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Purification and characterization of hyperthermotolerant leucine aminopeptidase from *Geobacillus thermoleovorans* 47b

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Abstract A thermophilic bacterium, which we designated as *Geobacillus thermoleovorans* 47b was isolated from a hot spring in Beppu, Oita Prefecture, Japan, on the basis of its ability to grow on bitter peptides as a sole carbon and nitrogen source. The cell-free extract from *G. thermoleovorans* 47b contained leucine aminopeptidase (LAP; EC 3.4.11.10), which was purified 164-fold to homogeneity in seven steps, using ammonium sulfate fractionation followed by the column chromatography using DEAE-Toyopearl, hydroxyapatite, MonoQ and Superdex 200 PC gel filtration, followed again by MonoQ and hydroxyapatite. The enzyme was a single polypeptide with a molecular mass of 42,977.2 Da, as determined by matrix-assisted laser desorption ionization and time-of-flight mass spectrometry, and was found to be thermostable at 90°C for up to 1 h. Its optimal pH and temperature were observed to be 7.6–7.8 and 60°C, respectively, and it had high activity towards the substrates Leu-*p*-nitroanilide (*p*-NA)(100%), Arg-*p*-NA (56.3%) and LeuGlyGly (486%). The K_m and V_{max} values for Leu-*p*-NA and LeuGlyGly were 0.658 mM and 25.0 mM and 236.2 μmol min^{-1} mg^{-1} protein and 1,149 μmol min^{-1} mg^{-1} protein, respectively. The turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m) for Leu-*p*-NA and LeuGlyGly were 10,179 s^{-1} and 49,543 s^{-1} and 15,470 mM^{-1} s^{-1} and 1981.7 mM^{-1} s^{-1}, respectively. The enzyme was strongly inhibited by EDTA, 1,10-phenanthroline, dithiothreitol, β-mercaptoethanol, iodoacetate and bestatin; and its apoenzyme was found to be reactivated by Co^{2+}.

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Introduction

Protein acid hydrolyzate is produced by hydrolyzing protein with 6 N hydrochloric acid at 180°C and 606 kPa. This extensive hydrolysis produces a high level of free amino acids and a savory flavor and is used in the food industry to prepare hydrolyzed vegetable protein (HVP) and hydrolyzed animal protein (HAP). In general, HVP and HAP are not seen as natural, rather as artificial flavorings. They also contain low levels of the carcinogens 3-chloropropane-1,2-diol and 1,3-dichloro-2-propanol. Thus, the formation of carcinogens is the most serious problem in the practical use of protein hydrolyzates.

Recently, consumers have begun to demand healthier and more natural products. Therefore, the enzymatic production of amino acid mixtures from proteins using a combination of proteases and peptidases has recently been paid considerable attention in the food protein processing industry. However, the enzymatic hydrolysis of proteins results in a bitter peptide taste, due to the formation of low molecular weight peptides composed mainly of hydrophobic amino acids [8–10, 20]. Further hydrolysis of bitter peptides is carried out using aminopeptidase, alkaline/neutral protease and carboxypeptidase. Although many industrial proteases have been developed, commercial peptidases (aminopeptidase, carboxypeptidase) are limited in number.

Aminopeptidase is an exopeptidase that catalyzes the cleavage of amino acids from the amino terminus of protein or peptide substrates. One of the most common aminopeptidases is leucine aminopeptidase (LAP). The LAPs are only commercially produced from two sources: lactic acid-producing bacteria and *Aspergillus oryzae* [21], which are only stable up to 40°C and 60°C, respectively. To develop a thermotolerant debittering enzyme, we screened microbes from hot springs in Oita

Prefecture, Japan, using bitter peptides as a sole carbon and nitrogen source. The object of this study was to purify and characterize the debittering enzyme LAP from *Geobacillus thermoleovorans* 47b to evaluate its usefulness as an alternative to commercial LAP in food protein processing.

Materials and methods

Materials

All the synthetic chromogenic substrates and bestatin were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). The MonoQ HR 5/5 column, Superdex 200 column and FPLC pump system were purchased from Amersham Biosciences (Piscataway, N.J., USA). Protein Marker (broad range, 2–212 kDa) for SDS-PAGE was obtained from New England Biolabs (Beverly, Mass., USA). All other chemicals used were of analytical grade.

