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Cloning, expression and characterization of a 46.5-kDa metallopeptidase from *Bacillus halodurans* H4 sharing properties with the pitrilysin family

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Abstract

A 1242 base pair DNA fragment from *Bacillus halodurans* H4 isolated from alkaline sediments of Lake Bogoria (Kenya) coding for a potential protease was cloned and sequenced. The hexa-histidine-tagged enzyme was overexpressed in *Escherichia coli* and was purified in one step by immobilized-metal affinity chromatography (IMAC) on Ni-NTA resin. The protease (ppBH4) presents an inverted zincin motif, HXXEH, which defines the inverzincin family. It shares several biochemical and molecular properties with the clan ME family M16 metallopeptidases (pitrilysins), as well as with database hypothetical proteins that are potential M16 family enzymes. Thus, like insulysin and nardilysin, but contrary to bacterial pitrilysin, ppBH4 is inactivated by sulfhydryl alkylating agents. On the other hand, like bacterial pitrilysin, ppBH4 is sensitive to reducing agents. The enzymatic activity of ppBH4 is limited to substrates smaller than proteins. In contrast to insulin, dynorphin and insulin B-chain are very good substrates for ppBH4 and several cleavage sites are common with those observed with well-characterized pitrilysins. As deduced from amino acid sequence, as well as determined by gel-filtration and SDS-polyacrylamide gel electrophoresis, ppBH4 is an active monomer of 46.5 kDa. This feature distinguishes ppBH4 from all other enzymes of the pitrilysin family so far described whose molecular masses range from 100 to 140 kDa.

Keywords: Pitrilysin; Metallopeptidase; Inverzincin; Dynorphin; Insulin B-chain; *Bacillus halodurans*

1. Introduction

The search for potential sources of proteases led us to isolate strains from alkaline sediments of Lake Bogoria (Kenya) which optimally grow above pH 8.0. Thirty-seven strains were identified after the sequencing of the 16 S rRNA gene and comparison with the databanks. Twenty-six strains could be attached to the Bacillus genus, namely *B. licheniformis*, *B. pumilus*, *B. flavothermus*, *B. halodurans*, *B. lautus*, *B. thuringiensis* and *B. pseudofirmus*. The strain H4 showed a 98% identity with *B. halodurans* C-125, the genome of which is known [1]. The latter contains a gene (BH 2405) showing a 75% homology with a putative insulysin from *B. anthracis*, a peptidase which belongs to the family M16 of the clan ME metallopeptidases according to the classification proposed by Barrett et al. [2].

The family M16 (pitrilysin family) can be divided into three subfamilies. The M16A subfamily contains oligopeptidases such as insulysin (EC 3.4.24.56) and nardilysin (EC 3.4.24.61) from animals [3,4] and pitrilysin (EC 3.4.24.55), also called protease III, from bacteria [5]. In *Escherichia coli*, pitrilysin shows structural and functional homologies with human insulysin [6,7] and cleaves the B-chain of insulin at the same bonds. The bacterial enzyme, like the human and other mammalian insulysins, consists of a single polypeptide of approximately 110 kDa. In addition, a

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molecular form of 50 kDa has been mentioned in *E. coli* [3,8].

The mitochondrial processing peptidase (MPP, EC 3.4.24.64) is a typical example of the second subfamily (M16B). This enzyme, which removes an N-terminal targeting signal from mitochondrial proteins during import, is composed of two non-identical α-and β-subunits of about 50 kDa, both of which are pitrilysin homologs [2,9]. Finally, the third subfamily (M16C) is essentially represented by eupitrilysin and falcilysin [10]. It should be noted that the three subfamilies share primary structural features including an inverted motif HXXEH rather than the more common HEXXH motif of zinc metalloendopeptidases [11].

In the present work, cloning and expression of the putative protease gene from *B. halodurans* H4 are reported. The main characteristics of the enzyme encoded show that it is a novel member of the pitrilysin family.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Strain H4 was isolated from the hot springs of Lake Bogoria (Kenya). It proved to correspond to the *Bacillus halodurans* species based on the rRNA 16 S gene homologies (98%). This strain was aerobically grown at 37 °C in the following medium (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. *E. coli* XL1 blue (Stratagene) and BL21(DE3) (Novagen) strains were used as host strains for cloning. They were grown at 37 °C in LB (Luria-Bertani) medium. Plasmid vector pET21d (Novagen) was used for overexpression and gave ampicillin resistance.

