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Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium *Bacillus lehensis*

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HIGHLIGHTS

- First report on the production of recombinant alkaline protease from *Bacillus lehensis*.
- A 4.1-fold increase in enzyme activity was achieved by cloning and expression.
- The enzyme is solvent tolerant and SDS-stimulated.
- The enzyme is useful as detergent additive and in silver recovery from used films.
- The enzyme is applicable in silk degumming and biocontrol of nematodes.

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ABSTRACT

A highly alkaline protease (BLAP) from a novel psychrotolerant and alkaliphilic bacterium, *Bacillus lehensis* was cloned and expressed in *Escherichia coli*. BLAP belongs to subtilase S8 family of proteases, comprising 27 aa secretion signal, 83 aa prosequence and 269 aa mature BLAP. The amino acids Asp 141, His 171 and Ser 324 form catalytic triad, while Ile 214, Leu 233 and Asn 267 are other active site moieties. Recombinant alkaline protease (rBLAP) is a monomeric protein of 39.0 ± 1.0 kDa, and it is active over broad pH (8–12) and temperature (30–60 °C) ranges, with optima at pH 12.8 and 50 °C. rBLAP is stimulated by SDS, Co^{2+}, Ca^{2+}, β-ME, and inhibited by Hg^{2+} and PMSF. The rBLAP is compatible with commercial detergents, useful in silk degumming and silver recovery from the used photographic films and a potent biocontrol agent for arresting the development of eggs of the nematode *Meloidogyne incognita*.

1. Introduction

Proteases occupy a pivotal status with respect to their commercial applications, which are hydrolytic enzymes that cleave peptide bonds in proteins and peptides. Due to their extensive use in a wide range of industrial applications, they hold a major share in the enzyme market with two-thirds in the detergent industry alone (Haki and Rakshit, 2003). These enzymes are utilized in leather, food, pharmaceutical and textile industries, in peptide synthesis, waste water treatment and biocontrol. For being applicable as detergent additive, proteases need to be active at high pH and moderate temperature, and in the presence of various additives like solvents, surfactants and oxidizing agents (Manachini and Fortina, 1998). Although the production of alkaline proteases has been reported from a wide range of microbes, a large proportion of the commercially available ones are derived from *Bacillus* spp. (Mehrotra et al., 1999). *Bacillus* strains are preferred because of their ability to produce enzymes extracellularly in a short time (Maurer, 2004). In order to produce alkaline proteases in a cost effective manner, attempts have been made to clone and express them in heterologous systems such as *Escherichia coli* (Fu et al., 2003; Karbalaei-Heidari et al., 2008).

Proteases have been found useful in degrading gelatin coating over the used X-ray and photographic films that liberates silver present in the gelatin layer. About 18–20% of the world's silver demand is met with that derived from waste products like X-ray and photographic films (Nakiboglu et al., 2003). According to Arami et al. (2007), 22–25% of natural silk is composed of sericin protein that gives harsh and stiff texture to the fibre, but diminishes the shine and whiteness of silk that affects the dyeing process. Enzymatic removal of sericin is an environment-friendly process of silk degumming (Arami et al., 2007).

Proteases have also been tested as biocontrol agents against numerous agricultural pests. Several nematode species are known to be pathogenic to crop plants leading to huge economic losses.

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Proteases from *Pochonia chlamydosporia* (Ward et al., 2012) and *Pseudomonas fluorescens* CHA0 (Siddiqui et al., 2005) have been found useful as biocontrol agents against root knot nematodes.

In this investigation, an alkaline protease from a novel psychrotolerant and alkaliphilic *Bacillus lehensis* MTCC7633 was cloned and expressed in biologically active form in mesophilic host *E. coli*. The recombinant alkaline protease was purified and characterized, and its applicability in degrading gelatin from the used X-ray/photographic films, degumming of raw silk, as a detergent additive and in the biocontrol of plant pathogenic nematode *Meloidogyne incognita* was tested.

2. Methods

2.1. Bacterial strains and vectors

B. lehensis, a psychrotolerant and alkaliphilic bacterium isolated from soil of Leh (India), was described as a novel species and maintained on nutrient agar plates at 20 °C (Ghosh et al., 2007) and deposited at Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India (MTCC 7633). *E. coli* DH5α (genotype: F⁻ *end*A1 *gln*V44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *deo*R *nup*G Φ80d*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169, *hsd*R17 ($r_K^- m_K^+$), λ-) was used for cloning and DNA manipulations. *E. coli* DH5α was maintained on LB agar plates. *E. coli* BL21 (DE3) (genotype: F- *omp*T *gal dcm lon hsd*S_B ($r_B - mB^-$) λ (DE3 (l*ac*I l*ac*UV5-T7 gene1 *ind*1 *sam*7 *nin*5) was used for expression studies and was grown in LB with kanamycin (50 μg/mL) for recombinant protein expression. TA cloning vector pGEM-T Easy (Promega) was used for primary cloning and sequencing purposes. pET28a (+) (Novagen CA, USA) was used as an expression vector.

