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Isolation, Purification and Characterization of a Surfactants-, Laundry Detergents- and Organic Solvents-Resistant Alkaline Protease from *Bacillus* sp. HR-08

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Abstract *Bacillus* sp. HR-08 screened from soil samples of Iran, is capable of producing proteolytic enzymes. 16S rDNA analysis showed that this strain is closely related to *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus mojavensis*, and *Bacillus atrophaeus*. The zymogram analysis of the crude extract revealed the presence of five extracellular proteases. One of the proteases was purified in three steps procedure involving ammonium sulfate precipitation, DEAE-Sepharose ionic exchange and Sephacryl S-200 gel filtration chromatography. The molecular mass of the enzyme on SDS-PAGE was estimated to be 29 kDa. The protease exhibited maximum activity at pH 10.0 and 60 °C and was inhibited by PMSF but it was not affected by cysteine inhibitors, suggesting that the enzyme is a serine alkaline protease. Irreversible thermoinactivation of enzyme was examined at 50, 60, and 70 °C in the presence of 10 mM $CaCl_2$. Results showed that the protease activity retains more than 80% and 50% of its initial activity after incubation for 30 min at 60 and 70 °C, respectively. This enzyme had good stability in the presence of H_2O_2, nonionic surfactant, and local detergents and its activity was enhanced in the presence of 20% of dimethyl sulfoxide (DMSO), dimethyl formamide (DMF) and isopropanol. The enzyme retained more than 90% of its initial activity after pre-incubation 1 h at room temperature in the presence of 20% of these solvents. Also, activation can be seen for the enzyme at high concentration (50%, *v*/*v*) of DMF and DMSO.

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Abbreviations

SDS	sodium dodecyl sulfate
PMSF	phenyl methyl sulphonyl fluoride
EDTA	ethylenediamine tetra acetic acid
DMSO	dimethyl sulfoxide
DMF	*N*,*N*-dimethyl formamide
DTT	dithiotheritol

Introduction

Proteases (serine-, cystein-, aspartic-, and metallo-proteases) constitute one of the most important groups of industrial enzymes and have applications in different industries. Alkaline serine proteases are the most important groups of enzymes exploited commercially [1]. Detergent industry has now emerged as one of the major consumers of hydrolytic enzymes working at alkaline pH [2]. In recent years the use of alkaline proteases has increased remarkably that are stable and active under high temperature, alkaline pH and in the presence of surfactants or oxidizing agents [3]. These enzymes are produced by a wide range of microorganisms including bacteria, moulds, yeasts, and also mammalian tissues, and are used as additives in detergents to facilitate the release of proteinacious materials in stains due to grime, blood, and milk, etc. Currently, a large proportion of commercially available alkaline proteases are derived from *Bacillus sp.* [4–6]. *Bacillus* strains have ability to secrete enzymes over a short period of time into the fermentation broth [7]. In this study, *Bacillus sp.* HR-08 was isolated from soil and the ability of protease production was examined on gelatin and skim milk agar medium. We purified and characterized an alkaline serine protease that was active at high temperature, alkaline pH and in the presence of organic solvents and stable against high temperature, nonionic surfactant, and local detergents. Also, DNA sequence information from the small subunit 16S rDNA has been used in identification and phylogenetic classification of HR-08 strain.

Materials and Methods

Microorganism and Culture Conditions

Soil samples were collected from rhizospher and rhizoplane zone of potato cultivated in Hamadan, Iran. Twenty grams of an air-dried soil samples were added to 100 ml sterile water in Erlenmeyer flask. The containers were incubated in a water bath at 80 °C for 10 min while the contents were agitated. Subsequently, 1 ml of these suspensions was added to 9 ml sterile water and a serial dilution (10^{-1} to 10^{-9}) were prepared. About 1 ml of each dilution was added and distributed on a sucrose-pepton agar (SP) medium. Plates were incubated at 37 °C for 24 to 72 h and different types of colonies were selected. These strains were screened by gelatin and/or casein hydrolysis tests. Gelatin hydrolysis was examined on agar medium containing 4 g/l gelatin. After incubation at 37 °C, plates were rinsed with 12% (*w*/*v*) $HgCl_2$ in 20% (*v*/*v*) concentrated HCl and hydrolysis was recorded when clear zone appeared around the colonies. Casein hydrolysis was determined by

