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# A Soluble Aggregated Thermophile Metalloaminopeptidase Produced by an Alcalophile Strain of *Bacillus halodurans*

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**Abstract:** H4 strain isolated from Lake Bogoria was found to be *Bacillus halodurans*. The Bacteria produced an extracellular peptidase activity toward substrates Ile-*p*NA, Met-*p*NA and Val-*p*NA. It also hydrolyzed small peptides. A purification procedure including ion-exchange chromatography ion exchange DEAE and size-exclusion chromatography followed by Sodium dodecyl sulphate-polyacrymalide gel electrophoresis (SDS-PAGE) revealed the aggregated form of the enzyme. The three substrates are hydrolyzed by a single catalytic site. The enzyme inactivated by bestatin, and 1,10-phenanthroline is a metalloaminopeptidase whose activity is maximal at pH 9.0 and 65ºC.

**Key words:** *Bacillus halodurans*, enzyme, extremophile environments, microbial peptidases, proteins

## INTRODUCTION

The inability of the production of peptidases by animals and plants to satisfy global demand has led to increased interest for microbial peptidases. Micro-organisms are indeed excellent sources of enzymes, because of their rapid growth, the limited space required for their culture and the ease with which new enzymes can be generated by genetic engineering.

The economic importance of the peptidases of bacterial origin dates from the 60s with the introduction of alkaline peptidases of bacteria of the genus Bacillus in the detergent industry. *Bacillus brevis* (Banerjee *et al*., 1999), Bacillus sp. SSR1 (Singh *et al*., 2001), and *Bacillus steorothermophilus* (Dhandapani and Vijayaragvan, 1994) are examples. Thermolysin is an example of metallopeptidase used in many industrial processes. It is capable of peptide synthesis under appropriate conditions (Jakubke and Konnecke, 1987; Clapes *et al*., 1995). It has been used for the production of a precursor of glutamate (Ooshima *et al*., 1985; Murakami *et al*., 1996).

Alkaline sediments of lakes contains many bacteria from bacillus gender as shown in previous work on the hypersaline lake Bogoria located in the Kenyan Rift Valley; its water has a pH value around 9.0 (Moallic *et al*., 2006). *Bacillus halodurans* H4 is among the strains isolated and identified from this lake after the sequencing of the 16 S rRNA gene and comparison with the databanks. Its genome encodes an original metalloendopeptidase belonging to the pitrilysin family, which was cloned and characterized (Dabonné *et al*., 2005). The in vitro culture of the same strain allowed us to explore its extracellular secreted peptidases. When screening the peptidases secreted by this bacterium, significant hydrolysis of the substrates Met-*p*NA, Val-*p*NA and Ile-*p*NA (MIV) were observed in the culture supernatant.

The present work describes the peptidase (pMIV) responsible for these enzymatic activities.

## MATERIALS AND METHODS

Experiments were conducted with the collaboration of laboratory biocatalysis of Nantes University (France)

**Bacterial strains and culture conditions:** Based on the rRNA 16 S gene homologies (98%) the strain isolated from the hot springs of Lake Bogoria (Kenya) was proved to correspond to the *Bacillus halodurans* sp. This strain was aerobically grown at 37ºC for 48 h in the following medium named BP13 (pH 9.0) : 10 g/L glucose, 5 g/L peptones, 5 g/L yeast extract, 0.120 g/L $Na_2CO_3$, 1.125 g/L $NaHCO_3$, 0.012 g/L $Na_2SO_4$, 0.0005 g/L $NH_4Cl$, 0.125 g/L NaCl, 0.095 g/L KCl, 0.0075 g/L $CaCl_2$, 0.0070 g/L NaF, 0.025 g/L silica. Growth was monitored by measuring the optical density at 600 nm.

