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Short communication

An extremely thermotolerant, alkaliphilic subtilisin-like protease from hyperthermophilic *Bacillus* sp. MLA64

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ABSTRACT

The current work is a report on a new extremely thermostable protease from newly isolated hyperthermophilic *Bacillus* sp. MLA64. The protease was purified with a 16.5-fold increase in specific activity and 93.5% recovery. The molecular weight of the enzyme was estimated to be 24 kDa. The enzyme was extremely stable and quite active over the temperature range from 40 to 100 °C with an optimal temperature at 95 °C as well as in a wide range of pH from 6.0 to 12.5, with a superlative at pH 9.5. The enzyme activity was not enhanced in the presence of $CaCl_2$, indicating that the enzyme is calcium-independent. The enzyme showed high stability towards non-ionic surfactants and anionic surfactant SDS. In addition, the enzyme was relatively stable with respect to oxidizing agents. The protease was inhibited by PMSF but not by TPCK and TLCK, suggesting that it can be a subtilisin-like protease. Moreover, the N-terminal sequencing of the first 20 amino acids of the purified protease showed less homology with other well-known bacterial peptidases. In conclusion, the enzyme can be considered as a novel protease which might be a candidate for industrial processes.

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1. Introduction

Proteases not only play an important role in the cellular metabolic processes, but have also gained considerable attention in the industrial communities [1,2]. Constituting more than 65% of the world market, they are becoming major industrial enzymes. Some big industries such as food, pharmaceutical, leather and textile are major consumers of these enzymes [3,4]. The new applications will be continued to grow in the present and future as the demands for stable biocatalysts capable of withstanding the harsh conditions of the operation increase [1,2,5,6]. The purified proteases from microbial sources constitute approximately 45% of the total worldwide production of enzymes [2,7]. Although the prominent producers of proteases are the microorganisms of the genera *Pyrococcus*, *Thermococcus* and *Staphylothermus* and also hyperthermophilic archaeum *Desulfurococcus* strains producing extremely thermostable serine proteases and metalloproteases [8], the genus *Bacillus* is in the focus of attention in biotechnology due to relative ease of isolation from diverse sources, such as soil, alkaline waters, and the deep sea, and the ability to grow on proteinaceous substrates as the source of carbon and/or nitrogen[9].

So far, a few thermophilic protease producers of *Bacillus* sp. have been isolated. *Bacillus stearothermophilus* was the first isolate [10], which was stable at 60 °C. However, other strains of this microorganism produce some different thermo-alkali-stable proteases as well as extracellular proteases, which are optimally active from 75 to 85 °C [11]. Additionally, newly isolated *Bacillus licheniformis* LBBL-11 has produced a thermostable protease that shows an optimum activity at 60 °C [12,13]. In addition to the thermo-alkaline stability, minimum loss of enzyme functions in the presence of chelating agents (such as EDTA) is another invaluable property of industrial enzymes, particularly proteases. This characteristic is of great advantage when an enzyme is used as a detergent additive due to the presence of such agents in the formulation of most detergents. Water softening and enhancing the removal of stain spots are the most important roles of chelating agents in detergents [3,14].

In the present study, we describe the purification and characterization of a new extracellular extremely thermostable protease with an alkaline pH profile produced by newly isolated *Bacillus* sp. MLA64 from a hot mineral spring in Iran that has never been reported.

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2. Materials and methods

2.1. Materials

All growth mediums and chemicals purchased from Sigma (Louis, MO, USA) were of analytical grade. Molecular biology reagents were bought from various international vendors. All chromatography reagents and corresponding equipment were purchased from Pharmacia (Pfizer and Pharmacia, Sweden).

2.2. Isolation of microorganism

Water sample from Dig Rostam hot spring was collected three times in a three-day interval in volume of 100 ml. Dig Rostam hot spring is located in south east of Iran (latitude 31°28′19″N, Longitude 56°49′22″E). The samples were frozen immediately by immersing on liquid nitrogen after adding 15% glycerol until further studies. The protease producing bacterium was isolated by screening on skim milk agar plates containing (g/l): peptone 7, yeast extract 4, bacteriological agar 20 and skim milk 300 ml[15]. Collected samples were plated onto skim-milk agar plates, and incubated for 48 h at 55 °C. Clear area around colonies on the plate gave an indication of protease producing strains.

2.3. Molecular identification

The isolated strain was identified as *Bacillus* strain based on the phenotypic characteristics and phylogenetic analysis of the 16S rDNA sequence. The 16S rDNA gene of the isolate was amplified using the upstream primer P1: 5′-CTCTCAAACTAGGACCGA-3′ and the downstream primer P2: 5′-ATGGCGAATCCGTAATG-3′ which generate a DNA fragment of approximately 1.3 kb. Amplification cycles were conducted by Techne FT Gene 2D thermocycler under the following conditions: denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 59 °C for 1 min, 72 °C for 90 s and final extension at 72 °C for 15 min [16]. The DNA nucleotide sequence was determined according to dideoxy chain-termination method. The PCR product was sequenced in both directions three times to confirm the fidelity of the sequence. Sequence comparison with the databases was performed using BLAST program through the NCBI website [17]. The highest hit was considered as the genus of the isolate.

