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A novel neutral protease from thermophilic *Bacillus* strain HUTBS62

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ABSTRACT

A novel neutral highly thermostable protease was detected in the culture medium of thermophilic *Bacillus* strain HUTBS62 isolated from hot-spring located near to the Dead Sea, Jordan. The enzyme was purified by precipitation with 55-60% ammonium sulfate, gel filtration on Sephadex G-100 and DEAE ion exchange chromatography. The enzyme was purified 53-fold with 2% yield. The optimum pH and temperature for catalytic activity of protease was pH 6.8 and 80ºC, respectively, and 31% activity of protease remained even after heat treatment at 100ºC for 60 min. The relative activity of the enzyme was highly stable (90%) at 50ºC for 2 h. The half-life of the enzyme at 90ºC, 80ºC and 70ºC was estimated to be 3, 4 and 6 h, respectively. The activation energy of denaturation of purified enzyme was 21.7 $kJmol^{-1}$. Iron, sodium, calcium, and manganese increased protease activity. On the other hand, magnesium, cobalt and zinc variably decreased the residual activity. But cadmium and copper drastically inhibited the enzyme activity. The enzymatic activity was highly stable in the presence of 1 and 2 mM EDTA at pH 6.8 and 80ºC. The neutral protease therefore could be defined as a highly thermostable with new properties make the present enzyme applicable for many biotechnological purposes.

Key words: *Bacillus*, enzymes, neutral pH, protease, thermophilic, thermostability

Introduction

The genus *Bacillus* produces a wide variety of extracellular enzymes, including proteases. Several *Bacillus* species involved in protease production, such as *Bacillus stearothermophilus* (Sookkheo et al., 2000), *Bacillus licheniformis* (Ferrero et al., 1996), *Bacillus subtilis* (Soares et al., 2005) and *Bacillus cereus* (Nilegaonkar et al., 2007). Proteases produced by *Bacillus* sp. find a wide variety of application in detergents, leather, food and pharmaceutical industries (Banerjee et al., 1999; Banik & Prakash, 2004). Optimal culture conditions for protease productivity were studied by many investigators (Kim et al., 2001; Miyaji et al., 2006; Sumantha et al., 2006).

Neutral proteases from high yielding strains have been studied extensively (Guangrong et al., 2006; Zhang et al., 2008). One of the major draw backs affecting the stability at neutral pH of enzymes recovered from thermophiles is that enzyme from thermophiles confer stability in a wide pH range but are usually thermolabile. Thus, it is desirable to search for new proteases with novel properties from as many different sources as possible.

Recently, a thermophilic *Bacillus* strain HUTBS62 was isolated from hot-spring located near the Dead Sea, Jordan. Phenotypic characteristics and genetic polymorphism by RAPD-PCR for *Bacillus* strain HUTBS62 were determined (Aqel & Atoum, 2003).

Here we report the efficiency of a highly thermostable enzyme, recovered from newly isolated strain of thermophilic *Bacillus* HUTBS62. Further characterization of this enzyme and the effect of various cofactors or additives on the stability at higher temperatures and in acidic and alkaline pH was performed.

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Materials and Methods

Bacterial strain and enzyme production

Bacillus strain HUTBS62, used in the present study, was isolated from water samples obtained from Main hot springs, Jordan. The strain was left to grow in a medium containing: 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 0.5 g/l glucose, 0.4 g/l Na_2HPO_4, 0.085 g/l Na_2CO_3, 0.02 g/l $ZnSO_4$, 0.02 g/l $CaCl_2$, 0.02 g/l $MgSO_4$. The culture was incubated in a bioreactor (Electrolab 351 EMC, Switzerland) at 50°C for 50 hours under a continuous stir (at 150 rpm) and then centrifuged at 14000g for 30 min at 25°C, and the clear supernatant was recovered. The crude enzyme supernatant was assayed for proteolytic activity and used for further purification.

