PRODUCTION AND PROPERTIES OF THERMOSTABLE PROTEASES FROM *BACILLUS THERMOLEOVORANS* STRAIN HSR

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ABSTRACT

The thermophilic bacterium Bacillus thermoleovorans strain HSR secretes thermostable extracellular proteases when cultivated in the presence of tryptone. The proteases which are found to be of the metallo-protease type, showed maximal activity when incubated at 80°C and pH 7.0. The enzymes are thermostable at temperatures between 60 and 80°C in the absence of substrate. Calcium is not required for thermostability. Cu^{2+}, Hg^{2+} and EDTA inhibit the protease activity while Fe^{2+} and Fe^{3+} ions enhanced the activity. SDS-PAGE studies indicate the presence of multiple protease activity bands with molecular masses between 40 and 57 kDa. The proteases show higher activity towards short synthetic peptides while a decrease in specificity was observed with longer peptides.

Keywords: *Bacillus thermoleovorans,* proteolytic, protease, thermophilic, thermostability

INTRODUCTION

Proteases catalyze the hydrolysis of peptide bonds in proteins. According to the mechanism of action they can be divided into four types (Kay, 1982); serine proteases (E.C. 3.4.21), cysteine proteases (E.C. 3.4.22), aspartate proteases (E.C. 3.4.23) and metal proteases (E.C. 3.4.24). Metal proteases are a group of metallo-endo-peptidases that exhibit maximal activity at or near pH7. These enzymes are characterized by their sensitivity to metal chelating agents such as ethylene-diamine-tetra-acetic acid (EDTA) indicating the importance of the presence of certain metal ions for the activity and stability of metallo-proteases (Priest, 1977). Proteases play an important role in different biotechnological processes representing the most important industrial enzymes that are produced in largest amounts on a commercial scale (Ladenstein and Antrankian, 1998). The main applications of proteases are in the detergent, natural leather and food industries (Cowan *et al.,* 1985). In the detergent industry, thermostable proteases active at alkaline pH values are required. Furthermore, the enzyme should be resistant against bleaching and chelating agents (Sonnleitner and Fiechter, 1983) . On the other hand, proteases with high keratonolytic and elastolytic activities are currently used for soaking purposes in the leather industry. Proteases can also be used as catalysts for peptide synthesis by employing their reverse reaction (Ladenstein and Antranikian, 1998). Therefore, the isolation of proteases that can catalyse reaction under extreme conditions will be of value for all of the previously mentioned applications.

Thermostable proteases have been reported in thermophilic bacteria and hyperthermophilic archaea (Klingeberg *et al.,* 1991; Leuschner and Antranikian, 1995; Friedrich *et al.,* 1996). Thermostable proteases with optimal temperature for activity between 55 and 95°C have been described from thermophilic bacteria of the genera *Thermus* (Cowan *et al.,* 1982; Matsuzawa *et al.,* 1983; Saravani *et al.,* 1989), *Bacillus* (Endo, 1962; Takii *et al.,* 1987; Coolbear *et al.,* 1991), *Fervidobacterium* (Friedrich *et al.,* 1996), *Thermotoga* (Friedrich *et al.,* 1996) and *Thermoshipo* (Friedrich *et al.,* 1996). *Fervidobacterium pennavorans* a keratin degrading thermophilic bacterium isolated from hot springs in Azores Island, degrades chicken feather completely to amino acids and peptides at 80°C within 48h (Friedrich *et al.,* 1996).

In the present study we report on the growth, production and general properties of thermostable proteases from the thermophilic *Bacillus thermoleovorans* strain HSR.

MATERIALS AND METHODS

Time course of growth and protease production

Bacillus thermoleovorans strain HSR (Sunna *et al.,* 1997) was grown in 300 ml flasks containing 100 ml of a medium prepared as described by Janssen *et al*. (1991) with some modifications. The modified medium was composed of (g l^{-1}): tryptone, 5.0; yeast extract, 1.5; NaCl, 5.0; $MgCl_2 . 6H_2O$, 0.05; $MgSO_4 . 7H_2O$, 0.2; KCl, 0.1; KH_2PO_4, 0.1; K_2HPO_4, 0.3; $CaCl_2 . 2H_2O$, 0.3; $FeCl_3$, 0.01; vitamin solution (Wolin *et al.,* 1964), 1.0 ml; trace element solution (Wolin *et al.,* 1964) 5.0 ml. The final pH was 7.0. The inoculated flasks were incubated at 55°C in a shaking incubator (New Brunswick, USA) with an agitation rate of 300 rpm. Samples were taken at intervals for determination of growth, pH and protease activity.