2.4. Phylogenetic analysis

The evolutionary distance of the isolated bacterium was calculated with regard to other well-known industrial strains, based on 16s rRNA gene sequencing data. The analysis involved 10 nucleotide sequences of 10 different strains. All positions containing gaps and missing data were eliminated. The evolutionary trees were inferred by using the maximum likelihood method based on the neighbor-joining algorithm and data specific model. Analyses were conducted using CLC Main Workbench program ver. 6.6.1 (CLC bio A/S, Denmark). The following strains were used for constructing the trees: *Bacillus amyloliquefaciens* (NC_009725), *Bacillus halodurans* (NC_002570), *B. licheniformis* (NC_006322), *Bacillus subtilis* (NC_000964), *Brevibacillus brevis* (NR_041524), *Brevibacillus thermoruber* (NR_026514), *Geobacillus stearothermophilus* (NR_040794), *Bacillus* sp. Ferdowsicous (*GQ365212), *B. subtilis* DR8806 (*JF309277), *Bacillus* sp. DR90 (*JN713925) and *Bacillus* sp. MLA64 (FJ763844). Our previously isolated bacteria are marked with an asterisk [16,18].

2.5. Culture and growth conditions

Production of protease from the isolated strain was carried out in a medium containing (g/l): hulled grain of wheat 40; yeast extract 8; $CaCl_2$ 2; $MgCl_2$ 0.5; K_2HPO_4 0.1 and KH_2PO_4 0.1; KCl 5; pH 9.5 [19]. The media were autoclaved at 120 °C for 20 min. Cultivations were accomplished on a rotator shaker (220 rpm) for 35 h at 85 °C, in 750 ml Erlenmeyer flasks with a working volume of 200 ml. Subsequently, the cultures were centrifuged for 10 min at 15,000 × *g*, and the cell-free supernatants were used for the estimation of enzyme activity.

2.6. Enzyme purification

2.6.1. PEG (polyethylene glycol) precipitation

The culture supernatant containing the extracellular enzyme was first subjected to PEG precipitation [20]. 750 ml of 50% (w/v) PEG (Sigma–Aldrich, USA; M_r 950–1050) was added to 500 ml of protein solution in a beaker with continuous gentle stirring. After the addition of PEG, stirring continued for a further 45 min, and then the suspension was centrifuged at 10,000 × *g* for 15 min at 4 °C. The pellet obtained in this step was air-dried and suspended in a minimal volume of buffer A (100 mM Tris–HCl, pH 8.0). To remove the residual amounts of PEG from the suspension, an ammonium sulfate precipitation step was accomplished. The precipitated material was dissolved in buffer B (25 mM Tris–HCl, pH 8.5) and dialyzed at 4 °C against the same buffer for 24 h, and then the solution was loaded on G-100 chromatography column.

2.6.2. Sephadex G-100 gel filtration

The dialyzed solution was subjected to gel filtration on a Sephadex G-100 column (2.6 cm × 90 cm) equilibrated with the buffer B containing 0.5% (v/v) Triton X-100. Fractions of 3 ml were collected at a flow rate of 20 ml/h with the same buffer. Protein contents (Absorbance at 280 nm) and protease activity were determined. Fractions showing the highest protease activities were pooled.

2.6.3. Q-Sepharose ion exchange chromatography (IEC)

The second round of chromatography was carried out by applying the active fractions from the previous step to a CM-Sepharose column (2 cm × 25 cm) equilibrated with 25 mM Tris–HCl, pH 8.5. Subsequently, the column was washed with the same buffer and bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–1 M in the equilibration buffer. Fractions (3 ml each) were collected at a flow rate of 50 ml/h and analyzed for protease activity and protein concentration. Active fractions were pooled and then subjected to third round of chromatography.

2.6.4. CM-Cellulose IEC

The third round of chromatography was performed by applying the pooled fractions from the previous step to a CM-Cellulose column equilibrated with 50 mM acetate buffer, pH 5.5. As the prior step, the column was washed with its equilibration buffer, and bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–1 M. Active fractions were pooled and stored for further analysis. All purification steps were conducted at temperatures not exceeding 4 °C for avoiding possible enzyme degradation.

2.6.5. Polyacrylamide gel electrophoresis and zymography

To assess the purity and molecular mass of the enzyme, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method described by Sambrook [21] using a 5% (w/v) stacking gel and a 12% (w/v) separating gel. The molecular weight of the enzyme was inferred using

a standard protein marker composed of bovine serum albumin (66,000 Da), chicken egg ovalbumin (45,000 Da), bovine carbonic anhydrase (29,000 Da), trypsin inhibitor (20,100 Da) and bovine α-lactalbumin (14,200 Da).