2.2. Chemicals

Chromogenic *p*-nitroanilide substrates, neurotensin, kemptide, leu-enkephalin and dynorphin A (fragments 1–10, 1–13 and 2–17) were purchased from Bachem. Porcine pancreas insulin and oxidized B-chain, glucagon, azocasein, bovine serum albumin, Na_2-EDTA, EGTA, 1, 10-phenanthroline, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), N-ethylmaleimide, iodoacetamide and bestatin were obtained from Sigma-Aldrich. Protein standards for molecular mass determination and the chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad. All other chemicals and reagents were of analytical grade.

2.3. Recombinant DNA techniques, cloning procedure, overexpression and purification

Chromosomal DNA of strain H4 was prepared as follows: cells were centrifuged, resuspended in water and treated with 1 volume of phenol/chloroform (1:1,v/v). The aqueous fraction was precipitated with ethanol and the corresponding pellet was resuspended in TE1X.

Amplification of putative protease (ppBH4): the PCR was carried out with the *pfu* polymerase from the chromosomal DNA of strain H4 with the primers: pp1 5′-ACGATCCATGGTTAACACGATGACGCTA-3′ and pp2 5′-TATACTCGGTGAATCAATGCTTTTGGAAG-3′ corresponding, respectively, to the 5′ and 3′ ends. They created restriction sites for endonucleases *Nco*I and *Xho*I.

The amplified 1242 bp DNA was digested by *Nco*I and *Xho*I and ligated in the plasmid vector pET21d by the T4 DNA ligase. The ligation mixture was then used to transform *E. coli* BL21(DE3). BL21(DE3)/pETpp was grown at 37 °C in LB containing ampicillin (100 μg/ml), induced with IPTG at OD_{600} of 1.4 and grown for 4 h. Cells were harvested and resuspended in lysis buffer consisting of 50 mM NaH_2PO_4, 500 mM NaCl and 12.5 mM imidazole (pH 8.0). Lysozyme was added (final concentration: 0.2 mg/ml) and the solution was incubated at 37 °C for 30 min. Sonication was carried out on ice for 30 min in an ultrasonic processor. The extract was clarified by centrifugation for 10 min at 10 000 × g and His-protein was purified by immobilized-metal affinity chromatography (IMAC) on Ni-NTA superflow (Qiagen). A volume of 8 ml of the clear lysate was added to 4 ml Ni-NTA previously equilibrated in lysis buffer. The mixture was shaken at 4 °C for 60 min before being loaded on a column. After washing the column with six bed volumes of lysis buffer containing 25 mM imidazole, the protein was recovered with a lysis buffer containing 200 mM imidazole. The purity of the preparation was checked by SDS-PAGE on 12% polyacrylamide gel.

2.4. Enzyme assays

The proteolytic activity of the enzyme was tested with potential substrates including porcine pancreas insulin; oxidized B-chain (insulin); bovine serum albumin; dynorphin A (fragments 1–10, 1–13 and 2–17); neurotensin; glucagon; kemptide; leucine-enkephalin; azocasein; *p*-nitroanilide amino acids. Under the standard test conditions, the enzyme activity was measured at 37 °C for different reaction times between 10 and 45 min with 50 μg of substrate in 50 mM sodium phosphate buffer (pH 8.0). The reaction was initiated by adding the protease (2.5 μg). The final volume was 200 μl and the reaction was stopped by heating the mixture at 100 °C for 3 min. The decrease in the amount of substrate and 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 μm ID) [12]. In the case of *p*-nitroanilide amino acids, the concentration of the chromogenic substrate was 1 mM in the reaction mixture and the hydrolysis was monitored by measuring

the increase in absorbance at 450 nm using a Labsystem iEMS spectrophotometer.

The kinetic parameters (K_m, k_{cat} and V_{max}) were determined from Lineweaver–Burk and Eadie representations using different concentrations (10–230 μM) of insulin B-chain and (60–380 μM) dynorphin A (fragment 1–13). In all cases, the initial rate was used for plotting.

Protein concentration was determined by the bicinchoninic acid method [13] 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 substrate per min at 37 °C under the conditions described above.