Isolation and identification of bitter peptide-degrading bacterium

The bacterium 47b was isolated from a hot spring at Oita Prefecture at 60°C, using a medium containing 0.5% bitter peptide, 0.02% yeast extract and 0.01% $MgSO_4 \cdot 7H_2O$ (pH 7.0). This strain was identified using 16S rRNA analysis. The 16S rRNA genes were amplified by PCR performed with the chromosomal DNA of the bacterium 47b as the template, using the method of Weisburg et al. [26]. The DNA sequence of the PCR product was identified by the dideoxy chain-termination method, using a 370A DNA sequencer (Applied Biosystems, Foster, Calif., USA).

Medium and growth conditions

For production of the enzyme, the bacterium was grown at 60°C for 18 h in 1 L of the medium containing 0.5% meat extract, 0.1% peptone, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.01% yeast extract (pH 7.0) in a 2-L Erlenmeyer flask on a reciprocal shaker (New Brunswick Scientific) at 150 rpm.

Preparation of bitter peptides from milk casein

A 10% bitter peptide solution was prepared by hydrolysis of 10% milk casein (Merck, Darmstadt, Germany) with protease (amanoN; Amano Enzyme Co., Japan).

Hydrolysis of bitter peptides using cell-free extract from *G. thermoleovorans* 47b

The enzymatic hydrolysis of bitter peptides was examined using the crude enzymes extracted from *G. thermoleovorans* 47b (at 60°C for 8 h). The reaction mixture contained 100 mM HEPS (pH 7.6), 80 μL of 10% bitter peptide solution and enzyme in a total volume of 200 μL. The bitter peptide solution was replaced by water in a blank sample. After incubating at 30°C for 1–24 h, the reaction was stopped by the addition of 200 μL of 0.25 M NaOH. The protein in the reaction mixture was removed by the addition of 2% sulfosalicylic acid followed by centrifugation.

Acid hydrolysis of bitter peptide was carried out using a PICO-TAG Workstation (Waters, Mass., USA) with 6 N HCl at 105°C for 48 h. After hydrolysis, the amino acid analysis was performed with a JLC-300 amino acid analyzer (JEOL, Tokyo, Japan).

Enzyme purification

All procedures were performed at 0–4°C. The cells (45 g wet weight) collected from 16 L of culture broth were washed twice with 10 mM potassium phosphate buffer (KPB; pH 6.8) and then suspended in 225 mL of 10 mM KPB. The suspended cells were disrupted by sonication, the cell debris was removed by centrifugation and the supernatant was used as a cell-free extract. The cell-free extract was dialyzed overnight against 10 mM KPB and solid ammonium sulfate was added to the enzyme solution to give 50% saturation. After 1 h, the precipitate was removed and the active enzyme was precipitated from the supernatant with ammonium sulfate (70% saturation) and collected by centrifugation. The precipitate was dissolved with 10 mM KPB and dialyzed overnight against 10 mM KPB. The dialyzed enzyme was loaded onto a DEAE-Toyopearl 650 M column (3.0×17.0 cm; Tosoh, Tokyo, Japan) previously equilibrated with 10 mM KPB. After washing the column with 10 mM KPB followed by 10 mM KPB containing 50 mM NaCl, the active enzyme was eluted with 10 mM KPB containing 100 mM NaCl. The active enzyme was pooled, concentrated by ultrafiltration with a YM-10 membrane (Amicon, Beverly, Mass., USA) and dialyzed against 1 mM KPB (pH 6.8). The enzyme (28 mL) was then applied to a hydroxyapatite column (3.0×7.0 cm, fast-flow type; Wako Pure Chemicals, Osaka, Japan) equilibrated with 1 mM KPB. After washing the column with 1 mM and 20 mM KPB (pH 6.8), the active enzyme was eluted with 40 mM KPB (pH 6.8). The enzyme was next concentrated by ultrafiltration and dialyzed against 10 mM KPB. The dialyzed enzyme (34 mL) was then placed on a Mono Q HR 5/5 column equilibrated with 10 mM KPB [fast protein liquid chromatography (FPLC) pump system]. The column was washed with 10 mM KPB and 10 mM KPB containing 150 mM NaCl. Following this, the enzyme was eluted with a linear gradient (150–200 mM, total volume 60 mL) in 10 mM KPB. The active fractions were combined, concentrated by ultrafiltration and dialyzed against 10 mM KPB. The dialyzed enzyme (0.6 mL) was applied to a Superdex 200 column (1.0×30.0 cm) equilibrated with 10 mM KPB containing 100 mM NaCl and