Zymography was performed by the method of Garcia-Carreno et al. [22] to visualize the enzyme activity. Following the electrophoresis, the gel was immersed in 100 mM Tris–HCl buffer (pH 8.5) containing 2.5% Triton X-100 at 4 °C, with shaking for 30 min to remove SDS. The gel was washed twice with 100 mM Tris–HCl buffer (pH 8.5) in order to extract residual Triton X-100, and then incubated with 2% (w/v) casein in 100 mM sodium tetraborate/sodium hydroxide buffer (pH 9.5) at 75 °C for 60 min. Finally, Coomassie Brilliant Blue R-250 (CBBR250) was used to stain the gel. Clear halo zone on the dark-blue background indicates the presence of enzyme.

2.6.6. N-terminal amino acid sequencing

N-terminal sequencing of the purified protease was performed by transferring the enzyme from SDS-PAGE to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was slightly stained with CBBR250 and the band corresponding to the enzyme was excised. Subsequently, the N-terminal amino acid sequence was determined by the Edman degradation method using an ABI Procise 492 protein sequencer (Applied Biosystems). The first 20 residues of the N-terminal sequence were determined.

2.6.7. Protein concentration

Bradford method was used to measure the protein concentration using bovine serum albumin as standard, in the final step and during the purification procedure [23].

2.7. Assay of protease activity

The enzyme activity was measured by the method of Kembhavi et al. [24] using casein as a substrate. A 0.25 ml aliquot of the purified enzyme was mixed with 0.75 ml of 100 mM sodium tetraborate/sodium hydroxide buffer (pH 9.5) containing 1% casein, and incubated for 15 min at 75 °C. The reaction was stopped by adding 0.5 ml of trichloroacetic acid (20%, w/v). The mixture was held at room temperature for 15 min, and then centrifuged at 15,000 × *g* for 15 min again, to remove the debris. The absorbance was measured at 280 nm. This procedure considered as "standard assay conditions". A standard curve was plotted using solutions of 0–50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to release 1 μg tyrosine per minute under experimental conditions.

2.8. Biochemical properties of the purified protease

2.8.1. Effect of pH on the enzyme activity and stability

To determine the optimal pH of the enzyme, it was incubated in a wide range of pH from 5 to 13.5 under standard assay conditions except that pH was changed in each step. Subsequently, the activity was measured as described before. The pH stability of the enzyme was studied by incubating the purified protease in buffers of various pH for 2 h, and then the residual proteolytic activity was determined under standard assay conditions. The buffer systems were as follows: 50 mM sodium acetate buffer, pH 5–6.0; 50 mM potassium phosphate buffer, pH 6.5–8; 50 mM Tris–HCl buffer, pH 8.5–9; 50 mM sodium tetraborate/sodium hydroxide pH 9.5–10.5; 50 mM glycine–NaOH buffer, pH 11–13.5.

2.8.2. Effect of temperature on the enzyme activity and stability

The effect of temperature on the protease activity was studied from 30 to 120 °C. The enzyme was first incubated at each temperature for 15 min at the optimum enzyme pH, and then the measurement was performed. The thermal inactivation of the enzyme was also tested by incubating the purified protease at temperatures up to 120 °C in the absence and presence of 2 mM $CaCl_2$. Aliquots were withdrawn at the 30-min intervals during 4 h, and the remaining activity was measured under the standard enzyme assay conditions.

2.8.3. Effect of metal ions, enzyme inhibitors and laundry additives

The effects of inhibitors on the enzyme activity were investigated using phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, dithio-bis-nitrobenzoic acid (DTNB), tosyl phenylalanyl chloromethyl ketone (TPCK) and tosyl lysinechloromethyl ketone (TLCK). TPCK and TLCK were used based on manufacture guidelines. The enzyme was incubated with inhibitors in the presence of 2% casein as a substrate for 30 min. Subsequently, the enzyme activity was determined by the method previously described. The activity of the enzyme in the absence of inhibitors was taken as 100%. The effect of various metal ions on the enzyme activity was investigated by the addition of the monovalent (Na^+ or K^+) and divalent metal ions (Mn^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}), all in 10 mM, to the reaction mixture. The measured activity in the absence of metallic ions was considered as 100%.

The effects of common laundry additives such as surfactants (SDS, Triton X-100 and Tween 80) and oxidizing agents (H_2O_2 and sodium perborate "$NaBO_3$") on the enzyme activity were studied at various concentrations. The purified protease was incubated with each agent at optimum pH and temperature conditions for 2 h, and the remaining activity was determined. The enzyme activity in the absence of any additive was assumed as 100%.

2.8.4. Substrate specificity of the enzyme

To check the substrate specificity of the enzyme, a solution of casein, bovine serum albumin, gelatin, ovalbumin and collagen, all in 2% (w/v), were incubated with the purified protease at the optimal reaction conditions for 15 min. The reaction was terminated with 0.5 ml of 10% (w/v) trichloroacetic followed by holding on ice for another 10 min and subsequently centrifuged at 15,000 × *g* for 15 min. Glycine was used as standard of the assay.