**Chemicals:** Chromogenic *p*-nitroanilide substrates (Ala-*p*NA, Arg-*p*NA, Asp-*p*NA, Benzoyl Arginyl-*p*NA, α-Glu-*p*NA, γ-Glu-*p*NA, Ile-*p*NA, Leu-*p*NA, Lys-*p*NA, Met-*p*NA, Pro-*p*NA, Val-*p*NA) were purchased from Bachem. Lys-Glu-Ile, Met-Arg-Phe-Gly, Ala-Ile-Val-Tyr-Gly-Gly and insulin oxidized B-chain were from Sigma. 1,10-

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phenanthroline, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), bestatin, pepstatin and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich. DEAE-Sepharose Fast Flow, Sephacryl S-300, S-200 and S-100 HR, were purchased from Amersham Biosciences. The chemicals used for polyacrylamide gel electrophoresis came from Bio-Rad. All other chemicals and reagents were of analytical grade.

**Chromatography process:** After the cultivation, cells were removed from the culture broth by centrifugation at 12,000 x g for 20 min and supernatants were concentrated by acetone precipitation at -20ºC and resuspended in 20 mM Tris-HCl buffer pH 7.8. The first step of the purification process was performed by ion-exchange chromatography on DEAE-Sepharose FF (2.4 x12.5). The crude extract (1.230 mL) was loaded onto a DEAESepharose Fast Flow (FF) column (gel bed, diameter of 5.0 cm and height of 14 cm) washed with 3 column volumes of starting buffer (20 mM Tris-HCl pH 7.8). Proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in starting buffer. The flow rate was 2.5 mL/min and fractions of 1.6 mL were collected. The fractions containing the Met-*p*NA, Ile-*p*NA and Val-*p*NA activities were pooled and concentrated by vacuum evaporation (from 9.6 to 3.5 mL). The concentrated extract is dialysed against 20 mM barbital buffer pH 9.0 and 1 mL is successively loaded onto Sephacryl S-100 HR, Sepahcryl S-200 and Séphacryl S-300 columns (column (1.6x57.0 cm) equilibrated with 20 mM barbital buffer pH 9.0. Sodium dodecyl sulphate-polyacrymalide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 10 and 7.5% as resolving gel and 4.5% as stacking gel.

**Enzyme assays:** The enzymatic activity was measured at 50ºC for 10 min in a final volume of 250 µL containing 0.8 mM of chromogenic substrate or 50 µg of peptide in 20 mM barbital buffer (pH 9.0); the reaction was initiated by adding 2.5 µg of the enzyme. The decrease in the amount of substrates di-peptide, tri-peptide and insulin oxidized B-chain, the release of the reaction products were easily monitored at 214 nm by capillary electrophoresis using a Beckman P/ACE System 5000 with an uncoated fused-silica capillary (47 cm total length, 40 cm effective length, 75 Am ID (Niamke *et al*., 1999). In the case of *p*-nitroanilide amino acids, the hydrolysis was monitored by measuring the increase in absorbance at 450 nm using a Labsystem iEMS spectrophotometer. Protein concentration was determined by the bicinchoninic acid method (Smith *et al*., 1985) using bovine serum albumin as the standard.

The enzymatic unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of Met-*p*NA, Ile-*p*NA and Val-*p*NA per min at 50ºC under the conditions described above. The kinetic parameters (*Km*, and *v*) were determined from Lineweaver–Burk and Eadie representations using different concentrations (50-800 µM) Met-*p*NA, Ile-*p*NA and Val-*p*NA. Each experimental point was determined at least in triplicate.

**Effect of pH and temperature on activity:** The effect of pH on the enzyme activity was determined at 50ºC by performing the incubations for 10 min according to the standard assay procedure, at various pH values in the following buffer systems: tris-HCl buffer (50 mM) from pH 7.0 to 9.0, phosphate buffer (50 mM) from pH 6.0 to 8.0, barbital buffer (50 mM) from 7.0 to 9.0 and Glycine-NaOH buffer (50 mM) from 8.5 to 10.6. For determination of the optimum temperature, the incubations were carried out for 5 min at various temperatures ranging from 35 to 85ºC in 50 mM barbital buffer (pH 9.0).