eluted with the same buffer at 0.2 ml min^{-1}, using a FPLC pump system. The active enzyme was concentrated by ultrafiltration and dialyzed against 10 mM KPB and then the dialyzed enzyme was loaded onto a second MonoQ HR 5/5 column equilibrated with 10 mM KPB (FPLC pump system). The column was washed with 10 mM KPB and 10 mM KPB containing 160 mM NaCl. The enzyme was next eluted with 165 mM NaCl in 10 mM KPB. The active enzyme was concentrated by ultrafiltration and dialyzed against 1 mM KPB and the dialyzed enzyme was applied to a Bio-Scale CHT2-I column (Bio-Rad Laboratories, Hercules, Calif., USA) equilibrated with 1 mM KPB (FPLC pump system). After the column was washed with 1 mM and 20 mM KPB (pH 6.8), the enzyme was eluted with a linear 20–40 mM gradient, using a volume of 60 mL. The active enzyme was finally concentrated by ultrafiltration and dialyzed against 10 mM KPB. Homogeneity of the final aminopeptidase preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli [12].

Aminopeptidase assay

Aminopeptidase activity was spectrophotometrically assayed by measuring *p*-nitroanilide (*p*-NA) release from L-Leu-*p*-NA at 60°C. The standard assay conditions were as follows. The reaction mixture consisted of 0.4 mM L-Leu-*p*-NA, 50 mM HEPES buffer (pH 7.6) and the enzyme in a final volume of 1.2 ml. After preincubation at 60°C for 5 min, the reaction was started by the addition of enzyme. The increase in absorbance at 405 nm was monitored for 15 min on a spectrophotometer (Hitachi model U 3000). One unit of aminopeptidase activity was defined as the amount of the enzyme that catalyzed the release of 1 μmol min^{-1} of *p*-NA under standard conditions. Specific activity was expressed as units per milligram of protein. The protein concentration was measured by the method of Lowry et al. [15] with crystalline egg albumin as the standard, or by measuring the absorbance at 280 nm.

Characterization of LAP

The molecular weight of the native form was measured by gel filtration on a Superdex 200 PC column using Sigma Chemical Co. molecular weight markers for gel filtration Chromatography. The N-terminal amino acid sequence was determined by automatic Edman degradation using a 491 Protein sequencer (Applied Biosystems). Molecular weight measurement based on matrix-assisted laser desorption ionization and time-of-flight (MALDI-TOF) mass spectrometry was also performed using a Bruker Autoflex spectrometer (Bruker Daltonik, Leipzig, Germany), with protein calibration standard II used for calibration.

The optimum pH for enzyme activity was measured under standard assay conditions using various 50 mM buffers and pHs [MES (pH 5.5–7.0), MOPS (pH 6.5–7.5), KPB (pH 7.0–8.5), HEPES (pH 7.0–8.5), Tris (pH 7.5–9.0), CAPS (pH 9.0–11.0), CHES (pH 9.0–11.0)]. Optimal temperature was also measured at various temperatures (30–85°C) under standard assay conditions. Enzyme thermostability was tested by preincubation of the enzyme in 50 mM HEPES buffer (pH 7.6) at various temperatures (40–100°C) for 1 h and then cooling on ice for 5 min followed by standard assay conditions to measure residual enzyme activity. The pH stability was tested by preincubation of the enzyme in various 50 mM buffers and at various pH values [MES (pH 5.5–7.0), MOPS (pH 6.5–7.5), HEPES (pH 7.0–8.5), KPB (pH 7.0–8.5), Tris (pH 7.5–9.0), CAPS (pH 9.0–11.0), CHES (pH 9.0–11.0)] for 30 min at 60°C before residual enzyme activity was measured.