supplementing nutrient agar with 10% skim milk and the consequent production of a white precipitate around the colonies was confirmed by the appearance of a clear zone after raising the plates with 10% (*v*/*v*) HCl [8]. Finally, a strain HR-08 exhibited prominent clear zones around its colonies on gelatin agar medium. This strain grew very fast and exhibited maximum production of extracellular proteases after 48 h of growth (data not shown). The strain was grown in liquid pre-culture medium containing 8 g/l nutrient broth, 10 g/l sucrose, 10 g/l yeast extract, and 5 g/l NaCl. Incubation was carried out with agitation at 180 rpm for 18 h. The liquid medium used for the protease production was composed of 5 g/l sucrose, 5 g/l citric acid, 10 g/l yeast extract, 1 g/l K_2HPO_4, 0.1 g/l $MgSO_4$ $7H_2O$ and 0.1 g/l $CaCl_2$ $2H_2O$. The pH of the medium was adjusted to 7.0 with 10% (*w*/*v*) Na_2CO_3 solution. The medium was inoculated with 5% (*v*/*v*) at 18-h-old culture and incubated at 37 °C in a shaker 180 rpm for 48 h.

Biochemical Test

Out of 93 isolates, the promising strains were examined for morphological, physiological, and biochemical characteristics with reference to Bergey s Manual of Systematic Bacteriology [8] and Color Atlas of *Bacillus species* [9]. Analysis such as Gram-stain reaction, KOH sensitivity, O_2 requirement, and catalase activity of the isolates were carried out using standard procedures [10].

PCR Amplification and 16S rDNA Sequencing

Genomic DNA was extracted according to Sambrook et al. [11] and its purity was assessed from the A260/A280. Universal 16S rDNA PCR forward primer (5′-AGTTTG ATCCTGGCTCAG-3′) and reverse primer (5′-GGC/T TACCTTGTTACGACTT-3′) were used in the amplification of 16S rDNA genes [12, 13]. A DNA thermal cycler (Eppendorf) was used and programmed as follows: (1) an initial temperature of 94 °C for 5 min, (2) a run of 30 cycles with each cycle consisting of 45 s at 94 °C, 45 s at 48 °C and 90 s at 72 °C, and (3) 5 min at 72 °C to allow for the extension of any incomplete products. The amplification products were purified using DNA extraction kit (Fermentas), then DNA sequencing on both strands directly was performed by SEQ-LAB (Germany) according to super long run. The 16S rDNA sequence obtained from HR-08 has been deposited in GeneBank under accession no. DQ092500.

Purification of the Protease

One hundred fifty milliliters of the culture was precipitated by ammonium sulfate saturation (up to 80%) by slow continuous stirring in a cold room. The saturated solution was centrifuged and precipitate was dissolved in minimum amount of 20 mM Tris–HCl, pH 8.5 containing 2 mM $CaCl_2$ and dialyzed against the same buffer for 24 h with four changes. It was then applied to DEAE-Sepharose column (1.6×10 cm) at a flow rate of 1 ml min^{-1}, previously equilibrated with 20 mM Tris–HCl buffer, pH 8.5. The enzyme was eluted in the unbound fractions using a continuous NaCl gradient (0–0.5 M). Unbound fractions that contained the enzyme activity were combined and loaded to Sephacryl S-200 column (0.8× 45 cm) previously equilibrated in 20 mM Tris–HCl, pH 8.5 containing 2 mM $CaCl_2$ and eluted at a flow rate of 0.25 ml min^{-1}. The active fractions were pooled and concentrated by ultrafiltration with the use of 10,000-Da memberane cut-off (Amicon, Bevery, MA, USA) and used for further characterization of the enzyme. During these experiments we used an

AKTA FPLC system (Amersham biosciences, Uppsala, Sweden) with both DEAE-Sepharose and Sephacryl S-200 columns.

Protease Assay and Determination of Protein Concentration

Protease activity was determined by the modified method of Kembhavi et al.[14]. The reaction mixture consisted of 0.5 ml casein 1% (*w*/*v*) and 0.25 ml Tris buffer, pH 9.0. The reaction was started by the addition of 0.25 ml enzyme solution. The reaction mixture was then incubated at 37 °C for 30 min and terminated by the addition of 1.0 ml trichloroacetic acid (10%, *w*/*v*) This mixture was further incubated at 4 °C for 1 h. After incubation the mixture was centrifuged at 12,000×*g* for 10 min and the absorbance of the supernatant was measured at 280 nm. One unit is the amount of enzyme which releases soluble fragments equivalent to 0.001 A_{280nm} per 30 min at standard condition. Protein concentration was determined by Lowry method [15].