Effect of nitrogen source on protease production

Bacillus thermoleovorans strain HSR was grown in 150 ml flasks containing 50 ml minimal medium, pH 7.0. This minimal medium consisted of the following (g l^{-1}): yeast extract, 6.0; K_2HPO_4, 1.0; $NaHPO_4$, 1.0; NaCl, 5.0. The medium was supplemented with 2% (w/v) of various organic and inorganic nitrogen sources. The inoculated flasks were incubated at 55°C in a shaking incubator with an agitation rate of 200 rpm. Cells were harvested after 36 h. For enzyme activity measurements, 50 ml culture broth was centrifuged at 6000 x *g* for 30 min at 4°C. The supernatants obtained were used to determine the protease activity as described in the standard assay.

Source of enzyme

Batch cultivation of *Bacillus thermoleovorans* strain HSR was performed in a 2.5-l membrane reactor (Bioengineering AG, Wald, Switzerland) containing 2 l of the above described medium. The medium was inoculated with 10% (v/v) of a 12-14 h preculture. The incubation temperature was 55°C and the agitation rate was 200 rpm. The pH was kept constant between 6.8 and 7.2, by the addition of 0.1 N sterile NaOH. Following the incubation for 18 h, the cells were collected by centrifugation at 6000 x *g* for 15 min at 4°C. The supernatant was concentrated by ultrafiltration with a PM 10 membrane (10-kDa cut-off, Amicon, Witten, Germany) and the sample was stored at -20°C.

Enzyme assay

The protease activity was determined by a modification of the method of Kunitz (1947). The assay mixture consisted of 0.25% (w/v) Hammarsten casein (Merck, Darmstadt, Germany) supplemented with 120 mM universal buffer (Britton and Robinson, 1931), pH 7.0 , and enzyme to give a final volume of 0.5 ml. The reaction mixture was incubated for 30 min at 80°C. The assay reaction was terminated by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid and was kept at room temperature for 30 min. After centrifugation for 20 min at 12,000 x *g*, the absorbance was read at 280 nm against a blank. One unit of protease activity is defined as the amount of enzyme that yields the equivalent of 1 µmol of tyrosine per min under the defined assay conditions.

Effect of pH and temperature on protease activity

The effect of pH and temperature on the enzyme activity was determined by incubating the ultrafiltrated crude extract at different temperature and pH values. The protease activity was assayed under standard assay conditions (with an assay time of 30 min).

Thermostability

The ultrafiltrated crude extract was dialyzed against 50 mM phosphate buffer, pH 7.0, and incubated at 60, 70, 80 and 90°C. Samples were removed at different time intervals and immediately cooled on ice. The residual protease activity was assayed under the standard assay conditions (with an assay time of 15 min).

Effect of protease inhibitors and metal ions

Dialyzed ultrafiltrated crude extracts were preincubated with various reagents and metal ions in a final concentration of 1 mM and 10 mM for 60 min at room temperature. The residual protease activity was measured according to the standard assay conditions.

Substrate specificity

The substrate specificty of the protease activity was determined by incubating 100 µl of the ultrafiltrated crude extract at 40°C for 10 min with 450 µl of 0.1% (w/v) chromogenic substrate prepared in 50 mM phosphate buffer, pH 7.0. The incubation was performed at a lower temperature in order to prevent the non-

enzymatic hydrolysis of the chromogenic substrate (Klingeberg *et al.,* 1991). The protease activity was detected by measuring the absorption of the released *p*-nitroaniline at 405 nm.

Electrophoresis

Sodium dodecyl sulfate poyacrylamide gel electrophoresis (SDS -PAGE) and activity zymogram staining was performed in 11.5% gels as described earlier (Klingeberg *et al.,* 1991). SDS low-M_r standard mixtures (Sigma, Deisenhofen, Germany) were used in order to determine the apparent molecular mass of the proteases. Proteins were stained with 0.25% Coomassie blue R-250.