After preincubating the enzyme with cations, chelating reagents or chemical reagents in 50 mM HEPES buffer (pH 7.6) at 60°C for 5 min, the remaining activity was measured under standard assay conditions. The metal ion requirement for the enzyme activity was tested by adding metal ions to the apoenzyme. The enzyme was dialyzed against three 1-L changes of 10 mM KPB (pH 6.8) containing 0.1 mM EDTA at 4°C for 3 h and was then further dialyzed against 10 mM KPB (pH 6.8) to completely remove EDTA. The apoenzyme obtained was preincubated with various cations in 50 mM HEPES buffer (pH 7.6) and enzyme activity was determined under standard assay conditions. The salt tolerance of the enzyme was examined by preincubation in the presence of 2–14% sodium chloride in 50 mM HEPES buffer (pH 7.6) at 60°C for 5 min.

Table 1 Hydrolysis of bitter peptides by enzymatic or acid methods

Amino acid	Enzymatic hydrolysis (mM)	Acid hydrolysis (mM)
Leu	7.3	20.6
Ile	0.4	11.8
Val	3.3	16.7
Ala	2.1	9.3
Met	1.0	2.5
Tyr	1.5	7.0
Phe	2.5	9.0
Trp	0.6	0
Ser	1.2	16.1
Thr	0.9	11.6
Cys	0	0
Pro	0	29.5
Gly	0.3	7.2
Asn	0.2	0
Gln	0.9	0
His	0	5.1
Lys	0.6	17.0
Arg	0.1	6.2
Asp	0.4	16.0
Glu	0.7	50.2

Results

Bacterial identification

The 16S rRNA sequence showed 98% similarity to that of *G. thermoleovorans* and strain 47b was tentatively designated as *G. thermoleovorans* 47b. Recently, thermophilic bacilli have been classified into the new genus *Geobacillus*. Therefore, *Bacillus stearothermophilus*, *B. thermoleovorans*, *B. thermocatenulatus*, *B. kaustophilus*, *B. thermoglucosidasius* and *B. thermodenitrificans* have been transferred to this new genus [19].

Hydrolysis of bitter peptides by *Geobacillus* cell-free extract

Table 1 shows the profile of free amino acids released during the enzymatic hydrolysis and acid hydrolysis. *Geobacillus* cell-free extract cleaved leucine in significant amounts, suggesting that *G. thermoleovorans* 47b produces LAP.

Enzyme purification

The purification of LAP from *G. thermoleovorans* 47b is summarized in Table 2. The enzyme was homogeneously purified about 164-fold for a 0.01% yield (Fig. 1). By MALDI-TOF mass spectrometry, the molecular mass of the purified enzyme was determined to be 42,977 Da, which corresponds closely with the relative molecular mass of the purified enzyme of 46.7 kDa determined by Superdex 200 PC gel filtration and 46.9 kDa found from SDS-PAGE. These results indicate that the native enzyme is active as a monomer.

The sequence of the first 17 amino acids from the amino-terminus was found to be MDREENE(T)L(V) XKYAE LAVK, where X indicates an unknown residue.

Characterization of the enzyme

The optimal pH and temperature of the enzyme activity were pH 7.6–7.8 and 60°C (Fig. 2), respectively. The enzyme was stable up to 90°C for 1 h (Fig. 3) and was stable within the range pH 7.5–8.5 in HEPES buffer. The enzyme was unstable in MES, Tris-HCl, MOPS, KPB, CHES and CAPS buffers; and enzyme activity was inhibited by Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+} and Ni^{2+} (Table 3). Stimulation of the enzyme activity by

Table 2 Enzyme purification

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Purification (*x*-fold)	Yield (%)
Crude extract	1924.0	731,0	0.38	1.0	100
$(NH_4)_2SO_4$	700.0	273.0	0.39	1.0	37.3
DEAE-Toyopearl	90.7	122.0	1.34	3.5	16.6
Hydroxyapatite	8.7	119.0	13.7	36.1	16.3
MonoQ	0.46	12.2	26.5	70.0	1.67
Superdex 200	0.17	12.2	72.3	190.3	1.66
Bio-scale CHT2-I	0.02	1.4	42.7	112.4	0.18
Second MonoQ	0.001	0.08	62.6	164.7	0.01