**Effect of protease inhibitors:** All inhibitors were prepared as stock solutions in the appropriate solvent: water for E-64, EDTA and EGTA; methanol for 1,10-phenanthroline, 1,6-phenanthroline, PMSF, bestatin and pepstatin. Inhibitors were preincubated at room temperature for 1 h with the enzyme. The reaction was initiated by addition of the substrate. Residual activity was determined as a percentage of the activity in control samples without inhibitor. Appropriate solvent controls were run in parallel when required.

**Mutual competition between substrates:** Experiments were conducted with each substrate separately and then combined in pairs. Theoretical calculations were made based on the equation of Dixon (1) (Dixon and Webb, 1979). Using this relation, we calculated the speeds where the enzyme has a single site.

$$v = \frac{v_a\left(1+\frac{a}{K_a}\right)+v_b\left(1+\frac{b}{K_b}\right)}{1+\frac{a}{K_a}+\frac{b}{K_b}} \qquad (1)$$

Equation of Dixon and Webb (1979).

$a$ or $b$ : Individual concentration of each substrate

$v_a$ or $v_b$ : Speed of reaction with respect to each substrate

$K_a$ or $K_b$ : Michaelis constant with respect to each substrate

$v$ : Overall rate of reaction for a single catalytic site

## RESULTS AND DISCUSSION

**Bacterial and growth conditions:** The *Bacillus halodurans* H4 was isolated from Lake Boria with six other species namely *B. licheniformis*, *B. pumilus*, *B. flavothermus*, *B. lautus*, *B. thuringiensis*and, *B. pseudofirmus.* It can be considered as a strictly alcalophile bacteria like most of the bacterium isolated in alcalophile lake are (Grant, 1992). *Bacillus halodurans* is the second species of the genus Bacillus whose genome was fully sequenced (Takami *et al*., 1992). Isolated since 1977, strain C-125 was identified after analysis of DNA-DNA hybridization as a member of the halodurans species by Takami and Horikoshi (1999). The strain *B. halodurans* H4 was well cultivated at pH 9.0, in BP13 an alcalophile medium. The MIV (enzyme hydrolysing Met-*p*NA, Ile-*p*NA and Val-*p*NA) activity appeared after about 20 h of the microorganism cultivation and increased until 48 h. As the cells began to lyse after 20 h, it is difficult to establish if the activities in the culture supernatant came from a secreted enzyme or from an intracellular enzyme that was liberated after cell lysis. Since we did not detect any intracellular activity after sonication of the bacterial cells, the MIV of strain H4 is probably an extracellular enzyme like the serine protease produced by *Bacillus clausii* GMBE 22 (Kazan *et al*., 2009). Monitoring both de bacterial growth and the enzyme activity led us to observe the pMIV production in the late exponential growth phase, when nutrients are limiting, suggests that the enzyme could have a nutritional function as previously reported for Gamma-Glutamyl Transpeptidase of *B. subtilis* (Kimura *et al*., 2004).

**Purification of pMIV:** The supernatant was carried out by acetone precipitation. The first step, which was performed on a DEAE-Sepharose FF column, enabled pMIV (eluted at 0.25 M NaCl) to be separated from most proteins eluted in the washing step Fig. 1A. The following step consisting in a size-exclusion chromatography on a