2.9. Nucleotide sequence accession number

The 16S rRNA gene sequence of *Bacillus* sp. strain MLA64 has been submitted to GenBank database and assigned an accession number (GenBank ID: FJ763844).

3. Results and discussion

3.1. Identification of the microorganism and phylogenetic comparison

The spring water sample properties were as follows: pH 9.7 ± 0.15; temperature 88 ± 1.5 °C; salt concentration (g/l): NaCl 12, sulfate 4, potassium 8, magnesium 0.5, calcium 2 and phosphate 1.5. 11 colonies were purified through repeated streaking on fresh agar plates. Finally, one colony was confirmed as a protease producer. The newly isolated strain was Gram-positive, spore forming, aerobic, rod-shaped, and also thermophilic with a growth temperature in the range of 60–90 °C. It could grow on the agar plate at pH 9.5.

To make molecular identification of the isolate, the nucleotide sequence of 16s rRNA gene was analyzed with the GenBank database using "BLAST" program. The result indicates that the microorganism is a species of *Bacillus* genus. Although, the pairwise comparison for already mentioned species demonstrated the maximum similarity of this isolate toward *B. licheniformis* (data not

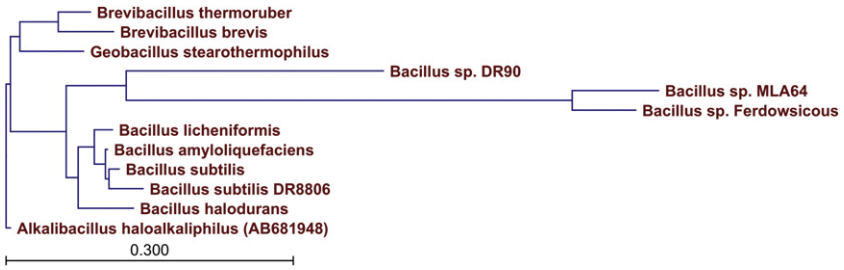

Fig. 1. Maximum likelihood phylogenetic relationship of *Bacillus* sp. MLA64 and related *Bacillus* species based on their 16S rDNA sequences. For further details about tree drawing parameters, please refer to Section 2. All sequences were retrieved from GenBank. The sequence of *Alkalibacillus haloalkaliphilus* was used as the out-group. Asterisk indicates our previously isolated bacteria. The scale bar represents 30% evolutionary difference. Analyses were carried out with CLC Main Workbench 6.6.1.

shown), the phylogenetic tree revealed no close relation between these strains and also other strains, such as *B. brevis*, *B. stearothermophilus*, *B. amyloliquefaciens, B. halodurans, B. subtilis* and *B. thermoruber* (Fig. 1), which are the common protease-producing candidates for industrial applications. However, the results demonstrated that the isolated strain is closely related to *Bacillus* sp. Ferdowsicous and *Bacillus* sp. DR90 previously identified by the same authors [16]. Surprisingly, the current isolate is far from *B. subtilis* DR8806 formerly isolated from Dig Rostam spring [18]. In conclusion, it was identified as *Bacillus* sp. MLA64 according to the characteristics of this strain, the 16S rRNA sequence and phylogenetic data.

3.2. Protease purification

A protease from *Bacillus* sp. MLA64 was purified by a four-step procedure as described in Section 2. In the first step, the culture supernatant was precipitated with PEG. The 50% (w/v) PEG precipitate showed specific activity of 70 U/mg $_{protein}$. In addition, no measurable activity was detected in the supernatant. The precipitate, which gave the significant specific activity, was then subjected to gel filtration on a Sephadex G-100 column. This procedure yielded four major peaks, among them, one peak related to protease activity. Fractions corresponding to the enzyme activity were pooled, and then loaded on a Q-Sepharose column equilibrated with 25 mM Tris–HCl, pH 8.5. Bound proteins were eluted with a linear gradient of NaCl from 0 to 1 M. The enzyme containing fractions were subjected to the third round of chromatography on a CM-Cellulose column in order to obtain more pure enzyme. The final chromatogram showed that the enzyme activity appeared as a clean single peak (figure not shown). At the end of purification, the enzyme was purified 16.5-fold with a recovery of 93.5% and a specific activity of 1000 U/mg$_{protein}$. The results of the purification procedures are summarized in Table 1.

The purified enzyme appeared as a single band on SDS-PAGE and its molecular weight was estimated to be 24 kDa (Fig. 2a), comparable with that determined by gel filtration. As shown in Fig. 2b, the protease activity was also observed as a unique clear band of casein hydrolysis appeared in non-denaturing gel electrophoresis. Almost all bacterial proteases have a molecular weight varying between 15 and 45 kDa [25,26]. The molecular weight of our protease is lower than those of Subtilisin Carlsberg (27.3 kDa), Subtilisin BPN0