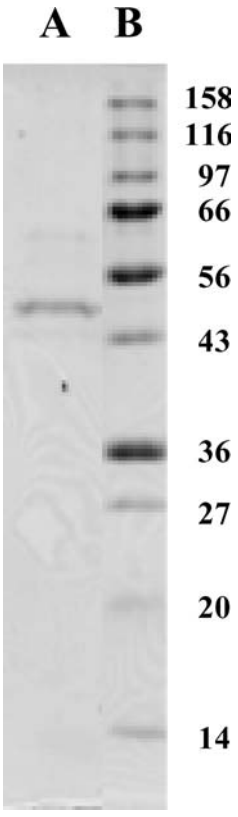

Fig. 1 SDS-PAGE of the purified enzyme. **A** The purified enzyme, **B** standards: lysozyme (14.3 kDa), trypsin inhibitor (20.2 kDa), triose phosphate isomerase (26.6 kDa), lactate dehydrogenase (36.5 kDa), maltose-binding protein 42.7 kDa), glutamate dehydrogenase (55.6 kDa), serum albumin (66.4 kDa), phosphorylase b (97.1 kDa), β-galactosidase (116.3 kDa) and MBP-β-galactosidase (158.1 kDa)

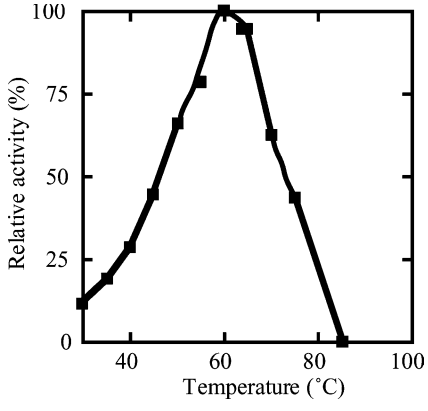

Fig. 2 Effect of temperature on enzyme activity. Enzyme activity was measured at various temperatures in 50 mM HEPES buffer (pH 7.6)

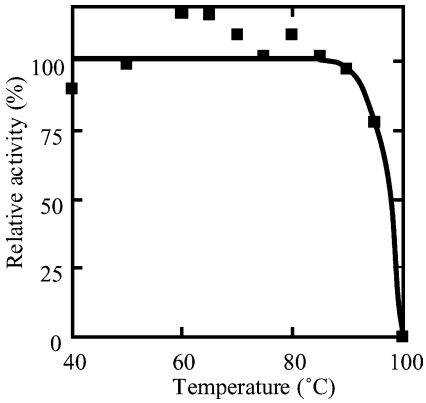

Fig. 3 Effect of temperature on enzyme stability. Enzyme preparations were preincubated in 50 mM HEPES buffer (pH 7.6) at various temperatures for 10 min

metal ions was not observed. Enzyme activity was inhibited by EDTA, 1,10-phenanthroline and α,α′-dipyridyl (Table 4), indicating that this enzyme probably requires a metal ion for either stability or activity. After complete inactivation of the enzyme by EDTA followed by dialysis, enzyme activity could be reactivated 219% or 150% by the addition of 0.2 mM or 0.4 mM Co^{2+}, respectively (Table 5), whereas the activity was not restored by the addition of Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Mn^{2+}. This result suggests that Co^{2+} is essential for the enzyme activity.

To identify the particular type or class of aminopeptidase, the effect of various chemical reagents on the enzyme was examined (Table 6). The thiol group reducers dithiothreitol (DTT) and β-mercaptoethanol and the sulfhydryl-directed reagent iodoacetate completely inactivated the enzyme. Two other sulfhydryl-directed reagents, *p*-chloromercuric benzoate (PCMB) and *N*-ethymaleimide were less effective inhibitors. Phenylmethanesulfonyl fluoride (PMSF), an inhibitor of a number of serine proteases, and *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), a specific inhibitor of chymotrypsin-like serine proteases, had no effect on enzyme activity. The aminopeptidase inhibitor bestatin strongly inhibited enzyme activity. Therefore, the enzyme is an aminopeptidase. As shown in Fig. 4, increasing the concentration of sodium chloride caused a decrease in enzyme activity.

