Journal of Scientific & Industrial Research
Vol. 72, March 2013, pp. 166-171

Characterization of thermostable serine protease from *Bacillus altitudinis* strain BR1

Brenda D'Costa, Kashif Shamim and S. K. Dubey*
Laboratory of Molecular Microbiology and Environmental Biotechnology,
Department of Microbiology, Goa University, Taleigao Plateau Goa- 403 206, India

Received: 14 May 2012; revised 28 September; accepted: 31 December 2012

This study is carried out for partially purified and characterized thermostable serine protease from alkaliphilic estuarine bacterium *Bacillus altitudinis* strain BR1 from Goa, India. The extracellular protease was stable at 50° C and alkaline pH 9-11. Protease activity was maximum at pH 9 and 50° C confirming it to be a thermostable, alkaline protease. Interestingly this bacterial strain possessed 5 distinct alkaline protease isozymes with approximate molecular mass of 17, 22, 43, 64 and 88 kDa which was clearly revealed by Zymogram. These isozymes are possibly encoded by five different genes. Phenyl methyl sulfonyl fluoride (PMSF) significantly inhibited protease suggesting it to be a serine protease. Interestingly protease production remained unaltered in presence of EDTA-Na_2 and β-mercaptoethanol. Significant morphological change as cell size reduction and transformation of rod shaped cells to oval cells at 50° C without any adverse effect on protease activity may prove a protective mechanism to temperature stress. These results clearly demonstrated stability and activity of these serine protease isozymes at high temperature and alkalinity which is advantageous for various industrial applications.

Keywords: Estuarine, isozymes, serine protease, protease inhibitor

Introduction

Microorganisms including bacteria are most common source of novel biocatalysts viz. protease, esterase, lipase, amylase, and chitinase due to their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation[1]. Proteases are enzymes which hydrolyse proteins into small peptides or free amino acids and represent one of the largest groups of industrial enzymes accounting for 60% of the total worldwide sale of enzymes[2]. Proteases are extensively used in various industries viz. detergent, food, leather, pharmaceutical, silk, leather, and for recovery of silver from used X-ray films[3-5]. The industrial demand for highly active novel protease enzymes with high specificity, biochemical diversity and stability with reference to pH, temperature, surfactants and metal ions have always stimulated the search for novel proteases and their sources.

Proteases have been classified according to their optimum pH as acidic, neutral and alkaline proteases. Alkaline proteases are enzymes that show optimal protease activity at alkaline pH range: 9-11. It is interesting to note that majority of commercially important proteases work at neutral and alkaline pH and are produced by microorganisms belonging to the genus *Bacillus*[6]. Under environmental stress viz. temperature, alkalinity and salinity microorganisms tend to adopt a variety of protective mechanisms for their survival[7, 8]. The mangrove and estuarine ecosystems also harbor alkaliphilic microorganisms including bacteria which are adapted to the unique environmental conditions and produce several industrially valuable biocatalysts viz. proteases, chitinases, cellulases and esterases. Interestingly, microbes including bacteria from estuarine ecosystems are less explored for novel proteases. Therefore it is worth exploring bacterial strains producing novel proteases from these extreme ecosystems. In the present communication, we have partially purified and characterized extracellular, thermostable, serine protease isozymes from a alkaliphilic estuarine bacterial strain from Goa, India.

Materials and Methods

Screening of protease producing bacteria

Surface water samples were collected from different locations of Zuari estuary, Goa, India for isolation of alkaliphilic bacteria producing alkaline protease. Serially

*Author for correspondence
E-mail: Santoshdubey.gu@gmail.com

diluted 0.1 ml water sample in sterile saline was spread plated on the skim milk agar (pH 9) and plates were incubated for 24 h at room temperature. Discrete colonies showing proteolytic activity as clear zone around the colony were picked up and purified through repeated streaking on fresh agar plates. Based on diameter of clearance zone the highest protease yielding strain was selected for further characterization.