Purification of thermostable proteases

The bacterial growth was carried out at 50ºC for 50 h, centrifuged at 14000g for 30 min and the supernatant retained as the source of extracellular enzyme. The purification steps were carried out at 25ºC. The supernatant was precipitated with ammonium sulfate. After 20 min, the precipitate was collected using centrifugation at 14000g and the pellet was resuspended in a minimum volume of 50 mM Tris-HCl buffered at pH 7.2 and dialyzed in the same buffer. The dialyzed sample was passed through a Sephadex G-100 column (Fluke, Switzerland; 1.5cm x 27cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.2. The flow rate was 0.5 $ml.min^{-1}$ and 3 ml fractions were collected and analyzed for protein content and protease activity. Fractions with high protease activities were pooled, concentrated by filter tube (Sartoruis, Germany) and passed through DEAE-Sepharose fast flow column (Pharmacia, Biotechnology, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl pH 7.6 and eluted with 50 mM Tris-HCl buffer, pH 8.6. The flow rate was 0.5 $ml.min^{-1}$ and 3 ml fractions were collected and analyzed for protein content and protease activity. Fractions of high protease activities were pooled and concentrated by filter tube. The pooled sample was subjected to sodium-dodecyl sulfate (7.5%) polyacrylamide gel electrophoresis (SDS-PAGE). The total protein was determined by the method of Lowry et al. (1951).

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out at 7.5% (w/v) isolation gel and 5% (w/v) concentration gel according to the method of Laemmli (1970). 2-mercaptoethanol was used as a reducing agent. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of the relative molecular mass versus the R_f value (relative mobility) on Bio-Rad Mini Protein Cell with Promega molecular weight standards 25, 35, 50, 75, 100, 150 and 225 kDa (Promega, Wisconsin, USA).

Protease assay

The proteolytic activity of the enzyme was assayed in triplicate using casein (Hi Media, India) as a substrate; initially a mixture of 400 µl casein solutions (2% (w/v) in 50 mM Tris-HCl buffer pH 7.2) and 100 µl of the sample were added to a tube. The reactions were carried out at 65°C in water bath (Memert, Germany) for 10 min and then terminated by the addition of 1 ml 10% trichloroacetic acid (w/v). The mixture was centrifuged at 14000g for 20 min. The supernatant of 500 µl was carefully removed to measure tyrosine content using a Folin-phenol method (Ledoux & Lamy, 1986). One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1.0 µM of tyrosine per minute at 65°C.

Effect of pH-values on protease activity

The activity of the purified protease was measured at different pH values. The pH was adjusted using the following buffers: 50 mM sodium acetate (pH 3.8-4.8); 50 mM sodium phosphate (pH 5.0-6.8); 50 mM Tris-HCl (pH 7.2 – 9.0); and 50 mM sodium carbonate (pH 9.2-10.8). The reaction mixtures were incubated at 80°C for 10 min, and the activity of the enzyme was measured.

Effect of temperature on protease activity and stability

The effect of temperature on the enzyme activity was determined at temperatures ranged from 20 to 100ºC, after incubating the enzyme for 10 min in sodium phosphate buffer, pH 6.8. The thermostability was determined by performing the standard assay procedure after incubating the purified enzyme at temperatures ranged from 50 to 100ºC for 1 to 8 h. The residual activity was assayed at standard assay conditions.

Effect of various metal ions on protease activity

The effect of different metal ions on protease activity was determined by the addition of the corresponding ion at a concentration of 5.0 mM to the reaction mixture, and the assays were performed under standard condition (pH 6.8 and 80ºC). The tested ions include the following corresponding salts: NaCl, $CaCl_2$, $MgSO_4$, $FeSO_4$, $CoCl_2$, $MnSO_4$, $CdCl_2$, $CuSO_4$ and $ZnSO_4$.

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Effect of different concentrations of EDTA on protease activity

Purified enzyme preparation was pre-incubated in 50 mM sodium phosphate buffer, pH 6.8 containing various EDTA concentrations (0 to 15 mM) in the assay mixture and activities of the protease enzyme were measured at 80ºC.

Results and Discussion

The purification of proteases is important from the perspective of developing a better understanding of the functioning of the enzyme (Tsai et al., 1988). Precipitation is the most commonly used method for the isolation and recover of proteins from crude biological mixtures (Bell et al., 1983). It also performs both purification and concentration steps. Precipitation by ammonium sulfate is used in acidic and neutral pH solutions (Aunstrup, 1980). Our data showed that the 55-60% ammonium sulfate saturation fractions correlated with highest proteolytic and specific activities compared with the crude protease and other fractions. Lopes et al. (1999) found that proteases from *Streptomyces alboniger* were precipitated by 40% saturation of ammonium sulfate. Moreover, protease from *Bacillus mojavensis* precipitated by 85% saturation of ammonium sulfate (Beg & Gupta, 2003).