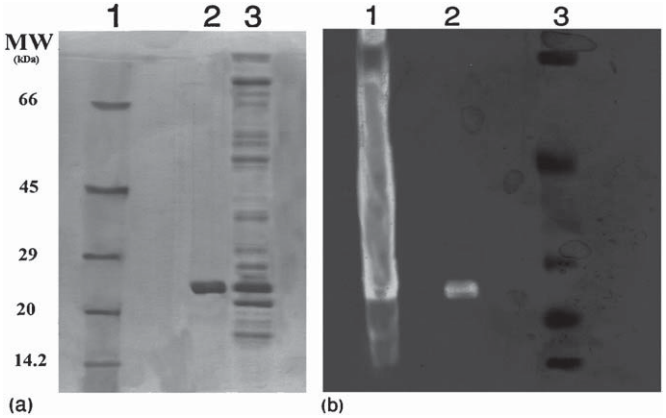

Fig. 2. (a) SDS-PAGE of the purified protease: lane 1: molecular size marker; lane 2: purified enzyme; lane 3: the crude enzyme extract of *Bacillus* sp.MLA64. (b) Zymography of samples, crude enzyme extract (lane 1), purified enzyme (lane 2) and molecular size marker (lane 3). The protein concentration and volume loaded in each well were as follows: for SDS-PAGE: lane 1: 10 μl [4 mg/ml]; lane 2: 5 μl [29 mg/ml]; lane 3: 5 μl [a dilution of 50 mg/ml from the crude extract]. For zymogram: lane 1:5 μl [a dilution of 50 mg/ml from the crude extract]; lane 2:5 μl [29 mg/ml]; lane 3: 15 μl [4 mg/ml].

(27.5 kDa) [27], alkaline protease from *B. mojavensis* (30 kDa) [28] and metalloprotease from *Pseudomonas* sp. DR89 (74 kDa) [29], but it is higher than those of the alkaline proteases from *B. subtilis* PE-11 (15 kDa) [30] and *Kurthia spiroforme* sp. Nov (8 kDa) [31].

3.3. N-terminal sequencing data

The first 20 N-terminal amino acids of the enzyme were determined to be MVKKRSTW LAMITGFMLLTF. The sequence alignment analysis showed not more than 18% similarity to other subtilisin- or bacillolysin-like proteases (Fig. 3a). Subtilisin from *Pyrococcus kodakaraensis*, EC 3.4.21.62 has the highest similarity to our enzyme (18%). Surprisingly, based on the phylogenetic tree (Fig. 3b), the enzyme had a greater similarity to subtilisin from *B. licheniformis* DSM13. These results indicate that the purified protease can be considered as a new enzyme in proteolytic enzyme family.

Table 1
Summary of the purification steps.

Purification steps	Crude extract	PEG precipitation	Sephadex G-100	Q-Sepharose	CM-Cellulose
Total protein (mg)	511.0	449.4	71.9	43.9	29.1
Total activity (Unit)	31,000	31,000	30,240	29,680	29,120
Fold	1.0	1.1	6.9	11.1	16.5
Recovery (%)	100.0	100.0	97.5	95.7	93.5
Specific activity	60.6	69.0	420.6	676.0	1000.7

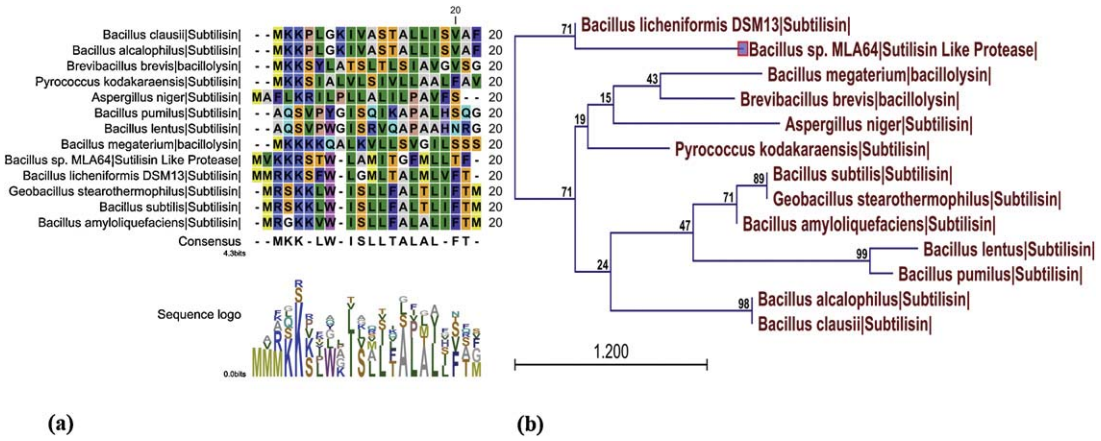

Fig. 3. (a) Alignment of N-terminal sequence of the purified protease with other peptidases. (b) Phylogenetic tree based on the first 20 N-terminal amino acids. The tree was constructed using the neighbor-joining algorithm and its confidence assessed by 100 bootstrap replicates. All analyses were conducted using CLC Main Workbench Ver. 6.6.1.