The ability of the enzyme to catalyze the hydrolysis of various *p*-nitroaniline derivatives is shown in Table 7. Among the substrates (*p*-NA derivatives) tested, the enzyme preferred Leu-*p*-NA and Arg-*p*-NA, indicating that it is a leucine aminopeptidase. In addition, LeuGlyGly was active as a substrate. The *K*m values for Leu-*p*-NA and LeuGlyGly were estimated to be 0.658 mM and 25.0 mM, respectively, and the V_{max} values for Leu-*p*-NA and LeuGlyGly were calculated to be 236.16 μmol min^{-1} mg^{-1} protein and 1,149.4 μmol min^{-1} mg^{-1} protein, respectively. The turnover rate (k_{cat}) and cata-

Table 3 Effect of cations on enzyme activity

Reagent	Concentration (mM)	Relative activity (%)
No addition	–	100
NaCl	1.0	97
KCl	1.0	95
$MnCl_2$	1.0	12
$MgCl_2$	1.0	84
$ZnCl_2$	1.0	17
$CuCl_2$	0.1	23
$CoCl_2$	1.0	17
$FeSO_4$	0.1	34
$FeCl_3$	0.1	40
$NiCl_2$	1.0	22

Table 4 Effect of chelating reagents on enzyme activity

Reagents	Concentration (mM)	Relative activity (%)
No addition	–	100
EDTA	0.1	25
EDTA	1.0	14
EDTA	10.0	21
o-Phenanthroline	0.1	32
o-Phenanthroline	1.0	17
o-Phenanthroline	10.0	11
α,α′-Dipyridyl	0.1	89
α,α′-Dipyridyl	1.0	21
α,α′-Dipyridyl	10.0	14

Table 5 Effect of bivalent ions on apoenzyme activity

Bivalent ion	Concentration (mM)	Relative activity (%)
Control	–	100
No addition	–	0.6
Zn^{2+}	0.2	24
Mg^{2+}	0.2	0.6
Ca^{2+}	0.2	8
Cu^{2+}	0.2	31
Mn^{2+}	0.2	4
Co^{2+}	0.2	219
Co^{2+}	0.4	150

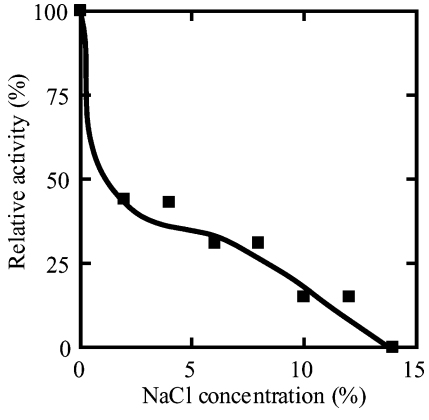

Fig. 4 Effect of sodium chloride concentration on enzyme activity. The reaction mixture contained 0–14% NaCl in 50 mM HEPES buffer (pH 7.6). Enzyme activity was measured as described in the Materials and methods

Table 6 Effect of chemical reagents on enzyme activity

Chemical reagent	Concentration (mM)	Relative activity (%)
No addition	–	100
PCMB	0.1	70
N-Ethylmaleimide	1.0	85
DTT	1.0	0.5
β-Mercaptoethanol	1.0	6.8
Iodoacetate	1.0	7.1
PMSF	1.0	116
TPCK	1.0	112
Bestatin	0.5	6.4

lytic efficiency (k_{cat}/K_m) for Leu-*p*-NA and LeuGlyGly were 10,179 s^{-1} and 49,543 s^{-1}, and 15,470 mM^{-1} s^{-1} and 1,981.7 mM^{-1} s^{-1}, respectively.

Discussion

The food industry has serious problems concerning the formation of bitter peptides during protein hydrolysis. Debittering of protein hydrolyzates has been carried out by methods involving masking, binding, extraction and enzymes. Debittering enzymes are classified into three groups: aminopeptidases, alkaline/neutral proteases and carboxypeptidases. The enzymatic hydrolysis of protein is carried out at 60°C to prevent microbial contamination. Therefore, to degrade bitter peptides formed during the course of enzymatic hydrolysis of proteins in food protein processing, we screened bitter peptide-degrading microbes at 60°C from a geothermal field in Oita Prefecture, Japan, using bitter peptides prepared from milk casein as a sole carbon and nitrogen source.