RESULTS AND DISCUSSION

Growth and protease production

Growth of *B. thermoleovorans* strain HSR and extracellular protease production were followed for 54 h, during which pH changes were also recorded *(Figure 1)*. Maximal protease activity was messured after 18 h, at the early stationary growth phase, reaching a value of 650 U l^{-1}. Similarly, in the genus *Bacillus* the maximal production of extracellular proteases has been reported to occur in the late exponential and early stationary growth phase (Priest, 1977). The protease activity, however, remained without any significant change up to 30 h of growth, after which a decrease to almost 50% was observed. A gradual increase in the pH from 7.2 to 8.5 was detected throughout the cultivation.

Figure 1. Growth enzyme production and pH change during cultivation of *B. thermoleovorans* strain HSR. ,optical density; □, protease, , pH.

Influence of nitrogen source on growth and protease production

The effect of different nitrogen sources in the production of protease by *B. thermoleovorans* strain HSR are shown in Table 1. Casein hydrolysate supported growth but not protease production while brain heart-infusion, tryptone and casamino acids supported both growth and protease production of *B. thermoleovorans strain* HSR. Furthermore, brain heart-infusion induced the highest protease activity reaching a value of 43 U l^{-1}. A similar enhancement of activity was reported with *B. stearothermophilus* (Fujio and Kume, 1991) and *B. thermoliquefaciens* (Klimov *et al.,* 1988). In addition, different inorganic nitrogen sources were tested, but *B. thermoleovorans* strain HSR was only able to grow and produce proteases (10 U l^{-1}) in the presence of KNO_3 .

TABLE 1
Effect of Nitrogen Sources on Growth and Protease Production of *B. thermoleoovorans* strain HSR

Nitrogen Source (2% w/v)	Growth (36 h)	Protease Activity (U L^{-1})
BHI broth[a]	+	43
Urea	-	0
Casein hydrolysate	+	0
Casamino acids	+	7
Peptone	+	13
Tryptone	+	33
Ammonium sulfate	-	0
Ammonim nitrate	-	0
Ammonium chloride	-	0
Potassium nitrate	+	10

[a]BHI = brain heart-infusion

General characterization of the proteases

Electrophoretic studies of the protease crude extract using SDS-PAGE and zymograms showed the presence of at least three activity bands with molecular masses of 40, 45 and 57 kDa *(Figure 2)*. Proteases from the genus *Bacillus* are usually characterized by having molecular masses in the range of 25 to 30 kDa [2].

The extracellular proteases from *B. thermoleovorans* strain HSR were active between 30 and 90°C, with an optimum at 80°C *(Figure 3)*. At 90°C around 40%

Figure 2. 11.5% SDS-PAGE zymogram staining of thermostable extracellular proteases from *B. thermoleovorans* strain HSR. Lane 1 low-M_r protein markers; lane 2, zymogram profile of *B. thermoleovorans* strain HSR crude extract.

Figure 3. Effect of temperature on the activity of the extracellular proteases produced by *B. theermoleovorans* strain HSR.

of the maximal activity was still detected. The enzyme was also active in a broad pH range, namely between pH 4.0-10, with maximal activity at pH 7.0 *(Figure 4)*. At pH values of 8.0, 9.0 and 10 the enzyme retained more than 85, 65 and 35% of its maximal activity, respectively. Among the most thermoactive and thermostable enzymes are those produced by hyperthermophilic archaea. Klingeberg *et al.* (1991) reported the production of thermostable proteases by different hyperthermophilic archaea growing optimally between 75 and 90°C. All these proteases were optimally active at temperature values between 80 and 95°C, while their optimal pH value ranged between 7.0 and 9.0. Although the optimum temperature of growth of *B. thermoleovorans* strain HSR is 55°C and the maximum is 70°C, yet the optimal temperature for its proteolytic activity lies 25°C above its optimal growth temperature. Similarly, the optimal temperature for the activity of the protease from *B. stearothermophilus* KP 1236, (optimal growth temperature between 55 and 65°C) has been reported to be 80°C (Takii *et al.,* 1987).

Figure 4. Influence of pH on the activity of the extracellular proteases produced by *B. thermoleovorans* strain HSR.