2.5. Determination of cleavage sites

Dynorphin A (fragment 1–13) or insulin B-chain (50 μg) was incubated at 37 °C for reaction times between 30 min and 12 h in 50 mM phosphate buffer (pH 8.0) with 2.5 μg of the purified protease (final volume: 200 μl). The separation of fragments obtained after digestion was performed by high performance liquid chromatography (HPLC) using a Waters system equipped with a Supelcosil LC-308 C8 (300Å) reversed phase column (4.6 × 250 nm) from Supelco. Elution was achieved with a 5–90% (v/v) linear gradient (50 ml) of acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min.

Peptidic fragments were collected then identified by the determination of their molecular weight obtained by mass spectra using a PE Biosystems Voyager-DE STR+ spectrometer in linear mode and by the analysis of their N-terminal amino acid sequence performed by automated Edman degradation using a Perkin-Elmer Procise 494-HT protein sequencer with the reagents and methods of the manufacturer.

2.6. Effect of pH and temperature on activity

The effect of pH on the enzyme activity was determined at 37 °C by performing the incubations for 20 min according to the standard assay procedure but at various pH values in the following buffer systems: sodium phosphate buffer (100 mM) from pH 6.5 to 8.0, sodium barbital (veronal) buffer (100 mM) from pH 6.8 to 9.2, Tris–HCl buffer (100 mM) from pH 7.4 to 9.0 and glycine–NaOH buffer (100 mM) from pH 9.0 to 10.0.

For the determination of the optimum temperature, the incubations were carried out at various temperatures ranging from 30 to 70 °C in 100 mM phosphate buffer (pH 8.0).

2.7. Metal analysis

The purified protease samples were dialyzed extensively against Milli-Q deionized water and the amount of metal in each preparation was determined by flame atomic absorption spectrophotometry (FAAS) (Varian SpectraAA 55 spectrophotometer). A zinc standard curve was established by using zinc solutions specific for atomic absorption spectrophotometry. The assays were performed from three different preparations.

2.8. Effect of ions and protease inhibitors

Studies with ions (1 mM of each ion in the reaction mixture) were performed under the standard test conditions. All inhibitors were prepared as stock solutions in the appropriate solvent: water for E-64, Na_2-EDTA and EGTA; methanol for 1, 10-phenanthroline and bestatin. Inhibitors were preincubated at room temperature for 1 h with the enzyme (2.5 μg). The reaction was initiated by the addition of insulin B-chain (50 μg). Residual activity was determined as a percentage of the activity in control samples without inhibitor. Appropriate solvent controls were run in parallel when required.

3. Results

3.1. Cloning and nucleotide sequence of the protease gene of B. halodurans H4 (ppBH4)

Analysis of the sequenced genome of *B. halodurans* (strain C-125) [1] allowed us to locate a putative pitrilysin. To identify its features, the purification and the determination of its properties had to be undertaken. In our laboratory, we had another strain of *B. halodurans* (termed H4) which we had isolated from Lake Bogoria (Kenya). This strain is slightly thermophilic (optimal growth temperature: 50 °C), which could provide a more stable enzyme, and is supposed to contain the same open reading frame with few modifications. We therefore cloned the gene encoding for ppBH4 using primers deduced from the genome of the strain C-125.

A single 1242-base pair DNA corresponding to the predicted size of the putative protease gene of *B. halodurans* C-125 was amplified by PCR from the chromosomal DNA of the strain H4, using the primers described in Materials and methods. The amplified DNA was sequenced and 98.8% identity was found over the entire length of the sequence with the putative processing protease of *B. halodurans* C-125. This protein belongs to the family M16 (clan ME) of metallopeptidases, which are characterized by the motif HXXEH in their active site, where X represents any residue. This motif was also found in the protease of the strain H4 (ppBH4) (Fig. 1).

3.2. Expression, purification and N-terminal sequence of ppBH4

The gene cloned in pET21d vector gave the pETpp plasmid, which was introduced into BL21(DE3). After IPTG induction, samples of *E. coli* BL21(DE3)/pETpp were