2.2. Primer synthesis for PCR and sequencing

Genomic DNA of *B. lehensis* was isolated according to Dulmau (1982). Isolated DNA was used as a template for amplification of alkaline protease (BLAP) gene. Various alkaline protease gene sequences, available at NCBI database, were aligned using ClustalW alignment tool. Conserved oligonucleotide sequences were identified from the aligned sequences. Based on the conserved nucleotide sequences, a pair of internal primers (BLAP Int F′: 5′TCCGTTGAACTTGATCCAGAAG3′ and Int BLAP Int R′: 5′GCCATAGATG-TACCATTGAAGC3′) was designed for amplifying alkaline protease encoding gene. The amplified partial fragment of alkaline protease gene was cloned into pGEM-T Easy vector and sequenced using T7 forward and SP6 reverse primers. Based on the homology of this partial sequence with the known proteases, a new set of primer pair (BLAP 'F 5′ATGAATAAGAAAATGGGG3′ and BALP R′ TTAACGTGTTGCCGCTTCTGCG) was designed to get full-length amplification of BLAP ORF. NCBI tool ORF finder was used for identifying BLAP ORF. A third pair of primers (BLAP NdeI F′: CGACATATGGCCG-AGGAAGCAAAGG3′ and BLAP XhoI R′: 5′CGACTCGAGACGTGTTGCCGCTTCTGCG3′) was designed to obtain BLAP gene, without secretion signal, for expression studies. Using BLAP NdeI F′ and BLAP XhoI R′ primers, BLAP gene was amplified using Pfu DNA polymerase (Fermentas) and Taq polymerase in 1:4 ratio under optimized PCR program (denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 50 s, annealing step at 52 °C for 40 s and extension step at 72 °C for 1 min 20 s) in 25 μL reaction with final extension step at 72 °C for 7 min in a C1000TM thermocycler (Biorad, USA).

2.3. Construction of recombinant rBLAP-pET28a

The ORF of the amplified BLAP was cloned into pGEM-T vector. Positive rBLAP-pGEMT clones were screened both by blue–white selection on IPTG-Amp-X-Gal LB agar plates and colony PCR. Plasmid from positive clones was double digested with NdeI and XhoI (New England Biolabs) restriction enzymes, and the fallout was cleaned up using Qiagen DNA purification kit (Qiagen, Germany). pET28a (+) was also double digested with NdeI and XhoI. Dephosphorylation of double digested pET28a (+) was done using Calf Intestinal Alkaline Phosphatase (CIAP). BLAP fallout and double digested pET28a (+) were ligated using T4 DNA ligase at 16 °C for overnight. The ligated product was transformed into competent *E. coli* DH5α cells. The positive rBLAP-pET28a clones were confirmed by colony PCR and double digestion of the construct with NdeI and XhoI. Ten positive clones were sequenced at the Nucleic acid Sequencing Facility, University of Delhi South Campus, New Delhi.

2.4. Gene expression in E. coli BL21 (DE3)

A total of ten clones were sequenced, and one having the same sequence was selected for further studies. Plasmid rBLAP-pET28a from the selected clone was isolated and transformed into *E. coli* BL21 (DE3). A 16 h inoculum of the recombinant *E. coli* BL21 (DE3) was prepared in 5 mL Luria–Bertani medium containing kanamycin with 50 μg/mL (LB kan). Fifty milliliter LB-kan was inoculated with 1% (v/v) inoculum and incubated at 37 °C to 0.5–0.6 OD_{600}, followed by expression of rBLAP was induced by 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 30 °C. After 16 h of induction, biomass was harvested by centrifugation, and localization of the expressed rBLAP was studied according to Verma and Satyanarayana (2012).

2.5. Bioinformatic analysis of rBLAP

The nucleotide and protein sequences of rBLAP were compared against the National Center for Biotechnology Information (NCBI) nucleotide/protein database, using Basic Local Alignment Search Tool for Nucleotides (BLASTn) and BLAST for proteins (BLASTp), respectively. Multiple sequence alignment (MSA) and phylogenetic analysis was performed by ESPript (2.2) and MEGA program (version 5, using neighbour-joining algorithm), respectively. The presence of a putative secretion signal peptide at N-terminus was predicted using SignalP 4.0 (http://www.cbs.dtu.dk/).

2.6. Homology modelling

rBLAP amino acid sequence without signal peptide was used for homology modelling. SWISS-MODEL workspace server was used to identify the template and model building (http://swissmodel.expasy.org/workspace). PyMOL software was used for analysing the model (http://www.expasy.ch/swissmod/SWISSMODEL.html).

2.7. Purification of recombinant alkaline protease

rBLAP was purified to homogeneity by Ni^{2+}-affinity chromatography (IMAC) under non-denaturing conditions using Novagen® Ni–NTA His•Bind® resin. The biomass of induced *E. coli* BL21 (DE3) cells was sonicated using Sonics® Vibra cell sonicator probe 630–0219: 13 mm at 25 amplitude (15 cycles of 1 min pulse, 2 s on/off) in binding buffer (containing 50 mM phosphate buffer (pH 8), 100 mM NaCl, 10 mM $MgCl_2$, 1 mg mL^{-1} lysozyme, 5 mM β-mercaptoethanol and 5% glycerol) for releasing intracellular rBLAP. After sonication, the supernatant containing rBLAP was collected for purification. A packed column volume (CV) of 5 mL resin was used for purification process. Ni^{2+}-NTA resin was first washed with 5 CV of deionized water and then equilibrated with 5 CV of binding buffer. Thereafter the supernatant was passed through the column for binding of the 6X histidine containing recombinant