Identification of the bacterial strain

Morphological, cultural and biochemical characteristics of the selected alkaliphilic protease producing bacterial strain were studied and recorded as per Bergey's Manual of Systematic Bacteriology[9]. Identity of selected bacterial strain was further confirmed by 16S rDNA sequencing and BLAST search analysis [10, 11].

Growth VS Protease activity of the bacterial strain

Growth and protease activity were measured by spectrophotometric method at different time intervals. The bacterial strain was inoculated in nutrient broth (pH 9) and incubated at room temperature (30°C) at shaker speed of 150 rpm. The culture suspension was withdrawn aseptically every 3 h and growth as well as enzyme activity was monitored spectrophotometrically.

Protease assay

Protease assay with casein as the substrate was performed according to modified Kunitz assay[12]. Enzyme solution with suitable concentration (200 µl) was added to 800 µl of substrate solution (0.5% Casein in 0.1 M Tris-HCl buffer, pH 9) and the mixture was incubated at 50° C. The reaction was terminated by adding 1 ml of 10% trichloroacetic acid (TCA) and incubated at room temperature for 15 min following centrifugation at 12,000 x g for 10 min. Absorbance of the supernatant was measured at 280 nm. Control assay was also done simultaneously by adding TCA prior to addition of enzyme solution. Standard curve of Tyrosine was plotted to estimate protease activity and all assays were done in triplicate. Proteolytic activity was expressed as enzyme units which is amount of enzyme responsible for liberation of 1 µg of tyrosine/ml/minute under the specific conditions of the assay. Protein content of the sample was also measured by Lowry's method using BSA as standard[13].

Partial purification of Protease enzyme

Protease enzyme in the cell free supernatant was concentrated using Ammonium sulphate in the range of 30% - 70% saturation for precipitation and stored overnight at 4° C. The precipitate was collected by centrifugation for 15 min at 4° C and 25,000 x g. The precipitate was re-suspended in 0.5 ml of 0.05 M Sodium carbonate buffer (pH 10) and dialysed against this buffer. The dialysed fraction was used for further characterization.

Characterization of extracellular protease

Effect of pH, temperature and Salinity on growth and protease activity

Effect of pH on growth and protease activity (Units/ml) of the bacterial strain was determined by growing cells in nutrient broth with different pH in the range of 7-12 respectively. Effect of temperature was also determined by growing the strain at different temperatures (i.e. 30° C, 35° C, 45° C and 50° C) under standard assay conditions. Similarly, effect of salinity (% NaCl) was also monitored using different concentrations of NaCl in the range of 0% - 8%.

Effect of inhibitors on protease activity

In order to determine the class of the protease several protease inhibitors were tested to check their action on the protease activity of the test bacterium. Enzyme activity was measured after incubating crude enzyme sample with selected protease inhibitors viz. EDTA-Na_2, β-mercaptoethanol, PMSF, SDS and Dithiothreitol **(**DTT) for 30 min at 50° C using standard assay procedure[12]. The level of protease inhibition was expressed as percentage residual enzyme activity compared to a control set which is devoid of protease inhibitor.

SDS- PAGE and Zymogram analysis

Crude protease sample was analyzed by SDS – PAGE using a 12% (w/v) resolving gel following standard method of Laemmli[14]. In order to check protease activity in the gel zymogram was done. Casein (0.5% w/v) was co-polymerized with the resolving gel and samples were loaded in the gel followed by electrophoresis at constant voltage i.e. 100 V at 4° C. Following electrophoresis the gel was incubated in 2.5% (v/v) Triton X-100 renaturing solution for 30 min at room temperature with gentle agitation. Renaturing solution was decanted and replaced with developing buffer (50 mM Tris, 0.2 mM NaCl and 5 mM $CaCl_2$, pH 8.0) and incubated at 37° C for at least 4 h. The gel was stained with Coomassie brilliant blue G-250 (0.5% w/v) for 30 min and finally destained using destaining solution (methanol, water, acetic acid in the ratio of 45: 45: 1).