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ppBH4          ------------------------------------------------------------
YEAST β-MPP    -----------------------------------MFSRTASKFRNTRRLLSTISSQI
E.COLI PTR     --------------------MPRSTWFKALLLLVALWAPLSQAETGWQPIQETIRKSDKDN
HUMAN INS      MRYRLAWLLHPALPSTFRSVLGARLPPPERLCGFQKKTYSKMNNPAIKRIGNHITKSPEDK

ppBH4          -MVNTMTLDNGVRIITEKMSTVRSVSIGIWVGTGSRYESAEENGISHFLEHMFFKGTNTR
YEAST β-MPP    PGTRTSKLPNGLTIATEYIPNTSSATVGIFVDAGSRAENVKNNGTAHFLEHLAFKGTQNR
E.COLI PTR     RQYQAIRLDNGMVVLLVSDPQAVKSLSALVVPVGSLEDPEAYQGLAHYLEHMSLMGSKKY
HUMAN INS      REYRGLELANGIKVLLMSDPTTDKSSAALDVHIGSLSDPPNIAGLSHFCEHMLFLGTKKY

ppBH4          -SAQEIAEFFDSIGGQVNAFTSKEYTCYYAKVLDDHAGQAIDTLSDMFFHSTFQKEELEK
YEAST β-MPP    -SQQGIELEIENIGSHLNAYTSRENTVYYAKSLQEDIPKAVDILSDILTKSVLDNSAIER
E.COLI PTR     PQADSLAEYLKMHGGSHNASTAPYRTAFYLEVENDALPGAVDRLADAIAEPLLDKKYAER
HUMAN INS      PKENEYSQFLSEHAGSSNAFTSGEHTNYYFDVSHEHLEGALDRFAQFFLCPLFDESCKDR

ppBH4          ERKVVFEEIKMVDDTPDDIVHDLLSSATYGKHSLGYPILGTVETLNSFN-------EEML
YEAST β-MPP    ERDVIIRESEEVDKMYDEVVFDHLHEITYKDQPLGRTILGPIKNIKSIT-------RTDL
E.COLI PTR     ERNAVNAELTMARTRDGMRMAQVSAETINPAHPGSKFSGGNLETLSDKP---GNPVQQAL
HUMAN INS      EVNAVDSEHEKNVMNDAWRLFQLEKATGNPKHPFSKFGTGNKYTLETRPNQEGIDVRQEL

ppBH4          RHYMDRFYTGNYVVISVAGNV-HDELIDKIKETFSQVKPTTFTY---------QGEKPMF
YEAST β-MPP    KDYITKNYKGDRMVLAGAGAVDHEKLVQYAQKYFGHVPKSESPVPLGS----PRGPLPVF
E.COLI PTR     KDFHEKYYSANLMKAVIYSNKPLPELAKMAADTFGRVPNKESKKPEITVPVVTDAQKGII
HUMAN INS      LKFHSAYYSSNLMAVCVLGRESLDDLTNLVVKLFSEVENKNVPLPEFPEHPFQEEHLKQL

ppBH4          LPN-RIVRKKETEQAHLCLGYPGLPIGDKDVYALVLLNNVLGG---------SMSSRLFQ
YEAST β-MPP    CRGERFIKENTLPTTHIAIALEGVSWSAPDYFVALATQAIVGNWDRAIGTGTNSPSPLAV
E.COLI PTR     IHYVPALPRKVLRVEFRIDNNSAKFRSKTDELITYLIGNRSPGTLSDWLQKQGLVEGISA
HUMAN INS      YKIVPIKDIRNLYVTFPIPDLQKYYKSNPGHYLGHLIGHEGPGSLLSELKSKGWVNTLVG

ppBH4          DIREKRGLCYSVFSYHSSFRDSGMLTIYAGTGHDQ--LDDLVYSIQETTSALAEKGLTEK
YEAST β-MPP    AASQNGSLANSYMSFSTSYADSGLWGMYIVTDSNEHNVQLIVNEILKEWKRIKSGKISDA
E.COLI PTR     NSDPIVNGNSGVLAISASLTDKGLANRDQVVAAIFSYLNLLREKGIDKQYFDELANVLDI
HUMAN INS      GQKEGARGFMFFI-INVDLTEEGLLHVEDIILHMFQYIQKLRAEGPQEWVFQECKDLNAV

ppBH4          ELENGKEQLKGSLMLSLESTNSRMS-RNGKNELLLKKHRSLDEMIEQINAVQKQDVSRLA
YEAST β-MPP    EVNRAKAQLKAALLLSLDGSTAIVE-DIGRQVVTTGKRLSPEEVFEQVDKITKDDIIMWA
E.COLI PTR     DFRYPSITRDMDYVEWLADTMIRVPVEHTLDAVNIADRYDAKAVKERLAMMTPQNARIWY
HUMAN INS      AFRFKDKERPRGYTSKIAGILHYYPLEEVLTAEYLLEEFRPDLIEMVLDKLRPENVRVAI