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed using 12% acrylamide gel according to Laemmli [16]. The protein samples were dissolved in sample solvent and denatured by heating at 100 °C for 3 min. The relative molecular mass of the protein was estimated using standard molecular weight (premixed protein molecular weight marker, Roche).

Zymogram Analysis

Zymography was carried out with 0.1% (*w*/*v*) of casein co-polymerized with the running gel and samples were loaded into the gel without heating. Following electrophoresis at 4 °C, gel was incubated in 2.5% (*v*/*v*) Triton X-100 for 30 min at room temperature with gentle agitation, and then the zymogram denaturing buffer was decanted and replaced with developing buffer (Tris 20 mM, NaCl 0.2 mM, $CaCl_2$ 5 mM, and Brij 0.02%). The gel was equilibrated for 30 min at room temperature with gentle agitation, then replaced with fresh developing buffer and incubated at 37 °C for at least 4 h. The gel was stained with coomasie brilliant blue R-250 (0.5%, *w*/*v*) for 30 min and finally destained [17].

Characterization of Purified Enzyme

Effect of pH and Temperature on Activity and Stability

Activity of the purified protease was measured at different pH values using a mixed buffer containing 50 mM of citric acid-sodium citrate, Tris and glycine-NaOH. The pH stability of the protease was established by incubating the enzyme in buffer of different pH in the range of 5.0–12.0 for 48 h at room temperature and then residual activity was determined under standard assay conditions. For determining optimum temperature of enzyme activity, the reaction mixture was incubated at different temperature ranging from 20 to 80 °C for 30 min in Tris buffer, pH 9.0. The thermal stability was determined by incubating the purified enzyme in 50 mM Tris buffer containing10 mM $CaCl_2$ (pH 9.0) at temperatures 50, 60, and 70 °C. Determination of the remaining activity was carried out through removal of some samples at regular intervals and cooling them on ice. Activity of the enzyme solution kept on ice was considered as the control (100%).

Effect of Various Metal Ions and some Inhibitors on Enzyme Activity

Metal ions with 5 mM final concentration were added to reaction and protease activity was determined. Inhibitors including phenylmethylsulphonyl fluride (PMSF), a classical serine protease inhibitor; iodoacetate, 2-mercaptoethanol and dithiotheritol, cystein protease inhibitors; and ethylenediamine tetra acetic acid (EDTA) chelator of divalent cations (metallo protease inhibitor) were pre-incubated with enzyme for 30 min at room temperature and residual activity was measured under standard assay.

Effect of Laboratory Surfactants, Bleach Agent, and Local Detergents on Protease

The enzyme was pre-incubated with 0.5% (*w*/*v*) SDS, 5% (*v*/*v*) H_2O_2 and 5% (*v*/*v*) Triton X-100 for 60 min at room temperature and 0.5% (*w*/*v*) of each laundry detergent in the presence of 10 mM $CaCl_2$ for 1 h at 40 °C and residual activity was measured under standard conditions.

Effect of Organic Solvent on Protease Activity

Organic solvents such as dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), 1-propanol, and isopropanol with various concentrations (10% to 30%, *v*/*v*) were added to reaction and protease activity was determined. Stability against these solvents determined with incubation at room temperature for 1 h and then residual activity was measured.

Results presented in this paper are the mean of at least three repeated experiments in a typical run to confirm reproducibility.

Results and Discussion

Identification of Microorganism and 16S rDNA Sequence

All collected strains (93 isolates) were spore forming, Gram positive and rod-shape bacteria. They were screened for protease activity and finally one isolate (HR-08) out of 93 which had good production of the enzyme was selected for further experimental work. The strain was catalase and oxidase positive and capable of using sodium citrate and sodium propionate as the sole carbon source. It was also able to grow in salt-containing nutrient media at different concentrations up to 7% (*w*/*v*). The strain was able to produce acid compounds from glucose, mannitol, xylose, and arabinose. Combinations of morphological, physiological, and biochemical data suggested that this strain belong to *Bacillus* species. To confirm its identity, PCR amplification and sequencing of the 16S rDNA gene of this isolate was done. Ribosomal RNAs are essential elements in protein synthesis and are therefore conserved in all living organisms [18]. Product of PCR amplification was about 1,400–1,500 bp (data not shown). 16S rDNA sequence was edited to a total length of 1,411 bp direct sequencing. 16S rDNA from *Bacillus* species were obtained from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and multiple sequence alignment was performed with ClustalW version (1.82). After homology searched with other *Bacillus* species more than 96% identity with 1% Gap obtained. The phylogenetic tree was constructed using neighbor-joining method (Fig. 1). Multiple alignment and phylogenetic tree showed that HR-08 strain belongs to the genus *Bacillus*