Morphological characterization of bacterial strain

Scanning electron microscope (SEM) was used to examine morphological changes at 30° C and 50° C respectively during their growth phase. Cells were grown in nutrient broth at two different temperatures and effect of temperature on cell morphology was analyzed by fixing bacterial cells in 3% glutaraldehyde overnight with 50 mM Potassium phosphate buffer at 4° C. Cells were washed thrice with phosphate buffer and dehydrated in gradually increasing concentration of ethanol i.e. 10, 20, 50, 70, 80, 90, 95 and 100% for 15 min each, air dried and stored in vacuum chamber prior to SEM analysis[15].

Results and Discussion

Identification of the protease producing bacterial strain

The alkaliphilic, facultative aerobic isolate was rod shaped, motile, spore forming which was identified as *Bacillus altitudinis* based on morphological and biochemical characteristics. This isolate also shows significant proteolytic activity at 30° C as well as 50° C (shown as supplementary data). BLAST search analysis of 16S rDNA sequence interestingly revealed that the strain shows 100% DNA sequence homology with *Bacillus altitudinis*. The data of 16 S rDNA sequence of the bacterial strain has already been submitted to GenBank (GenBank accession no JN 712305) and the strain has been designated as BR1.

Growth pattern VS Protease activity of the bacterial strain

Growth pattern VS protease activity of *Bacillus altitudinis* strain BR1 clearly revealed that enzyme activity was highest (89 Units/ml) during late log phase of growth (18 h) indicating that high level of extracellular alkaline protease enzyme is produced by this strain when cells are metabolically more active (data not shown).

Partial purification of protease enzyme

It is interesting to note that maximum protease production was observed at 70% ammonium sulfate saturation which was clearly evident from protease assay experiment (data not shown) using precipitate of different percentage of ammonium sulphate saturation. Zymogram analysis of the dialysed crude enzyme sample clearly demonstrated five distinct bands confirming presence of five protease isozymes. The approximate molecular mass of these isoenzymes were determined as 17, 22, 43, 64 and 88 kDa respectively (data not shown). Similarly five metalloprotease isozymes with molecular mass 30.5, 40, 41, 42.5 and 52 kDa were secreted by

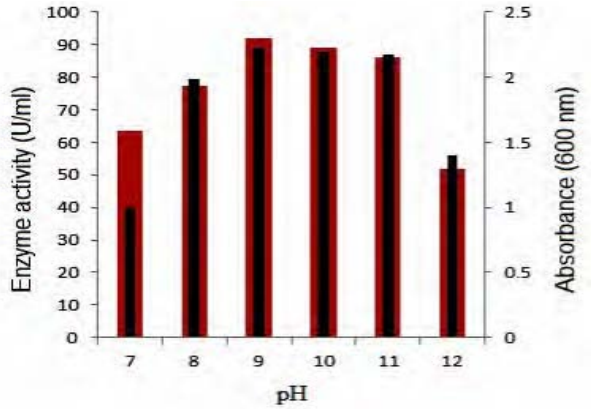


Fig. 1—Effect of pH on the growth and enzyme activity of *Bacillus altitudinis* strain BR1

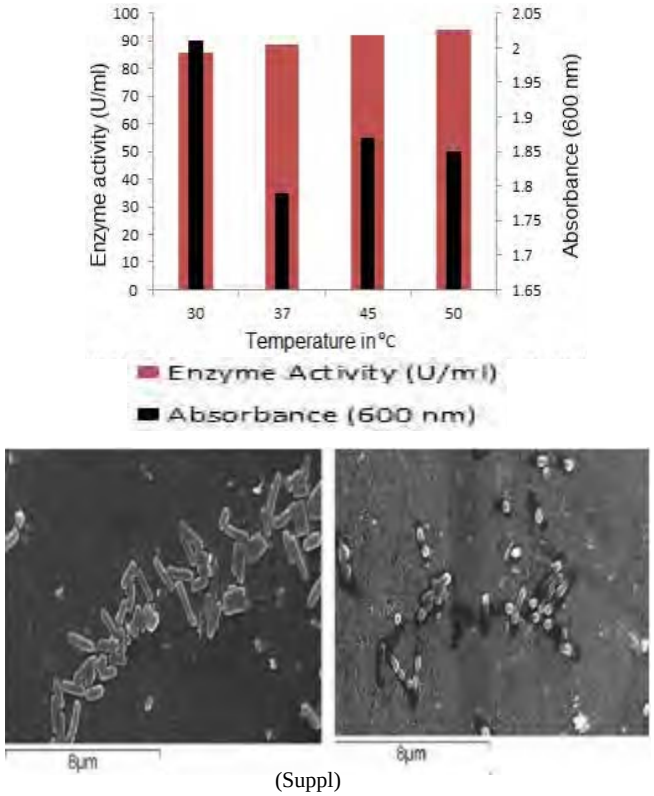
(Suppl)
Fig. 1– *Bacillus altitudinis* strain BR1 showing proteolytic activity on skim milk agar plate at 50°C.