3.4. Effect of pH on the activity and stability

The pH profile of the enzyme was determined using different buffers of varying pH values. The enzyme was highly active in a wide range of pH from 6.0 to 12.5(Fig. 4a). The relative activities at pH 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 were about 77, 84, 93, 88, 80 and 75%, respectively. The optimum pH for this protease was found to be around 9.5 which is greater than subtilisin from *G. stearothermophilus* and *Bacillus intermedius*, pH 9, *Alkalimonas collagenimarina*, pH 8.5, *B. licheniformis*, pH 8, and *B. subtilis*, pH 7.5 [32–35], but lower than those reported for *Bacillus* sp. DJ-4, pH 10, *Rhizopus microspores*, pH 10.5, and *Bacillus clausii* GMBAE42, pH 11.3 [36–38]. Other subtilisin-like proteases have a relatively similar pH range, for example: *B. licheniformis*, 3.5-11, *B. subtilis*, 4-8, *Bacillus* sp. DJ-4, 4-11, *R. microspores*, 5.5–12, and *Thermococcus kodakaraensis*, 7–11.5 [26,27,34,36,37]; however none of them have a significant activity over their range. Amazingly, the protease stability was also over a broad range of pH from 6.0 to 12.5. The enzyme maintained 100% of the initial activity even after 2-h incubation (Fig. 4b). Until now, just subtilisin-like serine protease from *T. kodakaraensis* had a wider range of pH stability, from 2 to 12 than our enzyme [26]. However, it is not fully stable over the whole of this range. Other subtilisin-like proteases from *Bacillus* species are stable in a narrow range of pH. For example, *B. subtilis*, *B.intermedius* and *B.clausii* are stable in pH from 5.5 to 9, 6.3 to 9.5 and 9 to 12, respectively [32,34,38].

3.5. Temperature effect on the activity and stability

The protease activity was evaluated at various temperatures. The enzyme was active in a broad range of temperature between 40 and 105 °C with an optimum at 95 °C that is the highest among proteolytic enzymes except subtilisin-like protease purified from hyperthermophilic archaeon *T. kodakaraensis* [26] (Fig. 5a).The relative activities at 75 °C and 85 °C were about 79 and 95%, respectively. Optimum temperatures at 70 °C, 67 °C and 60 °C which belongs to *B. clausii*, *B. licheniformis* and *B. stearothermophilus*, respectively, are the closest temperatures to our record [11,27,38].

The thermal stability study of the purified protease showed that the enzyme was highly stable at temperatures between 30 and 100 °C even after 240 min of incubation, but rapidly lost its activity at temperatures over than 110 °C. The enzyme remained fully active after 1-h incubation at 30–90 °C, suggesting that this enzyme could be used under mild/harsh heating conditions (Fig. 5b). Thus far, no record was found to introduce a wild-type of proteolytic enzyme with a similar or at least close range of thermal stability to our enzyme. A detailed description of recently isolated proteases [39–44] in comparison with our enzyme is presented in Table 2.

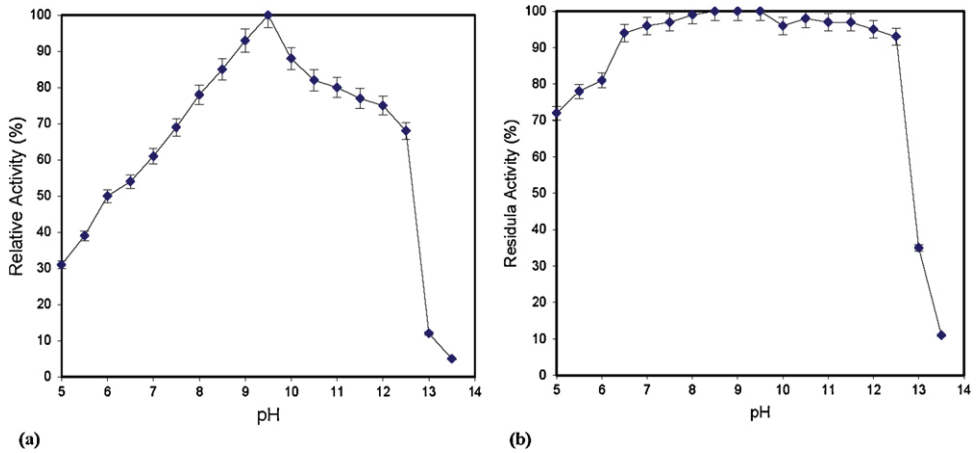

Fig. 4. Effect of pH on the activity (a) and stability (b) of the purified protease. The protease activity was assessed in the pH range of 2.0–13.5 using buffers of different pH values. The maximum activity obtained at pH 9.5 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 2 h, and then the residual activity was measured at pH 9.5. The activity of the enzyme before incubation was taken as 100%. The buffer solutions used for pH activity and stability are presented in Section 2. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

Table 2
Comparison of the some properties of isolated enzyme with previously published proteases.