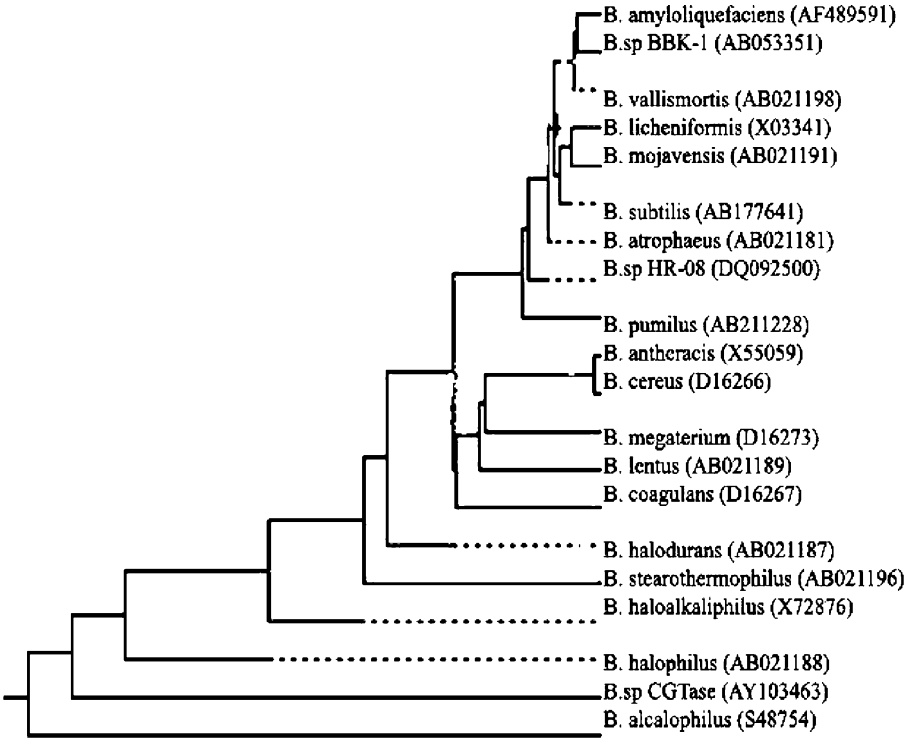

Fig. 1 Phylogenetic tree of HR-08 strain and related *Bacillus* inferred from sequence of 16S rDNA created by MEGALIGN

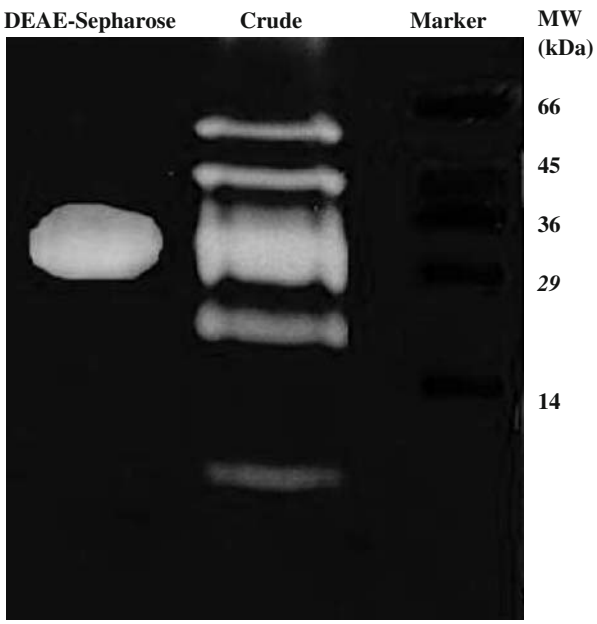

Fig. 2 Zymogram analysis of crude extract and active fraction alkaline protease HR-08 eluted from DEAE-Sepharose. For more detail please see Materials and Methods