Pseudoalteromonas sp. strain P 96–47 isolated from King George island, Antarctica[16] and two protease isozymes of 18 and 66 kDa have been reported from a *Bacillus* sp[17] whereas *Bacillus* sp. GUS1 produced three alkaline protease isozymes with molecular mass between 30 - 47 kDa[18].

Characterization of extracellular protease

Effect of pH, temperature and salinity on growth and enzyme activity

Although growth and enzyme activity was observed in the pH range 7 - 12, significant increase in enzyme activity was observed within pH range 8 - 11 with pH 9.0 being the optimum pH for growth and enzyme activity (92 Units/ml). This clearly indicates that bacterial strain BR1 is a alkaliphile producing alkaline protease (Fig. 1). Most of the microbial alkaline proteases reported so far have their optimal pH within the range of 7 - 9. Interestingly, when tested at different temperature



(Suppl)

Fig. 2—Effect of temperature on growth and enzyme activity of bacterium *Bacillus altitudinis* strain BR1

ranging from 30-50° C a sharp decline in cell biomass with increased protease enzyme activity was observed with a maximum activity (i.e. 95 Units/ml) at 50° C (Fig. 2), suggesting that the protease is thermostable and can be used as a promising detergent additive at alkaline pH and high temperature. Similar result on thermostable microbial protease production has also been observed within a broad range of temperature from 27° C to 50° C [19]. It is interesting to mention that majority of currently available detergent enzymes work optimally at alkaline pH and are thermostable in nature. Majority of commercially available subtilisin-type proteases are also active at pH and temperature ranging between 8.0 – 12.0 and 50° – 60° C respectively[20,21]. Protease activity as well as growth of the test bacterium was maximum in presence of 2% NaCl as the enzyme activity was recorded 86 Units/ml (Fig. 3). The growth and enzyme production was significantly repressed above 2% NaCl. *Bacillus altitudinis* strain BR1 appeared to be halotolerant as protease activity and growth was seen with and without NaCl in the growth medium. Halotolerance is an added advantage to the alkaline protease enzymes which enhances its potential in industrial processes where the concentration of salt varies[22].

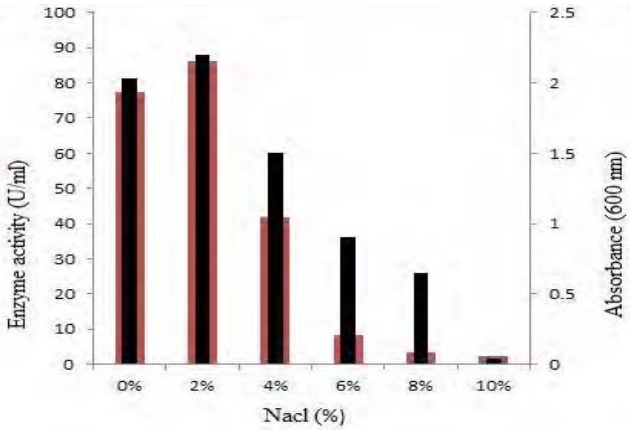


Fig. 3—Effect of NaCl on growth and enzyme activity of bacterium *Bacillus altitudinis* strain BR1

Table 1—Impact of protease inhibitors on residual protease activity (%) of *Bacillus altitudinis* strain BR1