Table 7 Substrate specificity

Substrate	Relative activity (%)
Leu-*p*-NA	100
Arg-*p*-NA	56.3
Phe-*p*-NA	9.2
Lys-*p*-NA	5.0
Met-*p*-NA	4.6
Ala-*p*-NA	2.0
Gly-*p*-NA	1.3
Pro-*p*-NA	1.2
Arg-Pro-*p*-NA	0
Gly-Pro-*p*-NA	0
Gly-Phe-*p*-NA	2.6
Gly-Arg-*p*-NA	2.5
Val-Ala-*p*-NA	0.7
Ala-Ala-Phe-*p*-NA	1.0
Ala-Ala-Phe-*p*-Na	0
Pyr[a]-Phe-Leu-*p*-NA	0
Suc[b]-Ala-Pro-Ala-*p*-NA	0
Suc-Ala-Ala-Leu-*p*-NA	0
CBZ[c]-Ala-Ala-Leu-*p*-NA	0
CBZ-Gly-Gly-Leu-*p*-NA	0
Leu-Gly-Gly	437

[a] Pyroglutamyl
[b] Succinyl
[c] Benzyloxycarbonyl

The isolate 47b bearing LAP activity was identified as *G. thermoleovorans*. Alignment of the 16S rRNA gene sequence with the sequences available in the Genbank database showed that strain 47b has 95.6, 83.5, 81.7 and 81.1% identity with *G. stearothermophilus*, *B. licheniformis*, *B. coagulans* and *B. subtilis*, respectively, which are used as industrial enzyme producers in food processing. Strain 47b has an optimal growth temperature of 60°C and produces hyperthermotolerant LAP, which retains 100% of its original activity even after 1 h of incubation at 90°C. The enzyme was strongly inhibited by DTT (Table 6), suggesting the presence of a disulfide linkage which is required to maintain its active conformation. The thermostability of the enzyme is therefore caused by the presence of a disulfide linkage (Fig. 3). The thermostability of aminopeptidase is due to disulfide bond formation [3].

Using a purification method consisting of seven steps, we homogeneously purified LAP from *G. thermoleovorans* 47b. Microbial LAP has been purified and characterized from *A. sojae* [4], *A. oryzae* [18], *Aeromonas proteolytica* [6], *Alteromonas* B-207 [16], *B. stearothermophilus* [11], *Pseudomonas putida* [7], *Streptomyces griseus* [2], *S. peptidofaciens* [24], *S. rimosus* [25] and *S. lividans* [1]. Gonzales and Robert-Baudouty [5] summarized that almost half (47%) of the 102 bacterial aminopeptidases are monomers and the remainder (53%) display a multimeric structure. The LAPs from *P. putida* and *Alteromonas* B-207 have multimeric structures composed of eight identical subunits of 53 kDa [11] and two identical subunits of 33 kDa [16], but the enzymes from *Aeromonas* and *Streptomyces* are monomers of 19–32 kDa. The LAP from *Geobacillus* 47b is a monomer of 42,977 Da. This molecular mass is similar to those reported for *Aeromonas* and *Streptomyces*.

Of the 22 LAPs examined, the sequence of the first 17 amino acids from *Geobacillus* 47b LAP had 79.0, 79.0, 28.6, 14.3, 28.6, 14.3, 21.4, 14.3 and 14.3% identity with LAPs from *B. stearothermophilus* NCIB8924 (GenBank D13385), *B. halodurans* (BA000004), *Aeromonas proteolytica* (M85159), *Aspergillus sojae* (AF 419160), *Aquifex aeolicus* (AE 000772), *Leishmania major* (AL 117384), *Vibrio cholerae* (D84215), cattle kidney (S65367) and *Arabidopsis thaliana* (X63444), respectively. Low (7.1%) identity was observed between *Geobacillus* 47b LAP and the LAPs from *Aeromonas punctata* (AB015725), *Agaricus bisporus* (AJ 271690), *B. kaustophilus* (AY308074), *Chlamydia muridarum* (AE 002299), *Chlamydophila pneumoniae* (AE 002199), *Mesorhizobium loti* (BA000012), *Petroselinum crispum* (X 99825), *Pseudomonas aeruginosa* (AE 004800), *P. putida* (AJ 010261), *Solanum tuberosum* (X 77015), *Synechocystis* sp. (CAE07077) and *V. proteolyticus* (Z 11993). These results indicate that LAP from *Geobacillus* 47b has high homology (79%) with those from *B. stearothermophilus* and *B. halodurans*.