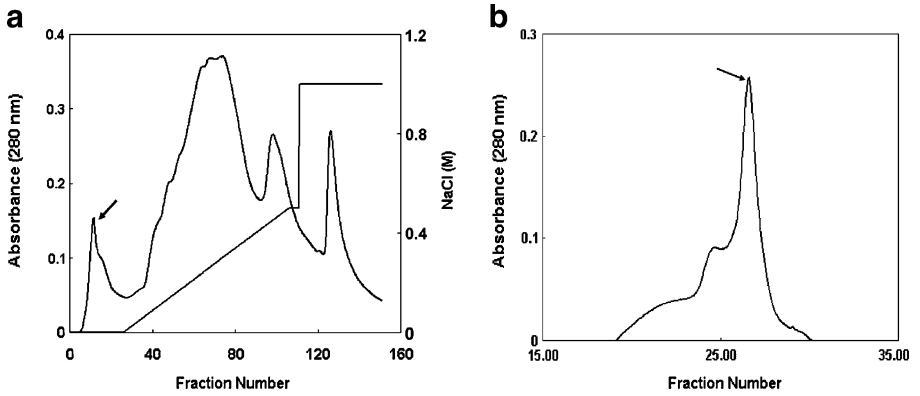

Fig. 3 Purification profile of alkaline protease from *Bacillus* HR-08 on DEAE-Sepharose (**a**) and gel filtration on Sephacryl S-200 (**b**) columns. **a** The enzyme was eluted with a linear gradient of NaCl (0–0.5 M). Protein content in the fractions was determined by absorbance at 280 nm. Fraction containing the protease activity (alkaline protease HR-08) is shown by *arrow*

and is closely related to *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus mojavensis*, and *Bacillus atrophaeus*.

Isolation and Purification of the Protease

The zymogram analysis of the crude extracellular enzymes precipitated by 80% saturated ammonium sulfate showed the presence of about five proteases (Fig. 2). The precipitate was subjected to ion-exchange chromatography on DEAE–Sepharose Fast Flow (Fig. 3). Serine alkaline protease eluted as unbound fractions with the specific activity of 2,306 U mg^{-1} of protein, 20-fold purification and 3.8% recovery of the enzyme (Table 1). Analysis by SDS-PAGE showed two bands with 20 and 29 kDa molecular weights (Fig. 4). The 20-kDa band did not contain protease activity as observed in zymogram analysis (Fig. 2). Therefore, this step provided an efficient method to separate the protease (29 kDa) from the other proteases (Figs. 2 and 4). This protease was further purified by chromatography on Sephacryl S-200. A final purification of 26-fold, 0.4% recovery of the enzyme, and a specific activity of 2,934 U mg^{-1} were obtained (Fig. 3b and Table 1). Purified protease migrated as a single band almost of 29 kDa in SDS-PAGE (Fig. 4). The results of purification procedure are summarized in Table 1. Results from zymogram have been showed the presence of five different proteases in the crude extract. As the purification proceeds toward the isolation of desired protease, the remarkable reduction in the yield of purification could be expected

Table 1 Summary of the purification procedures of the serine alkaline protease from *Bacillus* sp. HR-08.

Step	Total activity (unit)	Total protein (mg)	Specific activity (u/mg)	Purification fold	Yeild (%)
Crude extraction	60,000	525	114	1.0	100
$(NH_4)_2SO_4$ precipitation	15,200	76	200	1.8	25
DEAE-sepharose	2,306	1	2,306	20.2	3.8
Sephacryl S-200	235	0.1	2,938	26.0	0.4

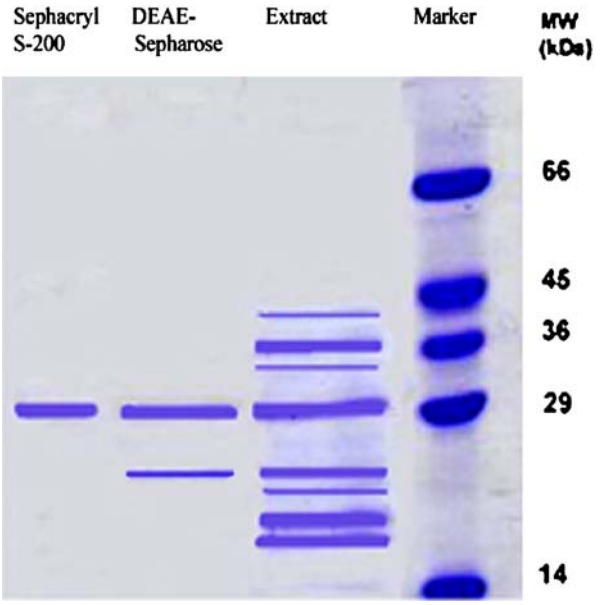

Fig. 4 SDS-PAGE analysis of crude extracellular (extract) and the active fractions after DEAE-Sepharose and Sephacryl S-200 columns

mainly due to the decrease in the number of total protease. Inactivation due to the auto-digestion could be another alternative explanation for the reduction in the yield as observed in Table 1.