Protease inhibitor (1 mM)	Residual activity (%)
Control (No inhibitor)	100
PMSF	41
SDS	60
DTT	89
EDTA-Na_2	82
β- Mercaptoethanol	50

Effect of inhibitors on protease activity

Among various protease inhibitors, 1 mM PMSF significantly inhibited protease activity as percent residual activity was only 40% as compared to control, suggesting it to be a serine protease (Table 1). PMSF generally causes sulphonation of the serine residues in the active site of the protease which results in the loss of protease activity. PMSF induced complete inhibition of alkaline protease activity has been reported in *Bacillus pumilus* and *Bacillus* sp. KSM - K16[23, 24, 25]. Whereas residual enzyme activity was 82% respectively in presence of chelating agent EDTA-Na_2, which is advantageous as it can be used as additives in the detergent industry since they contain high amounts of chelating agents which act by functioning as water softeners and aid in removal of stains [26]. Significant residual protease activity (i.e. 60%) was also observed in presence of 1 mM SDS which clearly suggested that it can successfully be used as a detergent additive. Similarly 89% and 50% residual enzyme activity was found in presence of β-mercaptoethanol and DTT respectively (Table 1).

Morphological characterization

Scanning electron microscope analysis (SEM) of the protease producing strain BR1 revealed significant reduction in cell size and rod shaped cells turned oval when cells were exposed to 50° C (shown as supplementary data). Morphological alteration of cells is one of several strategies bacteria adapt to withstand unfavourable environmental conditions[8, 27, 28]. It is also observed that morphological alterations disturb the production of enzymes and secondary metabolites[29]. In our investigation protease activity of the test organism was unaltered at 50° C making it a suitable candidate as detergent additive in high temperature washing cycle.

Conclusions

The alkaliphilic *Bacillus altitudinis* strain BR1 produced five distinct serine protease isozymes with approximate molecular mass of 17, 22, 43, 64 and 88 kDa respectively. The protease was thermostable as enzyme activity was observed at 50° C and worked well at alkaline pH range 9 - 11. It was also stable in presence of chelating agent EDTA-Na_2 and detergent SDS. These unique characteristics of the protease clearly suggest that it may be used as a potential detergent additive in detergent industry and also as a biocatalyst in other industrial processes which are performed at wide range of pH and high temperature.

Acknowledgments

Brenda D'Costa gratefully acknowledges U. G. C., Government of India for financial support as JRF. The authors are also thankful to Dr. M. S. Prasad and Mr. Vijay Khedekar from National Institute of Oceanography, Goa, India for scanning electron microscopy.

References

1 Patel R K, Dodia M S, Joshi R H & Singh S P, Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp., *Process Biochem*, **41** (2006) 2002- 2009.

2 Rao M B, Tanksale A M, Ghatge M S & Deshpande V V, Molecular and biotechnological aspects of microbial proteases, *Microbiol Mol Biol Rev*, **62** (1998) 597- 635.

3 Ainsworth S J, Soap and detergents, *Chem Engg News*, **72** (1994) 34-59.

4 Fujiwara N, Production of thermophilic alkaline protease from *Bacillus* sp.B18, *J Biotechnol*, **30** (1993) 245-256.

5 Outtrup H & C O L Boyee , Microbial proteinases and biotechnology, in *Microbial Enzymes and Biotechnology*, edited by W M Fogarty & C T Kelly (Elsevier Science, New York) 1990, 227-254.

6 Schallmey M, Singh A & Ward O P, Developments in the use of *Bacillus* species for industrial production, *Can J Microbiol*, **50** (2004) 1-17.

7 Shi B & Xia X, Morphological changes of *Pseudomonas pseudoalcaligenes* in response to temperature selection, *Curr Microbiol*, **46** (2003) 120- 123.

8 Naik M M & Dubey S K, Lead-enhanced siderophore production and alteration in cell morphology in a Pb- resistant *Pseudomonas aeruginosa* strain 4EA, *Curr Microbiol*, **62** (2011) 409- 414.

9 Seneath P H A, Mair N S, Sharpe E M & Holt J G, *Bergey's Manual of Systematic Bacteriology*, **9th edn** (Baltimore: Williams and Wilkins, New York, USA) 1986.

