms which produce enzymes with robust degradative properties. Surprisingly, tannery effluents have not yet been explored through metagenomic studies for the discovery of enzymes. We report the construction of a metagenomic library from the activated sludge of a tannery effluent treatment plant. From this library, an alkaline serine protease was identified and characterized which was resistant to oxidizing agents, metal ions and organic solvents, while also displaying high compatibility with SDS and commercial detergents.

MATERIALS AND METHODS

Bacterial strains, plasmids, and chemicals All chemicals of analytical and molecular biology grade were purchased from Sigma–Aldrich, India. pBluescript II KS(+) plasmid, pET 28a (+), *E. coli* TOP-10 cells, and *E. coli* BL21(DE3)pLysS were purchased from Invitrogen Bio Services India Pvt. Ltd.

General molecular procedures Restriction digestion, ligation, transformation and PCR amplification were performed according to standard protocols (8). Plasmid isolation and product purification were performed with EZ-10 Spin

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Column Plasmid Isolation kits/Gel Extraction Kit (Biobasic Inc., Canada). DNA sequencing was performed by Eurofins Genomics Pvt. Ltd., Bangalore, India.

Sample collection and construction of soil metagenomic library Activated sludge sample was collected from Common Effluent Treatment Plant (CETP) for tanneries, located at Pallavaram (12°57′44″N 80°8′8″E), Chennai, India, and stored at −70°C until use. Total DNA was extracted from the activated sludge sample using Fast DNA SPIN Kit (MP Biomedicals, Solon, OH, USA). The isolated DNA was partially digested with *Bam*HI, and fragments ranging from 1 to 8 Kb were gel eluted and cloned into *Bam*HI linearized and dephosphorylated pBluescript II KS(+) vector. The ligation products were transformed into competent *E. coli* TOP-10 cells and then plated in LB agar plates supplemented with ampicillin (50 μg/mL), X-gal (20 μg/mL), and isopropyl-β-D-thiogalactopyranoside (IPTG) (40 μg/mL) and incubated at 37°C overnight. The white recombinant clones were selected and maintained in microtiter plates as glycerol stocks and stored at −70°C.

Screening the metagenomic library for proteolytic activity and subcloning of the protease gene The recombinant clones were screened for proteolytic activity on LB agar plates supplemented with ampicillin (50 μg/mL) and 1% (w/v) skimmed milk (5) and incubated at 37°C for 48–72 h. Proteolytic clones were selected based on the zone of clearance surrounding the colony. A clone producing a significant clearance zone was further subjected to confirmation by digestion with *Bam*HI.

Sequencing and phylogenetic analysis Plasmid DNA was isolated from the positive clone and primer walking was used to sequence the insert. The insert sequence was analyzed for the presence of an open reading frame using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/). The ORF obtained was analyzed for sequence similarity using BlastP against NCBI database and also MEROPS (http://merops.sanger.ac.uk) database for identifying the protease class. Multiple sequence alignment was performed for the protein sequence with ClustalW (version 2.0) using the neighbor-joining clustering method (9). A bootstrapped phylogenetic tree with 1000 replicates was built using the neighbor-joining method with the Molecular Evolutionary Genetic Analysis (MEGA) version 4.0 (10). An N-terminal signal peptide was predicted with the Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP) (11). The physical and chemical parameters of the enzyme were analyzed using ExPASy Protparam tool (http://web.expasy.org/protparam/). The structure of the protein was predicted and a 3D model was constructed using SWISS-MODEL (http://swissmodel.expasy.org/) (12). Pymol was used to visualize and analyze the structure.

Cloning and overexpression of the protease encoding gene The full-length ORF was amplified using a forward primer (5′- GGG CAT ATG TCG CAT CGG CTG CTG AAG AAG -3′) and reverse primer (5′- GGG GGA TCC TTA GCG TGT TGC CGC TTC TGC-3′). The amplified DNA was digested with *Nde*I and *Bam*HI and cloned in pET 28a (+) digested with the same restriction enzymes. The recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS competent cells. The cells containing the recombinant plasmid were grown overnight at 37°C in an LB media containing kanamycin (50 μg/mL). The culture was inoculated in fresh media and incubated until the optical density at 600 nm reached 0.4. The culture was then induced with 0.1 mM IPTG at 20°C overnight. The induced cells were harvested by centrifugation at 12,000 ×*g* for 10 min at 4°C. Induced and uninduced fractions were analyzed by SDS-PAGE on a 12% polyacrylamide gel (13). The cell pellet was washed with buffer containing 50 mM NaH_2PO_4 and 300 mM NaCl (pH 8.0) and the cells were disrupted by sonication (5 times, for 30 s at 30 s intervals) (Q Sonica, United States). The soluble fraction was collected by centrifugation at 12,000 ×*g* for 10 min at 4°C and the protein was quantified using the method of Lowry et al. (14).

Enzyme assay for protease activity Protease activity was assayed by the method described by Sareen et al. (15). One mL of 1% casein was prepared in 50 mM glycine–NaOH buffer (pH 10) and 1 mL of diluted enzyme was prepared with the same buffer. The reaction mixture was incubated at 50°C for 20 min. The reaction was stopped by adding 10% trichloroacetic acid. The precipitate formed was removed by centrifugation at 12,000 ×*g* for 10 min at 4°C and the absorbance of the supernatant was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme that could release 1 μg of tyrosine per minute at 50°C. The soluble fractions were analyzed for the zone of clearance by a plate diffusion assay. Uniform wells were punctured into a 1% agar media containing 1% skimmed milk powder dissolved in 50 mM glycine–NaOH buffer (pH 10.0). 20 μL of both uninduced and induced fractions were dispensed into the wells and the plates were incubated at 37°C overnight and observed for a clearance halo around the wells.