The purification procedures of the protease secreted by tested bacteria are summarized in Table 1. The results showed that the enzyme was purified 6.5-folds with a specific activity of 83 $U.mg^{-1}$ proteins after ammonium sulfate fractionation. The enzyme was then purified using Sephadex G-100 column and resulted in 32-folds purification with a specific activity of 453 $U.mg^{-1}$ protein. The final purification step presented 53-fold enzyme purification with a specific activity of 744 $U.mg^{-1}$ proteins. These results indicated the effectiveness of the purification method applied in this research. However, the yield of the enzyme after purification was found to be low (2.0%). This might be due to the result of autolysis of the enzyme in each purification step.

Table 1. *Purification steps of protease from Bacillus strain HUTBS62.*

Purification steps	Volume (ml)	Protein (mg)	Enzyme activity (U/ml)	Total enzyme activity (U)	Specific activity (U/mg)	Purification folds	Yield (%)
Culture media	50	22.5	6.4	320	14.27	2.23	100
Ammonium sulfate 55%-60%	13	8	52	672	83	6.5	61
Sephadex G-100	12	1.5	57	679	453	32	12
DEAE Chromatography	10	0.63	47	468	744	53	2

Analysis of the purified enzyme by SDS-PAGE revealed a monomer band with a molecular mass of 48 kDa (Figure 1). Sumantha et al. (2006) reported that the molecular mass for protease range from 30 to 45 kDa. Different molecular masses for different neutral proteases have been reported by gel filtration: 40 kDa for *Bacillus nematocida* (Niu et al., 2006), 38 kDa for *Arthrobotrys oligospora* (Minglian et al., 2004), and 35 kDa for neutral protease from *Bacillus stearothermophilus* in *Bacillus subtilis* (Zhang et al., 2008).

The protease activity with the relative activity of more than 70% was found in the buffer pH ranging from 6.6 to 8.2 (Figure 2). However, the relative activities were observed to be less than 20% in sodium acetate and sodium carbonate buffers. The optimum pH for present enzyme activity was found to be 6.8 in the sodium phosphate buffer (Figure 2). Sumantha et al. (2006) reported that the optimum pH ranges of proteases were 5-7. On the other hand, the other optimum pH ranges were reported to be suitable for maximum protease production including pH 8 (Beg & Gupta, 2003), pH 8.5 (Sanchez-Porro et al., 2003), pH 10.5 (Banik & Parakash, 2004), pH 11 (Kobayashi et al., 1989) and pH 12 (Olivera et al., 2006). The enzyme was stable over a broad pH range of 6.0 to 8.6 at 80ºC, indicating that it is neutral enzyme.

The enzyme activity increased with temperature within the range of 55ºC to 90ºC but was inactive in the ranges of 25ºC-50ºC and 95ºC-100ºC, respectively (Figure 3). The optimum temperature for protease activity was 80ºC. Sumantha et al. (2006) reported that the optimum temperature for proteases was 65-85ºC. While, other optimum incubation temperature for protease production by *Bacillus* species were 55ºC (Niu et al., 2006), 65ºC (Zhang et al., 2008), and 70ºC (Kim et al., 2001). These results were not in agreement with our results.

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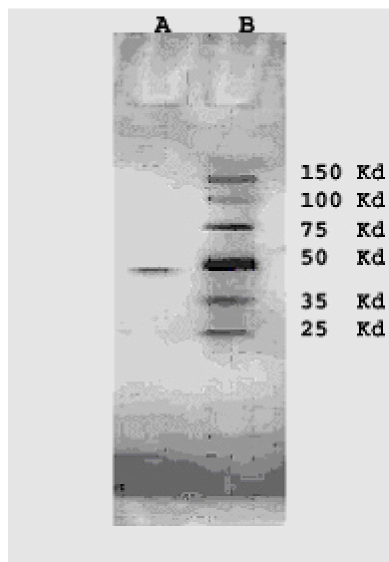

Figure 1. *SDS-Polyacrylamide gel electrophoresis of protease from thermophilic Bacillus strain HUTBS62 (Lane A - purified protease from thermophilic Bacillus strain HUTBS62; Lane B - protein markers for 25, 35, 50, 75, 100 and 150 kDa).*

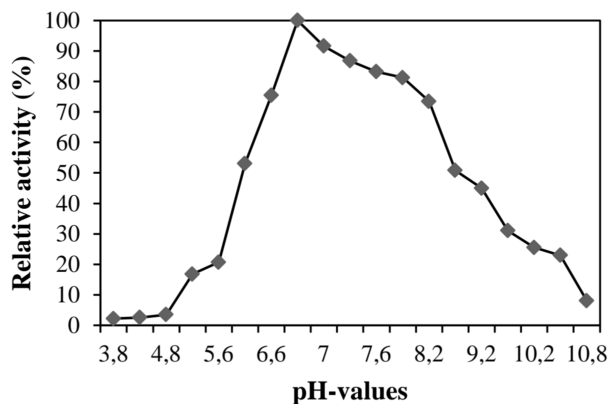

Figure 2. *Effect of pH-values on the protease activity from Bacillus strain HUTBS62.*