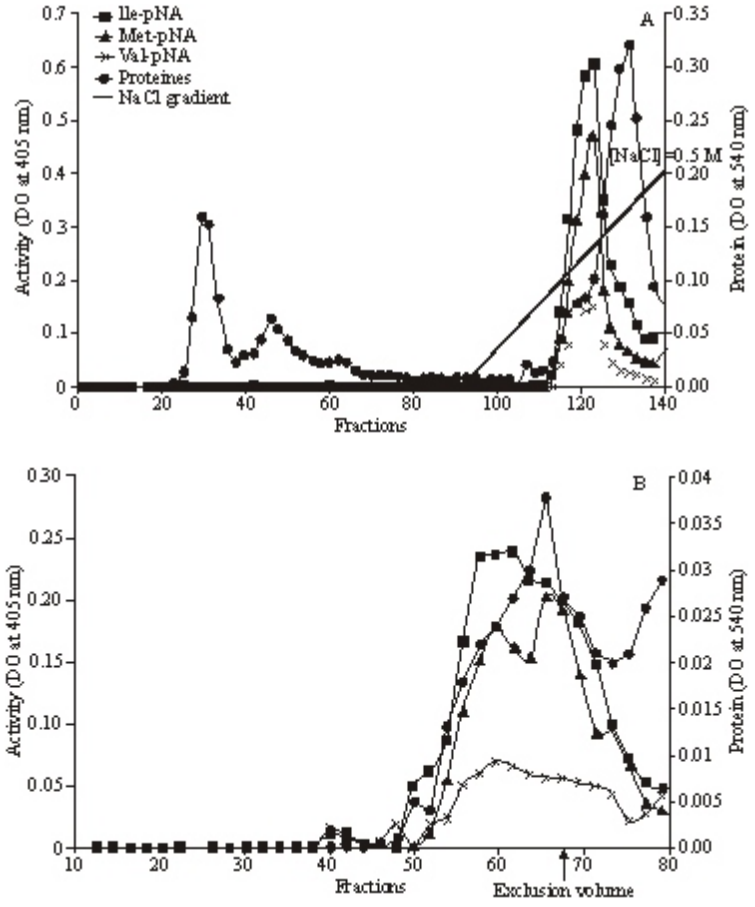


Fig. 1: Purification of *B. Halodurans* pMIV. (A) Anion exchange chromatography on DEAE-Sepharose FF column. (B) Gel filtration chromatography on Sephacryl S-300 HR column. The hydrolytic activity towards Ile-*p*NA, Met-*p*NA and Val-*p*NA is collected in fractions of the exclusion volume. Enzyme activity was measured at pH 9.0 and 50ºC

Table 1: Mutual competition between substrates Ile-*p*NA, Met-*p*NA and Val-*p*NA The calculated values in the case of distinct catalytic sites are obtained by the sum of the two activities. The values calculated in the case of a common site are determined by the equation of Dixon (Dixon and Webb, 1979). The results reported in tablecl indicated that a single catalytic site is responsible for the hydrolysis of three substrates

| Experimental values with single substrate (DO at 405 nm) | | | | | |
|---|---|---|---|---|---|
| Ile-*p*NA | Met-*p*NA | Val-*p*NA | Experimental values with substrates in pair | Values for a single site (Dixon and Webb, 1979) | Values for two substrates (sums) |
| 0.097 | 0.096 | | 0.090 | 0.100 | 0.193 |
| | 0.096 | 0.57 | 0.095 | 0.100 | 0.153 |
| 0.097 | | 0.57 | 0.099 | 0.098 | 0.155 |