ppBH4          KILLSASPSISLINANGELPKALIH*
YEAST β-MPP    NYRLQNKPVSMVALGNTSTVPNVSYIEEKLNQ*
E.COLI PTR     ISP--KEPHNKTAYFVDAPYQVDKISAQTFADWQKKAADIALSLPELNPYIPDDFSLIKS
HUMAN INS      VSKSFEGKTDRTEEWYGTQYKQEAIPDEVIKKWQNADLNGKFKLPTKNEFIPTNFEILPL
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Fig. 1. Alignment of sequences of ppBH4 with yeast mitochondrial processing peptidase β-subunit (YEAST β-MPP), *E. coli* pitrilysin (E.COLI PTR) and human insulysin (HUMAN INS). The alignment was carried out from the N-terminal extremity of ppBH4. The framed residues correspond to amino acids involved in zinc coordination and in catalysis. (-) Indicates an insertion in a sequence. (*) Indicates the end of the amino acid sequence.

analyzed by SDS-PAGE (Fig. 2) and the His-tagged enzyme was purified in one step on Ni-NTA resin.

The N-terminal amino acid sequence determined by Edman degradation corresponded to the sequence M-V-N-T-M-T-L-D-N-G-V-R (Fig. 1). The Genpeptide database identified this sequence as being the N-terminal sequence of a putative protease of *B. halodurans*.

3.3. Molecular properties

The molecular weight of 46,485 deduced from the amino acid sequence (413 residues) is in good agreement with that obtained by electrophoresis on polyacrylamide gels in the presence of SDS (Mr 47500±1500) (Fig. 2) and also with that determined by gel-filtration through a calibrated Sephacryl S-100 column in the absence of any denaturing agent (Mr 47000±2000). The fact that denatured and non-denatured proteases display the same molecular weight confirms a monomeric structure for the enzyme molecule.

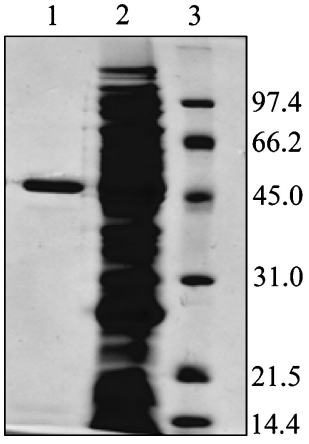

Fig. 2. SDS-PAGE of ppBH4 expressed in *E. coli*. The gel (12% acrylamide) was stained with Coomassie Brilliant Blue R-250. Lane 1, ppBH4 obtained after purification by immobilized-metal affinity chromatography (IMAC) on Ni-NTA resin; lane 2, crude cell extract of BL21 (DE3)/pETpp; lane 3, molecular mass markers (values in kDa are indicated at the right).

Its theoretical isoelectric point was found to be 5.36 as estimated by the DNASIS program.

The relative degree of the polar or non-polar character of a protein depends on the proportion of both hydrophilic (H) and apolar (A) sets of amino acids. We have classified Asx, Glx, Lys, Arg, Ser, Thr and His as hydrophilic residues (H) and Val, Ile, Leu, Phe and Met as apolar residues (A). The H/A ratio, calculated from the amino acid composition, is 1.7. This value is lower than that of most soluble proteins (mean value, H/A=2.1) and indicates a relatively large proportion of apolar residues.

3.4. Physicochemical properties

The effect of pH on the catalytic activity of the enzyme was studied at 37 °C between pH 6.5 and 10.0. The pH curve displayed a maximal activity around pH 8.0 in the veronal buffer, which covers the pH range 6.8–9.2 (Fig. 3). The effect of the nature of the buffer was very significant as the enzyme showed a 3- to 4-fold higher activity at pH 8.0 in the sodium phosphate buffer than in the veronal and Tris–HCl buffers.

The activity of the enzyme was also studied at various temperatures between 30 and 70 °C. Under the standard test conditions, the activity was optimal around 45 °C (Fig. 4). The effect of temperature on the enzyme stability was also investigated by incubating enzyme solutions for 10 min at different temperatures (inset Fig. 4). The enzyme retained 100% of its activity up to 40 °C. The half-life of the enzyme was about 20 min at 50 °C and 10 min at 55 °C.