Enzyme	Optimal pH	Optimal temp. (°C)	Half-life time (min)	M_r (kDa)	Ref.
Subtilisin like protease	9.5	95	240/100 °C	24	This work
SAPB	10.6	65	220/50 °C	34.4	[39]
BPP-A	8–11	50	5/50 °C	33	[40]
DHAP	10	55	30/50 °C	32	[41]
Subtilisin E	7	50	5/60 °C	28	[42]
Subtilisin Carlsberg	10	60	2.5/60 °C	27.5	[43]
Subtilisin Novo	9	48	90/50 °C	31	[44]

Table 3
Effect of inhibitors on the protease activity.

Inhibitor	Control (mM)	EDTA (mM)	DTNB (mM)	PMSF (mM)	2-ME (mM)	TPCK (mM)	TLCK (mM)
Concentration	0	2	2	2	2	2	2
Residual activity (%)	100	100	100	25	100	100	100

The enzyme activity measured in the absence of any inhibitor was taken as 100%. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means). 2-ME: β-mercapto-ethanol.

In addition, the incorporating of 2 mM $CaCl_2$ in the protease reaction medium did not significantly enhance the thermostability of the purified protease (data not shown). This clearly indicated that the enzyme is calcium-independent and doubtless takes advantage of other strategies to retain its native structure in non-permissive temperatures.

Finally, the half-lives of the enzyme at 90, 100,110 and 120 °C were estimated to be 190, 150, 25 and 10 min, respectively (Fig. 5b). This is higher than those of other proteolytic enzymes which retain lower amount of their initial activity even in a shorter period of time and also lower temperatures.

3.6. Effect of various inhibitors and metal ions on the protease activity

To determine the nature of the purified protease, the enzyme activity was measured in the presence of different enzyme inhibitors (Table 3). Thiol reagent, DTNB, and the chelating agent, EDTA, had practically no influence on the enzyme activity indicating that the enzyme was not neither a cysteine-like protease nor a metalloproteinases. In the other side, the enzyme was strongly inhibited by serine protease inhibitor, PMSF, it revealed that the enzyme is a serine-protease, but TLCK and TPCK (irreversible inhibitors of trypsin- and chymotrypsin-like protease) had no effect on the activity suggesting that the enzyme should be a subtilisin-like protease [45]. The full activity of the protease in the presence of EDTA is highly valuable for application as a detergent additive because chelating agents are integral components for most of the detergents. Stain removal and water softening by chelating metal ions are the common roles of such agents in the detergent formulation.

Table 4
Effect of different metal ions on the purified protease activity.

Metal ion	Concentration (mM)	Relative activity (%)
Control	0	100
Mn^{2+}	10	33.5
Ca^{2+}	10	102.1
Zn^{2+}	10	60.8
Mg^{2+}	10	125
Cu^{2+}	10	19.6
Na^{+}	10	100
K^{+}	10	100

Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

The effects of different metal ions, at a concentration of 10 mM, on the activity of the protease were also studied at 95 °C and pH

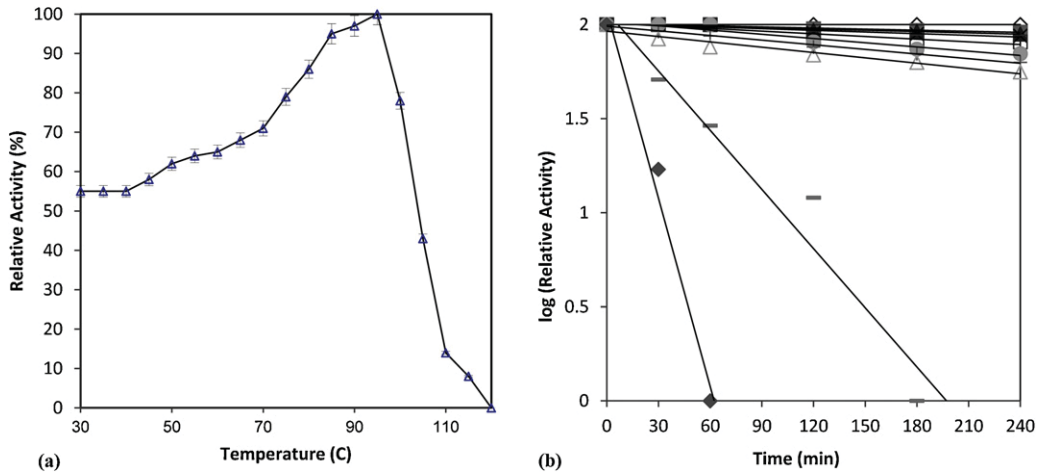

Fig. 5. Effect of temperature on the activity (a) and thermal inactivation (b) of the purified protease. The temperature profile was determined by assaying protease activity at temperatures between 30 and 120 °C. The activity of the enzyme at 95 °C was taken as 100%. The temperature stability was also determined by incubating the purified enzyme at temperatures from 30 to 120 °C for 240 min. The residual enzyme activity was measured for each 30 min under the standard assay conditions. The original activity at the beginning of the test was taken as 100% for each temperature. In (b)<!– legends are as follows: 30 (◊), 40 (■), 50 (▲), 60 (×), 70 (□), 80 (●), 90 (+), 100 (△), 110 (—), 120 (♦). Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

Table 5
Stability of the purified protease in the presence of various surfactants and bleaches.