The heat stability of the protease in a range of 50 to 100°C was studied for 24 h and results are shown in Figure 4. Thermostability profile indicated that the enzyme was stable at 50ºC for 2 h while at 60 and 100ºC, 28% and 73% of the original activities were lost, respectively. The half-life of the enzyme estimated from the decreases in the fraction of native enzyme as a function of increasing temperature was found to be 6 h, 4 h and 3 h at 70, 80 and 90ºC, respectively. Nascimento & Martins (2004) reported that the neutral protease from *Bacillus* sp. SMIA2 lost 80% and 100% of the original activity after 2 h treatment at 60ºC and 80ºC, respectively. Fujio & Kume (1991) showed that neutral protease produced from *Bacillus stearothermophilus* maintained 90% of its activity at 90ºC for 30 min.

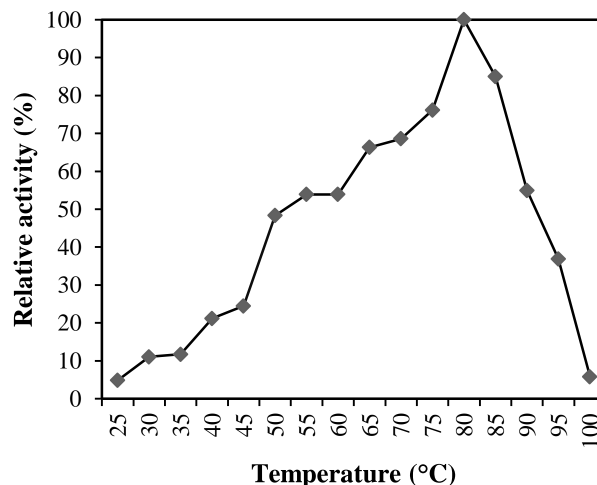

Figure 3. *Effect of temperature on Bacillus strain HUTBS62 protease activity. The activity of the purified enzyme was measured in 50 mM sodium phosphate buffer pH 6.8 at 80ºC temperatures for 10 min.*

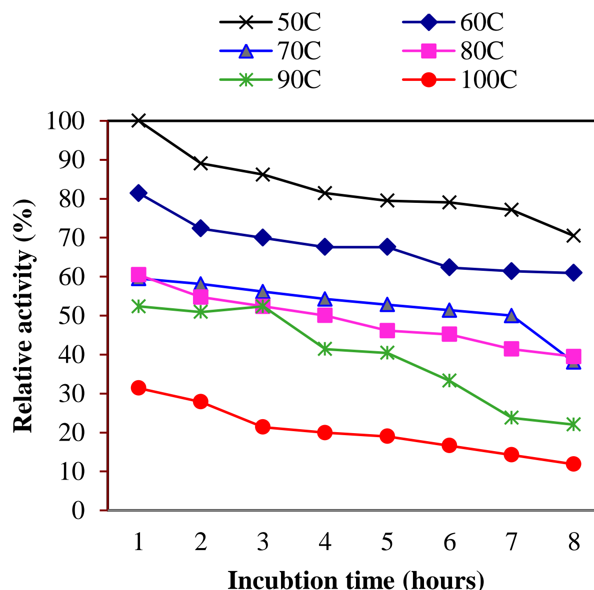

Figure 4. *Effect of temperature on the thermostability of the protease purified from Bacillus strain HUTBS62. Enzyme activity was measured after enzyme incubation for designated time and perform enzyme assay as usual in 50 mM sodium phosphate buffer pH 6.8 at 80ºC.*

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On the other hand, a neutral protease called thermolysin produced by *Bacillus thermoproteolyticus* (now considered as a strain of *Bacillus stearothermophilus*) retained 86% of its activity after 30 h at 70ºC (Endo, 1962). While the neutral enzyme obtained from *Bacillus stearothermophilus* KP 1236 had maximal activity at 80ºC and at pH 7.5 and was stable for 10 min at 80ºC at pH 7.5 and for 18 h at 60ºC at pH 6 to 8.8 (Takii et al., 1987).