The activator or inhibitor effects of cations (1 mM) on the enzyme activity were studied (Table 1). A marked activator effect was observed with Mn^{2+} whereas Zn^{2+}, Co^{2+} and Cu^{2+} totally inhibited the activity. Fe^{2+} and Ca^{2+} had a more limited inhibitory effect.

To confirm the class of the protease, a series of inhibitors were tested on the enzyme activity with insulin B-chain as substrate. The high sensitivity to metal inhibitors, such as EDTA, EGTA and 1,10-phenanthroline indicates the metalloprotease character of this enzyme (Table 2). Metal analysis by atomic absorption spectrophotometry showed that the protease contains one zinc atom per molecule. Bestatin, which is a metalloprotease inhibitor selective for aminopeptidases, had no significant effect.

In other respects, the peptidase was very sensitive to reducing agents such as cysteine as well as to sulfhydryl group blocking reagents such as iodoacetic acid or N-ethylmaleimide. As for the inhibitor E-64, which is an active-site titrant of cysteine proteases, its effect on the enzyme activity was limited (Table 2).

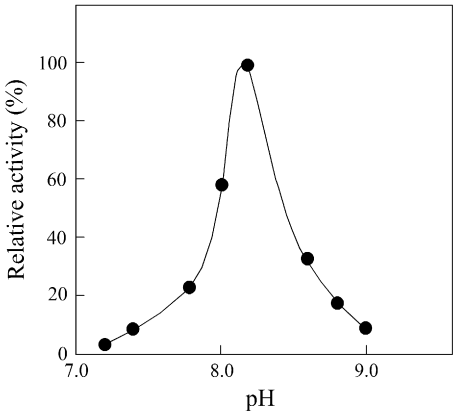

Fig. 3. Effect of pH on the activity of ppBH4. The experiments were carried out at 37 °C with insulin B-chain as substrate using a 100 mM sodium barbital (veronal) buffer at the indicated pHs.

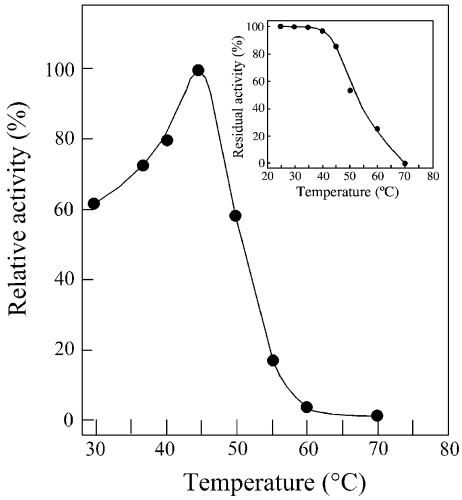

Fig. 4. Effect of temperature on the activity of ppBH4. The experiments were carried out at the indicated temperatures for 20 min in 100 mM sodium phosphate buffer (pH 8.0). Inset: thermal stability of ppBH4. The enzyme was maintained for 10 min at different temperatures in the above buffer. The residual activity was then measured under the standard assay conditions.

3.5. Substrate specificity and kinetic parameters

A variety of substrates, including a series of chromogenic *p*-nitroanilide (*p*NA) substrates, natural peptides and

Table 1
Effect of cations on the activity of $ppBH_4$

Cation (1 mM)	Proteolytic activity (percent of control)
None	100
Ca^{2+}	38±3
Co^{2+}	0
Cu^{2+}	0
Fe^{2+}	23±2
Mg^{2+}	92±3
Mn^{2+}	209±10
Zn^{2+}	0

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

Table 2
Effect of potential inhibitors on the activity of ppBH4

Potential inhibitor (1 mM)	Proteolytic activity (percent of control)
None	100
EDTA	0
EGTA	0
1, 10-Phenanthroline	30±3
Bestatin	91±5
N-Ethylmaleimide	5±3
β-mercaptoethanol (1%, w/v)	5±2
Cysteine	0
E-64	54±4

The enzyme was preincubated with the potential inhibitor for 1 h at room temperature before the reaction was initiated by addition of substrate (insulin B-chain). The values reported represent the means and standard errors of four determinations.