The most remarkable property of the extracellular proteases produced by *B. thermoleovorans* strain HSR is their extreme thermostability. The proteases were thermostable, with full activity being retained in the absence of substrate and calcium even after an incubation period of 24 h at 60, 70 and 80°C (data not shown), while at 90°C it still showed half-life of more than 120 min. In contrast, thermolysin from *B. thermoproteolyticus* (Dalquist *et al.,* 1976) and caldolysin from *Thermus aquaticus* (Cowan *et al.,* 1987) were also stable up to 80°C, however, they required calcium ions for thermostabilization. The protease from *B. thermoruber* is less thermostable showing only 40% residual activity after 5 min incubation at 80°C (Manachini *et al.,* 1988). Some of the most thermostable proteases, with half-lives at 90°C between 30 and 50 h, are those reported from the crude extracts of *Thermococcus* strain AN1, *T. stetteri*, *T. celer*, *T. litoralis* and *Staphylothermus marinus* (Sonnleitner and Fiechter, 1983).

Effect of metal ions and inhibitors

The effect of metal ions and different protease inhibitors are shown in Table 2. The protease activity was enhanced by 2- and 5-fold in the presence of 1 mM Fe^{3+} and Fe^{2+}, respectively.Ferric and ferrous ions are probably cofactors bound to the active-site and consequently are involved in enzyme catalysis and

Table 2
Effect of Various Metals and Inhibitors on the Proteases of *B. thermoleovorans* Strain HSR

Addition[a]	Concentration (mM)	Class of Inhibitor	Residual Activity (%)
Without	1	-	100
Mg^{2+}	1	-	90
Mn^{2+}	1	-	65
Zn^{2+}	1	-	70
Co^{2+}	1	-	80
Ni^{2+}	1	-	40
Cu^{2+}	1	-	20
Ca^{2+}	1	-	90
Fe^{2+}	1	-	485
Fe^{3+}	1	-	190

Hg^{2+}	1	Cysteine	5
EDTA	1	Metal Chelator	100
EDTA	10	Metal Chelator	15
DFP	1	Serine	95
DFP	10	Serine	45
PMSF	1	Serine	95
PMSF	10	Serine	85

[a] Dialyzed ultrafiltrated crude extracts were preincubated with various inhibitors and metal ions for 60 min at room temperature. Residual protease activity was measured according to the standard assay conditions. DFP, diisopropylflouro-phosphate; PMSF, phenylmethylsulfonyl flouride.

structural stability (Dalquist *et al.,* 1976). When incubated with 1 mM Cu^{2+} and Hg^{2+}, only 20 and 5% of the initial protease activity was measured, respectively. The extracellular proteases produced by *B. thermoleovorans* strain HSR belong to the group of the metalloproteases (Kay, 1982). Like most of the known metalloproteases, the proteolytic activity exhibited by *B. thermoleovorans* strain HSR was inactivated in the presence of 10 mM EDTA showing only 15% of its maximal activity. This loss of activity could be due to the removal of an active site-bound cofactor involved in enzyme catalysis or to the removal of a metal ion from a site of structural stability (Dalquist *et al.,* 1976).

Substrate specificity

The specificity of the thermostable protease of *B. thermoleovorans* strain HSR to different synthetic peptides are presented in Table 3. The *pNA* of the basic amino acid arginine was preferably cleaved by the protease. A decrease in the protease specificity was observed with the increase of the peptide length. Thus, the lowest protease activity was measured against *N*-Suc-Ala-Ala-Pro-Phe-*pNA*. On the

Table 3
Specificity of *B. thermoleovorans* Strain HSR Proteases Towards Different Synthetic Peptides.

Substrate[a]	Relative Activity (%)
Z-Arg-pNA	100
Z-Lys-pNA	73
Suc-Phe-pNA	72
N-a-Bz-DL-Lys-pNA	48
N-Suc-Ala-Ala-Pro-Phe-pNA	9

[a] Abbreviations: Z, N-benzyloxycarbonyl; Suc, Succinyl; Bz, benzoyl; pNA, p-nitroanilide.

contrary, the same long peptide was the best substrate for the protease activity of the thermophilic bacterium *Thermobacteroides proteolyticus* and the hyperthermophilic archaea *Thermococcus* strain AN1, *T. litoralis* and *S. marinus* (Klingeberg *et al,* 1991).

This is the first report dealing with the characterization of proteases from *B. thermoleovorans*. The ability of this species to secrete extreme thermostable proteases makes this thermophilic bacterium a good candidate for the production of industrial relevant enzymes. However, further optimization of the cultivation conditions may be necessary in order to improve the production of its extracellular enzymes.

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