pH Activity and pH Stability Profiles

The protease exhibited its highest activity at pH 10.0 (Fig. 5a). This finding was in accordance with previous studies that reported the optimum pH of 10.0–10.5 for proteases from *Xanthomonas maltophila*, *Vibrio metschnikovii, B. licheniformis* NH1, and *B. mojavensis* [19–22]. The important detergent enzymes, subtilisin Carlsberg and subtilisin NOVO or BPN [23] also showed maximum activity at pH 10.5. The stability of the purified protease was also determined by the pre-incubation of the enzyme in various buffers of different pH values. With 48 h pre-incubation, the enzyme was stable over a broad range of pH 7.0 to pH 12.0 (Fig. 5b). In earlier studies, the protease from a *Thermus* sp strain Rt41A [24] exhibited stability for at least 4 h over a pH range of 5.0 to 10.0 and in the case of the 20-h pre-incubation, the protease from *B. subtilis* PE-11 was stable between pH 8 and pH 10 [25]. Alkaline serine protease from *Bacillus sp.* SSR1 was stable over pH range from 8.0 to 11.0 at 1 h [26].

Effect of Temperature on Enzyme Activity and Stability

The activity of purified enzyme was determined at different temperatures ranging from 20 to 80 °C. The optimum temperature of the enzyme was at 60 °C and declined at temperatures beyond 60 °C (Fig. 5c). A similar result was observed by other investigators where a maximum temperature of 55 and 60 °C was recorded for an alkaline protease from *Bacillus stearothermophilus* AP-4 [27] and *B. subtilis* PE-11 respectively [25]. Also, Joo et al. reported an alkaline serine protease with optimum temperature around 45–50 °C [28]. The irreversible thermoinactivation of the purified protease was tested at temperatures of 50, 60, and 70 °C for different incubation times (0 to 180 min) in the presence of 10 mM

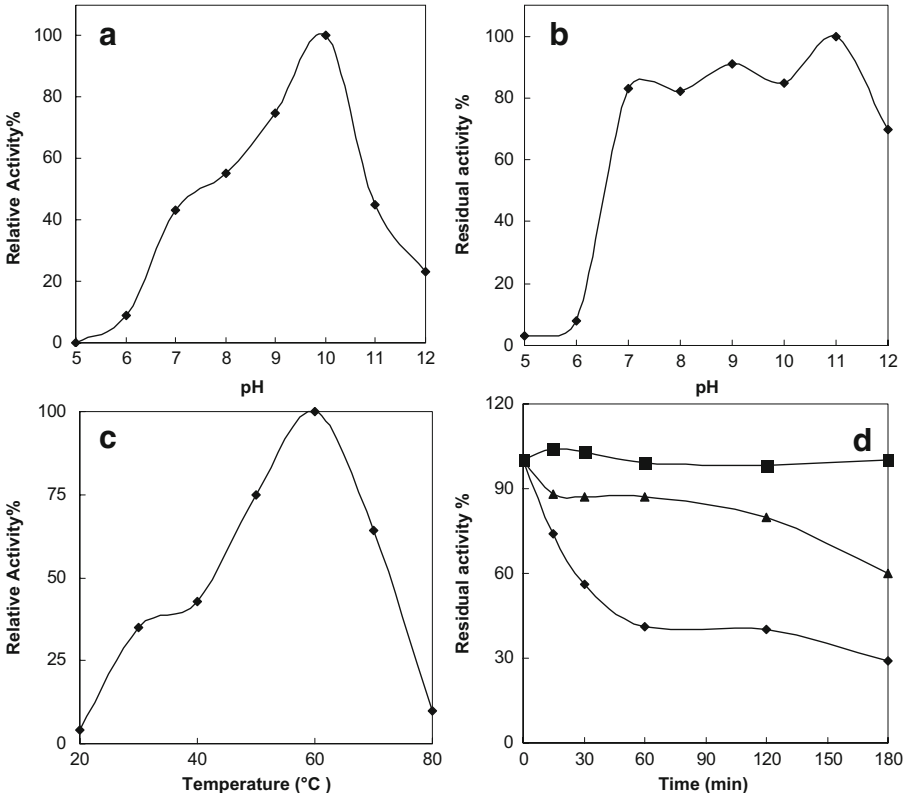

Fig. 5 Effect of pH on activity (**a**) and stability (**b**) of alkaline protease from *Bacillus* HR-08. **c** Effect of temperature on activity and **d** irreversible thermoinactivation of HR-08 protease in 50 (*filled square*), 60 (*filled triangle*), and 70 °C (*filled diamond*). Standard deviations were within 5% of the experimental values. For more details please see Materials and Methods