10 Sambrook J, Fritsch E F & Maniatis T, *Molecular Cloning: a Laboratory Manual*, **2nd edn**, (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) 1989.

11 Altschul S F, Gish W, Miller W, Myers, E W & Lipman D J, Basic local alignment search tool, *J Mol Biol*, **215** (1990) 403-410.

12 Kunitz, Crystalline soyabean trypsin inhibitor II- General properties, *J Gen Physiol*, **30** (1947) 291-310.

13 Lowry O H, Rosebrough N J, Farr A L & Randall R L, Protein measurement with the folin phenol reagent, *J Biol Chem*, **193** (1951) 265-273.

14 Laemmli U K, Cleavage of structural proteins during the assembly of head of bacteriophage T4, *Nature*, **227** (1970) 680-685.

15 Neumann G, Veeranagauda Y, Karegoudar T B, Sahin O, Mausezahl I *et al*, Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size, *Extremophiles*, **9** (2005) 163-168.

16 Vazquez S C, Hernandez E & Cormack W P, Extracellular proteases from the Antarctic marine *Pseudoalteromonas* sp. P96- 47 strain, *Rev Argent Microbiol*, **40** (2008) 63- 71.

17 Mala M & Srividya S, Partial purification and properties of a laundry detergent compatible alkaline protease from a newly isolated *Bacillus* species Y, *Ind J Microbiol*, **50** (2010) 309- 317.

18 Seifzadeh S, Reza H S & Reyhaneh S, Isolation and characterization of thermophilic alkaline proteases resistant to sodium dodecyl sulphate and ethylenediaminetetra acetic acid from *Bacillus* sp. GUS1, *Iranian J Biotechnol*, **6** (2008) 214 – 221.

19 Kasana R C & Yadav S K, Isolation of a psychotropic *Exiguobacterium*sp.SKPB5 (MTCC 7803) and characterization of its alkaline protease, *Curr Microbiol*, **54** (2007) 224–229.

20 Singh J, Batra N & Sobti R C, Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1, *Process Biochem*, **36** (2001) 781- 785.

21 Gupta R, Beg Q K, Khan S & Chauhan B, An overview on fermentation, downstream processing and properties of microbial alkaline proteases, *Appl Microbiol Biotechnol*, **60** (2002) 381- 395.

22 Mohaparta B R, Banerjee U C & Bapuji M, Characterization of a fungal amylase from *Mucor* sp. associated with the marine sponge *Spirastrella* sp., *J Biotechnol*, **60** (1998) 113 - 117.

23 Kobayashi T, Hakamada Y, Adachi S, Hitomi J *et al*, Purification and properties of an alkaline protease from alkalophilic *Bacillus* sp. KSM- K16, *Appl Microbiol Biotechnol*, **43** (1995) 473- 481.

24 Beynon R & Bond J S, (Ed.), *Proteolytic Enzymes-A Practical Approach.* (Oxford University Press, New York, USA) 2001.

25 Feng Y Y, Yang W B, Ong S L, Hu J Y & Ng W J, Fermentation of starch for enhanced alkaline protease production by constructing an alkaliphilic *Bacillus pumilus* strain, *Appl Microbiol Biotechnol,* **57** (2001) 153- 160.

26 Walsh G, *Proteins: Biochemistry and Biotechnology.* (Wiley & sons, Chichester, UK) 2002.

27 Beg Q K, Saxena R K & Gupta R, Kinetic constants determination for an alkaline protease from *Bacillus mojavensis* using response surface methodology, *Biotechnol Bioeng,* **78 (**2002) 289-295.

28 Bihong S & Xuhua X, Changes in growth parameters of *Pseudomonas pseudoalcaligenes* after ten months culturing at increasing temperature, *FEMS Microbiol Ecol,* **45** (2003) 127- 134.

29 Yun T, Yan X & Dong W, Changes in morphology of *Rhizopus chinensis* in submerged fermentation and their effect on production of mycelium – bound lipase, *Bioprocess Biosyst Eng,* **32 (**2009) 397- 405.