Agent	Control	H_2O_2		Triton X-100		$NaBO_3$		SDS			Tween 80	
Concentration (%)	0	1	4	1	10	0.5	1	1	5	10	1	10
Residual activity (%)	100	75	40.8	91	53	57	31	95	69.4	36	100	86

The activity in the absence of any additive was taken as 100%. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means). $NaBO_3$: Sodium perborate.

Table 6
Substrate specificity of the enzyme.

Substrate	μmol as glycine h^{-1} μg^{-1}
Ovalbumin	0.18 ± 0.01
Gelatin	0.31 ± 0.018
Bovine serum albumin	0.24 ± 0.031
Collagen (purified protease)	0.14 ± 0.008
Collagen (collagenase)	0.09 ± 0.026
Casein	0.63 ± 0.031

Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means).

9.5 by the addition of the respective cation to the reaction mixture (Table 4). The addition of $MgSO_4$ increased the protease activity by 125% of the control, but $CuSO_4$, $MnSO_4$ and $ZnSO_4$decreased the enzyme activity up to 19.6, 33.5 and 60.8%, respectively. The effect of $CaCl_2$ on the enzyme activity was also studied in already mentioned conditions, but no considerable change was obtained in the presence and absence of $CaCl_2$.

3.7. Effect of surfactants and oxidizing agents on the protease stability

Proteases have a long history of applications in laundry industry. Bleaches, surfactants and oxidizing agents are the most common additives of laundry detergents formulation. Therefore, this is a distinguishing characteristic for a protease to be compatible with such compounds [46]. As shown in Table 5, non-ionic surfactants such as Triton X-100 and Tween 80 had ignorable effects on the enzyme stability. Furthermore, the purified protease was pretty stable in the presence of the strong anionic surfactant (SDS), retaining approximately 95, 69.4 and 36% of its initial activity after 1-h incubation at 95 °C in the presence of 1, 5 and 10% SDS, respectively. The stability of the protease against 5% SDS was lower than that reported for subtilisin-like protease from *T. kodakaraensis*, although the effects of higher concentrations were not determined. Other proteases had a lower stability than the enzyme reported here. The stability towards SDS is a remarkable characteristic because SDS-tolerant enzymes are not widely available [46,47]. In addition, the enzyme was little influenced by oxidizing agent as retained 75 and 40.8% of its initial activity after 1 h of incubation at 95 °C in the presence of 1 and 4% H_2O_2, respectively. Sodium perborate ($NaBO_3$) also had the same effect by 68 and 42% activity corresponding to 0.1 and 1% concentrations.

3.8. Substrate specificity

Among all investigated substrates, the enzyme was extensively active toward casein and gelatin as well as considerably acting on bovine serum albumin, ovalbumin, and collagen (Table 6). Surprisingly, the enzyme had a higher activity toward collagen than the collagenase from *Clostridium histolyticum.*

4. Conclusion

The present work involves the isolation and characterization of a novel extremely thermostable subtilisin-like protease with natural and alkaline pH profile from *Bacillus* sp. MLA64. The enzyme was purified to homogeneity with a 16.5 fold increase in specific activity and 93% recovery. One of the prominent features of the purified enzyme is the high activity and stability in a broad range of pH. The enzyme retained 100% of its original activity at pH between 6.0 and 12.5 after 2-h incubation at 95 °C. Another outstanding characteristic of the purified enzyme is the optimum temperature at 95 °C.This optimum temperature has not been yet reported for any kind of bacterial proteinase except subtilisin-like protease from *T. kodakaraensis* as well as Boilysin, an engineered, highly thermostable neutral protease of *B. stearothermophilus* [48]. Furthermore, the enzyme remained fully active over a range of temperature from 30 to 90 °C which is unique among proteolytic enzymes. Moreover, the enzyme had a calcium-independent mechanism for the thermostability. This is an uncommon feature within the serine protease family members in which the contribution of calcium ions towards their stability against thermal denaturation is of critical importance [49]. In spite of some benefits, calcium dependency possess a limitation in the use of enzymes in detergents containing chelating agents to remove magnesium and calcium, the water hardness ions [50].

Considering its high activity and stability in a broad range of pH, tolerance and stability in the presence of anionic surfactant, including SDS, stability in the presence of various commercial laundry detergents, and last but not least, stability and activity in extreme temperatures without calcium dependency, the purified protease may find potential application in laundry detergents and other industrial processes that take advantage of such enzymes at non-permissive conditions. Further studies regarding this protease can be performed concerning the medium optimization for overproduction at ambient temperatures. In addition, identifying the gene coding this protease can be useful to elucidate the 3D structure as well as the precise nature of the enzyme. Furthermore, *Bacillus* sp.MLA64 can certainly produce other enzymes which might have common properties with the purified protease. Therefore, it is invaluable to explore the other beneficial aspects of this microorganism for the subsequent work.

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