The activation energy (Ea) for denaturation of purified enzyme was calculated from the slope of Arrenhenius plots to be 5.18 $kcal.mol^{-1}$ or 21.7 $kcal.mol^{-1}$ (data not shown). The Ea of our present protease was comparable to other proteases from other *Bacillus* species. Ahmed et al. (2008) reported that the Ea of the free enzyme was 6.206 $kcal.mol^{-1}$, whereas Guangrong et al. (2006) reported that the Ea for the purified protease enzyme from thermophilic *Bacillus* HS08 was 29.17 $kJ.mol^{-1}$.

The protease activity was enhanced with addition of 5 mM $FeCl_3$, NaCl and $CaCl_2$, resulting in the relative activity of 124, 107 and 122%, respectively (Table 2).

Table 2. *Effect of metal ions on protease activity for Bacillus strain HUTBS62. Activity of protease was determined in the presence of 2.0 mM of metal ions, 50 mM sodium phosphate buffer pH 6.8 and assayed at 80ºC. Control for each was the activity in the absence of comparable ion.*

Ions	Units/ml	Relative activity (%)
Control	59	100
$FeCl_3$	73	124
$CaCl_2$	72	122
NaCl	63	107
$MnSO_4$	61	103
$MgSO_4$	58	98
$CoCl_2$	47	81
$ZnSO_4$	39	66
$CdCl_2$	15	26
$CuSO_4$	12	21

On the other hand, the protease was unaffected or inhibited upon addition of 5 mM $MnSO_4$, $MgSO_4$, $CoCl_2$, and $ZnSO_4$ resulting in relative activity of 103, 98, 81 and 66%, respectively. A strong inhibitory effect on protease activity was observed in the presence of 5 mM $CuSO_4$ and $CdCl_2$. The relative activities were 21% and 26%, respectively (Table 2). Most previous studies showed that the effect of sodium, calcium and manganese was to increase protease activity, which was similar to our results (Adinarayana et al., 2003; Beg & Gupta, 2003; Nascimento & Martins, 2004). These metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature. In addition, protease required a divalent cation like Ca^{2+} and Mn^{2+} or combination of these cations for its maximum activity (Kumar et al., 1999; Beg & Gupta, 2003). In addition, these cations enhanced the stability of a *Bacillus* protease (Durham et al., 1987).

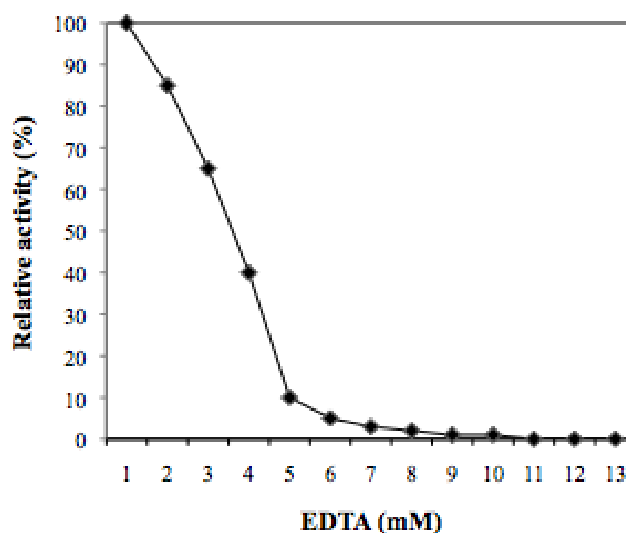

Figure 5. *Effect of different concentrations of EDTA on protease activity from Bacillus strain HUTBS62. Assay was performed in 50 mM sodium phosphate buffer, pH 6.8 containing 0-15 mM EDTA and 80ºC assay temperature. The absence of EDTA from the reaction mixture was used as a control.*

Our results indicated that the presence of EDTA had an inhibitory effect on protease activity at concentration over 4 mM (Figure 5), but the enzyme activity retained 85% and 65% activity in the presence of 2 mM and 3 mM EDTA, respectively. The relative activity of the enzyme was highly stable in the presence of 1 mM EDTA. The stability of the enzyme in presence of EDTA is advantageous for use of enzyme as detergent additive. This might be due to detergents that contain high amount of chelating agents, which function as water softeners and also assist in stain removal. These agents specifically bind to and chelate metal ions making them unavailable in the detergent solution (Beg & Gupta, 2003).

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The results of pH and temperature stability in the presence of such high salt and ionic metals concentration of the investigated neutral protease from thermophilic *Bacillus* strain HUTBS62 suggesting that it may have potential application in detergent pharmaceutical formulations, leather, laundry, food and waste processing industries. Further work on this protease towards cloning and expression of gene responsible for stability is currently underway.

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