$CaCl_2$ (Fig. 5d). The HR-08 protease was almost 100% stable at 50 °C even after 180 min of incubation. The enzyme had a half life of >180 min at 50 °C and less than 50 min at 60 °C and 70 °C. Previous reports on thermostability have shown half-lives of >200 min at 50 °C and 22 min at 60 °C for heat stable serine proteases and half-lives of 3.4 min and

Table 2 Effect of various metal ions and some inhibitors[a] on protease activity from *Bacillus* sp. HR-08.

Metal ion/inhibitor	Concentration (mM)	Residual activity (%)
PMSF	10	5
Iodoacetate	2	96
EDTA	5	93
2-Mercaptoethanol	10	96
Dithiotheritol	5	94
$CaCl_2$	5	128
$ZnCl_2$	5	69
NaCl	5	115
$CuSO_4$	5	130
$FeCl_2$	5	25
$MgCl_2$	5	79
$MnSO_4$	5	46

[a] Enzyme were pre-incubated with inhibitors for 30 min at room temperature and residual activity was measured under standard conditions

2.4 min at 50 °C have been recorded for subtilisin Carlsberg and subtilisin BPN, respectively [21, 29]. Also, in subtilisin Calsberg and thermophilic *Bacillus* sp. JB-99 the thermal stability was enhanced in the presence of calcium. [30–32]. SSR1 protease had a half-life of 300 and 60 min at 50 and 60 °C, respectively [26] and an alkaline serine protease showed more than 85% of initial activity after 60 min at 50 °C [28].

Effect of Inhibitors and Metal Ions

The effects of different inhibitors and metal ions on enzymes are shown in Table 2. Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of active site [33]. Among the inhibitors tested, PMSF was able to inhibit the protease completely. PMSF strongly blocks the serine residue in the active site causing complete loss of enzyme activity. Inhibitory effect of EDTA, iodoacetate, DTT, and 2-mercaptoethanol was not observed suggesting that the enzyme was not metallo and/or cysteine protease. Results indicated that HR-08 protease was a serine type alkaline protease [26, 34]. The results of metal ion experiments showed that 5 mM of Ca^{2+}, Na^{+}, Cu^{2+} stimulated the protease activity more than 10% while Fe^{2+} and Mn^{2+} inhibited the activity about 70% and 50%, respectively (Table 2). Divalent cations like Ca^{2+}, Mn^{2+}, and Mg^{2+} were found to enhance the thermal stability of *Bacillus* alkaline proteases [35]. Ca^{2+} increased not only the enzyme activity but also the thermal stability in this study and in many reports [32, 36]. Most alkaline proteases have been reported to be significantly stablilized by Ca^{2+} [37–39] and extracellular enzymes produced by thermophilic microorganisms require Ca^{2+} for their stability [40]. The effect of Ca^{2+} on the thermal stability of the enzyme may be explained by the strengthening of interactions inside protease molecules and by the binding of Ca^{2+} to the autolysis site [36]. The use of subtilisin BPN in chelating detergent conditions causes a loss of bound calcium from the high affinity site A and results in destabilization and loss of activity [41].

Compatibility with Detergents and H_2O_2

After pre-incubation with 0.5% SDS, 5% Triton X-100 and 5% H_2O_2 for 60 min at room temperature protease activity was determined and residual activity was calculated. Residual activities of the enzyme were 54%, 91%, and 109% in the presence of SDS, Triton X-100

Table 3 Effect of laboratory surfactants, bleach agent and local detergents[a] on protease activity from *Bacillus* sp. HR-08.