proteins, were tested for cleavage by the enzyme. No hydrolysis was detected using any of the *p*NA derivatives of the amino acids tested (i.e., Gly, Ala, Val, Leu, Ile, Met, Pro, Thr, Phe, Tyr, Lys, Arg, His, Asp and Glu). The enzyme failed also to hydrolyze the proteins such as azocasein and bovine serum albumin. Rates of cleavage of a number of peptides were determined and expressed relative to that for dynorphin A (fragment 1–13) (Fig. 5). The latter was cleaved at a multitude of points in the interior of the chain with preferential cleavage sites between residues 4–5 (Phe–Leu), 5–6 (Leu–Arg), 6–7 (Arg–Arg) and 7–8 (Arg–Ile). The enzyme also showed a very low exopeptidase activity since, after prolonged incubation times, the fragments 1–12, 1–11, 1–10 and 2–13, 3–13 and 4–13 were detected in very low quantities as determined by sequencing and mass spectrometry. Digestion of the insulin B-chain by the enzyme was also efficient and led to the breaking of the bonds between residues 25–26 (Phe-Tyr), 16–17 (Tyr-Leu) and 10–11 (His-Leu). The k_{cat}/K_m ratio, known to be the more significant parameter with respect to catalytic efficiency, was of the same order of magnitude for dynorphin A (fragment 1–13) and insulin B-chain (Table 3).

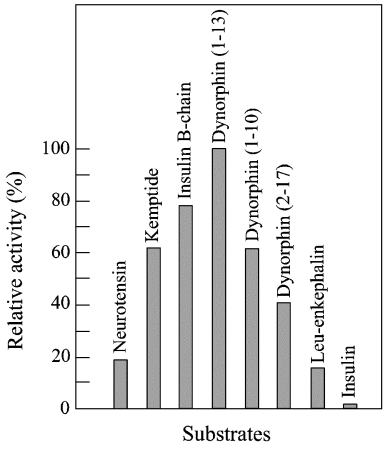

Fig. 5. Relative rates of degradation of peptides by ppBH4. The different activities are expressed relative to that of dynorphin A (fragment 1–13) (100%).

Table 3
Kinetic parameters for degradation of dynorphin A (fragment 1–13) and insulin B-chain by ppBH4

Substrate	K_m (mM)	*k*cat (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Dynorphin A (1–13)	0.120	33	275
Insulin B-chain	0.050	11	220

4. Discussion

The bacterial strain H4 isolated from sediments of Lake Bogoria (Kenya) was identified as *B. halodurans*, the genome (strain C-125) of which is known [1]. The gene encoding a protein showing a 75% homology with a putative insulysin from *B. anthracis* was cloned in *E. coli* and sequenced. The overexpressed enzyme, ppBH4, is a zinc metallopeptidase and a member of the inverzincin family [11], characterized by an inverted active site motif, HXXEH (His 46 and 50, Glu 49), rather than the common HEXXH motif found in most zinc metallopeptidases. In the classification proposed by Barrett et al. [2], the inverzincins correspond to the clan ME (families M16 and M44) of metallopeptidases. The protease ppBH4 shares several biochemical and molecular properties with the enzymes of the pitrilysin family (M16). The latter contains, in particular, pitrilysins (EC 3.4.24.55) identified in bacteria and previously known as protease III [5] and protease Pi [14,15], animal insulysins (EC 3.4.24.56) formerly named insulinase and insulin-degrading enzyme (IDE) [3], and nardilysins (EC 3.4.24.61) from mammals, also called N-arginine dibasic (NRD)-convertase [16].

In the HXXEH motif, it has been demonstrated for *E. coli* pitrilysin as well as for human (Fig. 1) and *Drosophila* insulysins that the two histidine residues (His 88 and 92 for *E. coli* pitrilysin; His 108 and 112 for human insulysin) coordinate the essential zinc atom while the glutamate residue (Glu 91 for pitrilysin and 111 for insulysin) is involved in catalysis [17,18]. By mutagenesis studies, another glutamate residue (Glu 169 for pitrilysin and 189 for insulysin) has been shown to be a zinc-binding residue also [19,20]. Thus, the sequence $HXXEH(X)_{76}E$ is characteristic of these enzymes (i.e., pitrilysins, insulysins but also nardilysins) [21–23]. Another glutamate residue (Glu 162 for pitrilysin and 182 for insulysin) might also be involved in catalysis, the final motif $HXXEH(X)_{69}E(X)_6E$ representing the characteristic sequence of this peptidase family. In ppBH4, the motif $HXXEH(X)_{68}E(X)_6E$ is found (Fig. 1). Sequence comparisons which were based only on amino acid sequence length of ppBH4 showed a homology of 46% with *E. coli* pitrilysin and of 44% with human insulysin using the BLAST program.