Purification of the recombinant enzyme Purification of the recombinant protease was performed under non-denaturing conditions by Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography (Sigma–Aldrich). The cell biomass was dispersed in binding buffer containing 50 mM NaH_2PO_4 and 300 mM NaCl (pH 8.0). The suspension was sonicated and centrifuged at 12,000 ×*g* for 30 min at 4°C. The His-Select Nickel Affinity Gel (Sigma–Aldrich) was thoroughly suspended by gentle inversion and 1.5 mL of the gel was poured into a clean chromatography column. The column was washed twice with deionized water to remove the ethanol bound to the affinity gel. The column was then equilibrated with 5 mL of binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8). The soluble fraction containing the recombinant enzyme was passed through the column. The column was washed twice with 5 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, pH 8). The purified protein was eluted with 1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8) and analyzed in SDS-PAGE (12%).

Effect of pH and temperature on protease activity The effect of temperature on protease activity was examined at a range of temperatures (20°C–65°C) in 50 mM glycine–NaOH buffer (pH 11.0) using 1% casein as substrate. To determine its thermal stability, the purified Prt1A protease was incubated at 50°C, 60°C, and 70°C for 6 h. Activity before incubation was taken as 100% and residual activity for each hour was determined. The effect of pH on Prt1A activity was determined by standard assay at 50°C using 1% casein as substrate. The assay was performed in different pH buffer systems such as sodium acetate buffer (pH 4.0, 5.0), sodium phosphate buffer (pH 6.0), Tris buffer (pH 7.0, 8.0), and glycine–NaOH buffer (pH 9.0, 10.0, 11.0, 12.0, 12.5).

Effect of different additives on protease activity The effect of different additives was assessed at the optimum temperature and pH by pre-incubating the reaction mixtures for 30 min with various surfactants such as sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), Tween 80, and Triton X 100 (1% and 5%); oxidizing agent H_2O_2 (1% and 5%); organic solvents such as methanol, ethanol, isopropanol, hexane, and toluene (5% and 10%); metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+} and Zn^{2+} (1 mM and 5 mM); inhibitors such as EDTA, DTT, 2-mercaptoethanol and PMSF (1 mM and 5 mM), and NaCl (0–2 M). In all the experiments, the specific activity of the control without any of the above-mentioned additives was taken as 100% activity and residual activity (%) of the enzyme in the presence of the additives was determined in accordance with the control.

Kinetic analysis Effect of substrate concentration on enzyme activity was studied using as a casein substrate (0.1 mg/mL–50 mg/mL) at optimized pH (11.0) and temperature (55°C). The K_m, V_{max} of saturation kinetics were determined through a combination of non-linear fitting and regression of linearized forms to allow for more rigorous assessment of the kinetic parameters and their associated standard error and noted below under statistics. Protease kinetics was assumed to fit saturation kinetics:

$$v = \frac{v_{max} \cdot S}{K_m + S} \quad (1)$$

Effect of different commercial detergents The protease activity in the presence of commercial detergents such as Tide, Ariel (Procter & Gamble, India), Rin, Surf excel (Hindustan Unilever Ltd.) and Henko (Jyothi Laboratories Ltd., India) was tested by pre-incubating the enzyme with detergent at two different concentrations (0.5% and 1%) for 1 h. Prior to the assay, the detergent solutions were incubated in boiling water bath for 1 h to inactivate their native proteases. Reaction without added detergent was taken as control and its specific activity was considered to be 100%, while residual activities were calculated for the test samples measured at pH 11.0 and 55°C.

Statistical analysis All the assays were performed in triplicate and the mean and standard deviations were estimated for each assay. Student's *t*-test was performed with the software GraphPad Prism 5.0. The values were considered significant if $p < 0.05$.

Parameters were assessed by least squares non-linear fitting (using MicroSoft Excel Solver) as well as various linearized forms of saturation kinetics to allow for rigorous assessment of standard error of the kinetic parameters (using Excel LINEST function) and associated propagation of error for calculated parameters. Linear regressions were performed for the popular linearized forms of saturation kinetics: double-reciprocal (Lineweaver–Burk), Hanes–Woolf and Eadie–Hofstee plots. In addition, a non-linear fitting and assessment of standard error was also conducted with Hyper32.exe written by Dr. John Easterby, U. Liverpool.

RESULTS

Construction and screening of metagenomic library for proteolytic activity A clone library was constructed from the metagenomic DNA of tannery activated sludge. Restriction digestion of the resulting library was found to contain a wide range of inserts (1.5–6 Kb) with the average insert size of ~3 Kb. Screening of 10,000 clones from the library for protease enzyme resulted in a single positive clone showing a clear halo zone in skimmed milk agar. The insert size of the clone after *Bam*HI digestion was found to be 1.7 Kb.

Nucleotide and amino acid sequence analysis To identify the gene conferring proteolytic activity, the plasmid isolated from the positive clone was sequenced by primer walking. The sequence revealed the presence of 1143 bp ORF (designated as *prt*-1A) which