Detergent/bleach agent	Concentration (%)	Residual activity[b] (%)
SDS	0.5	54±10
H_2O_2	5	109±2
Triton X-100	5	91±5
Sepid 3	0.5	88±5
Shoma	0.5	85±3
Tage	0.5	85±5
Yekta	0.5	85±4

[a] Local detergents such as Sepid 3 (Paksan, Iran), Shoma (Toolipers, Iran), Tage (Behdad, Iran) and Yekta (Pakname, Iran) were used

[b] The enzyme was pre-incubated with surfactants and bleach agent at room temperature and with local detergents at 40 °C for 60 min then residual activity was measured under standard conditions. All experimental results represent the mean of at least three experiments

and H_2O_2, respectively indicating that this protease is also bleach stable (Table 3) although hydrogen peroxide is a strong oxidizing agent and usually inactivates proteins oxidatively. An alkaline protease from a *Bacillus* sp. retained 40%, 60%, 61%, and 75% of its activity in the presence of 5% and 1% H_2O_2 and 0.1% and 0.5% SDS, respectively [1, 21, 32, 42]. However, many of the available alkaline proteases exhibit low activity and stability towards oxidants like peroxides and perborate which have been the common ingredients in modern bleach based detergent formulation [43]. HR-08 protease showed high stability and compatibility with local detergents at 40 °C for 1 h. The enzyme retained more than 80% of its initial activity in the presence of 0.5% commercial detergents (Table 3).

Effect of Organic Solvents on Protease Activity and Stability

In recent years, several reports showed that peptide synthesis could be enhanced by the addition of organic solvents in the reaction mixture [44–47]. However, a major drawback of this approach is the strongly reduced activity of enzymes under anhydrous conditions. It has become a new area in enzymology to search for proteases, which are naturally stable in the presence of organic solvents [42, 44, 48]. The activity of the protease exposed to different concentrations of organic solvents (Fig. 6a). The protease HR-08 was activated by 20% (*v*/*v*) of DMSO, DMF, and isopropanol, whereas this proteolytic enzyme was partially inactivated in presence of 1-propanol. Similar result was reported for an alkaline serine protease by Hadj-Ali et al. [21]. Effects of different percentages of various organic solvents on the stability of protease were also studied (Fig. 6b). The enzyme was not only stablilized at 50% DMSO and DMF but also demonstrated increased activity by 1.1 and 1.3, respectively. Alkaline serine protease from *B. licheniformis* NH1 was just stable at 25% DMSO [21]. However, the protease activity decreased as the concentration of 1-propanol and isopropanol in the pre-incubation mixture was increased. In addition, the enzyme retained more than 90% of its initial activity when incubated 1 h at room temperature in the presence of 20% DMSO, DMF, and isopropanol (data not shown).

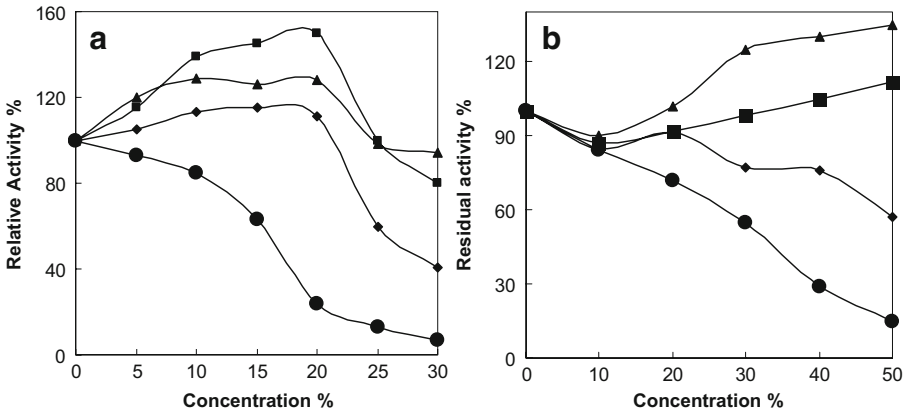

Fig. 6 Effects of different percentages of DMSO (*filled square*) DMF (*filled triangle*) isopropanol (*filled diamond*), and 1-propanol (*filled circle*) on the activity and stability of protease HR-08. For evaluation of the stability, the enzyme was incubated at room temperature in the presence of 10%, 20%, 30%, 40%, and 50% (*v*/*v*) organic solvents for 60 min. Standard deviations were within 6% of the experimental values. For more details please see Materials and Methods

Conclusion

The most important features of HR-08 protease are high activity and stability at high temperature and pH as well as in the presence of H_2O_2, commercial detergents and organic solvents such as isopropanol, DMF, and DMSO without any mutation. Considering these properties, HR-08 protease may find potential application in peptide and oligopeptide synthesis and/or in laundry detergents. Although there are many microbial sources available for producing proteases only a few are recognized as commercial producers. Further experiments on this protease towards molecular genetic analysis are currently underway.

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