Besides the inverted active-site sequence (HXXEH), the sequence NQLRTEEQLGY of *E. coli* pitrilysin has been

suggested to further define related enzymes [2]. Indeed, this sequence is 81% identical to the comparable region of human insulysin [6] and is also conserved in the insulin-degrading enzyme from *Drosophila* [24], but it is not found in ppBH4. Nor does the latter contain the extended acidic domain, which is a distinctive feature of nardilysins [25] consisting of a 71-amino acid acidic stretch in the rat enzyme [21] and 53 amino acids in the human one [26].

Like insulysin [18,27] and nardilysin [28] but in contrast to pitrilysin [7], ppBH4 is inactivated by sulfhydryl alkylating agents such as N-ethylmaleimide suggesting a possible implication of a cysteine in the catalytic process. In addition, ppBH4, which contains three cysteine residues, is sensitive to reducing reagents such as β-mercaptoethanol or cysteine, indicating that a disulfide bond might play an essential role in the native conformation of the protease. Pitrilysin from *E. coli* is also sensitive to reducing agents [5,29] but the inhibition is only significant at high concentrations of reagent (DTT, 5 mM; β-mercaptoethanol 10 mM). In this case, since pitrilysin contains only a single residue of cysteine [30], the inhibition by the reducing agent is probably due to binding to the zinc atom [29].

The enzymatic activity of ppBH4 appears limited to substrates smaller than proteins. From our assays, dynorphin A was found to be the best peptide tested. The protease ppBH4 cleaved at several points in the interior of the chain; in particular, the bond Arg-Arg (6–7) was hydrolyzed as is also the case with nardilysin [22,28] and insulysin [31]. It should be noted that dynorphin A is not a substrate for bacterial pitrilysin [29]. Whereas intact insulin, which is the primary substrate for insulysin, was cleaved very slowly by ppBH4 as is also the case with bacterial pitrilysin [29], insulin B-chain was rapidly degraded. The ppBH4 shares two cleavage sites, the bonds Tyr-Leu (16–17) and Phe-Tyr (25–26) of insulin B-chain, with bacterial pitrilysin [5] and insulysin [32,33]. Finally, like most proteases of the pitrilysin family, the specificity of ppBH4 appears directed essentially towards the amino side of hydrophobic and basic residues.

The ppBH4 protease behaves as a monomer around 47 kDa when determined by gel-filtration as well as by SDS-polyacrylamide gel electrophoresis. This value is consistent with that deduced from the amino acid sequence and distinguishes ppBH4 from all other well-characterized enzymes of the pitrilysin family whose molecular masses range from 100 to 140 kDa and which can exist under active multimeric forms as is the case with insulysin [34,35]. It should be noted that the β-subunit (49 kDa) of the yeast mitochondrial processing peptidase (about 100 kDa) which belongs to the M16B subfamily of pitrilysins [36,37] contains an identical zinc binding motif (HFLEH) and the same sequence $HFLEH(X)_{68}E(X)_6E$ of those found in ppBH4 (Fig. 1) and also shows a 49% sequence homology with ppBH4.

Comparing the predicted and observed properties of proteins encoded in the genome of *E. coli* K-12, the *yhjj* gene [38] encodes an insulysin-like protein of about 55 kDa as found in the Swiss-Prot database. The sequence of the *yhjj* gene presents a 39% homology with that of ppBH4. This *E. coli* protease of 55 kDa is different from that of the 50 kDa identified previously in *E. coli.* Analysis of the cloned protease III gene (*ptr*) demonstrated that, in addition to the well-characterized 110 kDa protease III (pitrilysin), a second 50 kDa polypeptide (p50) is derived from the N-terminal end of the coding sequence [8,30].

In conclusion, this study reports the cloning and expression of a novel metallopeptidase belonging to the pitrilysin family by most of these physicochemical and molecular properties. However, it has a molecular mass (46.5 kDa) much lower than that of the well-characterized enzymes of this family. The substrate specificity of ppBH4 is complex and its role is unknown but, like most enzymes of the pitrilysin family, it might fulfil a variety of physiological functions.

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