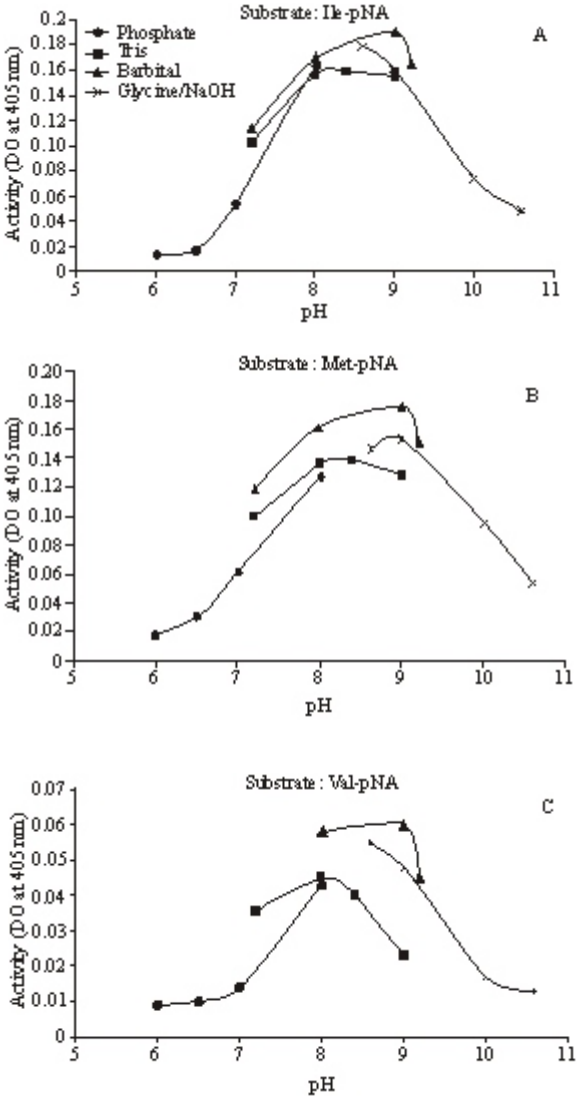


Fig. 2: Effect of pH on the extracellular peptidase activity on substrates (A) Ile-*p*NA, (B) Met-*p*NA and (C) Val-*p*NA

Sephacryl column showed pMIV activity successively with S-100, S-200 and S-300 in the exclusion volume of the column (Fig. 1B). Séphacryl S-300 is used to separate proteins whose size are between 10 000 and 1 500 000 Da. This result suggests that the molecular weigh of

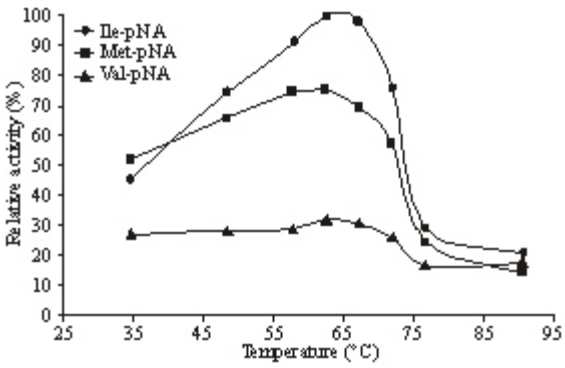


Fig. 3: Effect of temperature on the extracellular peptidase activity on substrates Ile-*p*NA, Met-*p*NA and Val-*p*NA

pMIV is above 1 500 000 Da revealing its aggregated form. Sodium dodecyl polyacrymalide (10%) gel electrophoresis (SDS-PAGE) performed on the active fractions of S300 did not show any band confirming the hypothesis of an aggregated form (Data not shown).

**Single catalytic site:** During the chromatographic steps, pMIP was found in the same fractions. These initial results raised the question of whether these three substrates are hydrolyzed by a single protein with one or more catalytic sites or by or several proteins forming an enzymatic complex in the aggregated form.

Results of competition tests between substrates helped to answer this question. The values calculated in a case of distinct sites are obtained by the sum of the two activities. The values calculated in the case of a common site are determined by the equation of Dixon. They are compared to the experimental values obtained when the substrates are combined in pairs. These values are closed to those determined by the equation of Dixon and Webb (1979) (Table 1). pMIV is a non specific peptidase like the one isolated from *Aeromonas veronii* PG01 (Divakar *et al*., 2010).

**Effects pH, temperature and inhibitors:** pMIV was analyzed for its hydrolytic pH an temperature dependence. The maximum activity was observed at pH 9.0 (Fig. 2) and 65ºC (Fig. 3) in a barbital buffer for the three

Table 2: Effect of different inhibitors on peptidase activity responsible for extracellular hydrolysis of Ile-*p*NA, Met-*p*NA and Val-*p*NA

| | | | Relative activity (%) Substrates | | |
|---|---|---|---|---|---|
| Inhibitors | Class | Concentration (mM) | Ile-*p*NA | Met-*p*NA | Val-*p*NA |
| None | | | 100 | 100 | 100 |
| 1,10-phenanthroline | Metallopeptidase | 1.0 | 32±2 | 52±3 | 46±2 |
| 1,10-phenanthroline | - | 5.0 | 10±3 | 15±2 | 25±3 |
| Bestatin | Aminopeptidase | 0.1 | 11±2 | 21±1 | 35±3 |
| Bestatin | - | 0.5 | 9±2 | 13±3 | 30±2 |
| E64 | Cystein peptidase | 0.5 | 96±2 | 95±1 | 86±1 |
| PMSF | Serine peptidase | 1.0 | 100 | 100 | 100 |
| Pestatin | Aspartic peptidase | 0.5 | 78±2 | 80±2 | 82±1 |

All activities were measured under conditions of initial velocity. The values reported represent the means and the standard errors of four determinations

Table 3: Hydrolytic activity of pMIP on some substrates

| Substrates | Hydrolytic activity (%) |
|---|---|
| Ile-*p*NA | 100.0 |
| Met-*p*NA | 97.3±2.8 |
| Val-*P*na | 89.8±2.2 |
| Benzoyl Arginyl-*p*NA | 00.0 |
| Ala-*p*NA | 00.0 |
| Asp-*p*NA | 00.0 |
| Arg-*p*NA | 00.0 |
| α-Glu-*p*NA | 00.0 |
| γ-Glu-*p*NA | 00.0 |
| Leu-*p*NA | 00.0 |
| Lys-*p*NA | 00.0 |
| Pro-*p*NA | 00.0 |
| Lys-Glu-Gly | 21.4±2.1 |
| Gly-Gly-Arg-Leu | 19.4±1.3 |
| Met-Arg-Phe-Gly | 16.6±0.4 |
| Ala-Ile-Val-Tyr-Gly-Gly | 09.5±1.8 |
| Insulin oxidized B-chain | 00.0 |

All activities were measured under conditions of initial velocity. The values reported represent the means and the standard errors of four determinations

substrates. This result reinforces the hypothesis of the involvement of a single catalytic site. With this temperature and pH of activity, pMIV can be considered as thermo-alcalophile enzyme if compared with an alanine aminopeptidase from bovine skeletal muscle which activity was optimal at pH 8.0 ant 37ºC (Xiu and Tzi, 2011).

Several inhibitors targeting different classes of proteases were assayed on pMIV activity.

The cystein, serine and aspartic protease inhibitors had no significant effect on the activity. On the other hand, the peptidase was significantly inactivated by 1,10-phenanthroline, which is known as a specific inhibitor of metallopeptidases. Bestatin which specifically inhibits aminopeptidase, produced a strong inhibition of the activity (Table 2). The enzyme responsible for hydrolysis of three substrates would therefore belong to the metalloaminopeptidase calss. An other metallopeptidase with an endopeptidase activity was cloned from the same bacterial strain (Dabonné *et al*., 2005).

**Substrates hydrolytic specificity**: pMIV from *Bacillus halodurans* was essayed for his capability to hydrolyze a variety of substrates. The results in Table 3 showed that pMIV hydrolyses a broad range of substrates. Only Met-*p*NA, Ile-*p*NA and Val-*p*NA were hydrolyzed among the chromogenic *p*-nitroanilide substrates. The highest activity was observed with Ile-*p*NA as substrate (100±2.3%), followed by Met-*p*NA (97.3±2.8) and Val-*p*NA (89.8±2.2). With these results, pMIV seemed to recognize some specific residues, Isoleucine (Ile), Methionine (Met) and Valine (Val) at the N-terminus like aminopeptidase isolated from chicken intestine which exhibited activity only toward chromogenic substrates containing residues Leu, Phe or Ala (Sushma *et al*., 2010). Thereafter the hydrolysis of various peptides including some that did not contain residues Ile, Met and Val showed a much broader specificity. pMIV failed to hydrolyses the Insulin oxidized B-chain, a peptide containing 30 amino acid. The enzyme hydrolytic activity is therefore limited to small substrates and showed a nonspecific peptidase that can be used to form banks of peptide sequences from protein (Kanoh *et al*., 1993; De Laureto *et al*., 1995).

## CONCLUSION

This study highlights and describes a novel thermostable peptidase activity produced by a strain of *B. halodurans*. The physicochemical properties of the enzyme make it a good candidate for possible application in protein chemistry.

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