Aminopeptidases can be sub-divided into three groups based on their sensitivity to various inhibitors:

metallo-aminopeptidases, cysteine-aminopeptidases and serine-aminopeptidases. We tested several inhibitors of *Geobacillus* 47b enzyme (Table 6) and found that it was strongly inhibited by bestatin. Its activity was not affected by PMSF and TPCK, classic inhibitors of serine protease and chymotrypsin-like serine protease, and was slightly decreased in the presence of PCMB and *N*-ethylmaleimide. Enzyme activity was strongly inhibited by EDTA, 1,10-phenanthroline, and α,α′-dipyridyl, indicating that the enzyme is a metallo-enzyme. The LAP can be classified as a Zn^{2+}-metalloenzyme or Co^{2+}-metalloenzyme on the basis of metal requirement. The LAPs from *Aeromonas proteolytica* [6], *Alteromonas* B-207 [16], *Streptomyces griseus* [2], *S. peptidofaciens* [24] and *B. kaustophilus* [14] possess Zn^{2+}, while LAP from *B. stearothermophilus* contains Co^{2+} [11, 17, 23]. At present, it is not clear which a kind of metal *Geobacillus* 47b LAP contains. Stimulation of holo-LAP by Co^{2+} was not observed (Table 3) and apo-LAP was reactivated by Co^{2+} but not by Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Mn^{2+} (Table 5), indicating that *Geobacillus* 47b LAP is a Co^{2+}-dependent aminopeptidase. However, the activity of *Geobacillus* 47b LAP was inhibited by 1.0 mM Co^{2+} (Table 3). The excess amount of Co^{2+} may change the conformation of its metal ion-binding site to inhibit its activity, just as D-aminoacylase is inhibited by the excess amount of its catalytically essential metal ion [13].

Geobacillus 47b LAP was active against Leu-*p*-NA (100%) and Arg-*p*-NA (56.3%), but very little active for Phe-*p*-NA, Lys-*p*-NA, Met-*p*-NA, Ala-*p*-NA, Gly-*p*-NA, Pro- *p*-NA. However, *Aspergillus sojae* LAP was active against Leu-*p*-NA (100%), Phe-*p*-NA (98.6%), Lys-*p*-NA (41.3%) and Arg-*p*-NA (20.3%) [4]. *B. stearothermophilus* LAP was most active against Leu-*p*-NA (100%), followed by Arg-*p*-NA (46.3%), Lys-*p*-NA (37.8%) and Ala-*p*-NA (15.2%) [11]. The LAP from *Thermotoga maritima* was active toward Leu-*p*-NA (100%), Lys-*p*-NA (68%), Ala-*p*-NA (27.3%) and Pro-*p*-NA (7.3%) [22]. These results indicate that bacterial LAPs have relatively broad substrate specificity.

This is the first report on the purification and characterization of a hyperthermostable LAP. The *Geobacillus* LAP described here has potential value for the food industry as an alternative to commercial *A. oryzae* LAP due to its hyperthermostability.

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References

1. Aphale JS, Strohl WR (1993) Purification and properties of an extracellular aminopeptidase from *Streptomyces lividans*. J Gen Microbiol 139:417–424
2. Ben-Meir D, Spungin A, Ashkerazi R, Blumberg S (1993) Specificity of *Streptomyces griseus* aminopeptidase and modulation of activity by divalent metal ion binding and substitution. Eur J Biochem 212:107–112
3. Chen G, Edwards T, D'Souza Y, Holz R (1997) Mechanistic studies on the aminopeptidase from *Aeromonas proteolytica*: a two metal ion mechanism for peptide hydrolysis. Biochemistry 36:4278–4286
4. Chien H-CR, Lin LL, Chao SH, Chen CC, Wang WH, Shaw CY, Tsai YC, Hu HY, Hsu WH (2002) Purification, characterization, and genetic analysis of a leucine aminopeptidase from *Aspergillus sojae*. Biochim Biophys Acta 1576:119–126
5. Gonzales T, Robert-Baudouy J (1996) Bacterial aminopeptidase: properties and functions. FEMS Microbiol Rev 18:319–344
6. Guenet C, Lepge P, Harris BA (1992) Isolation of the leucine aminopeptidase gene from *Aeromonas proteolytica*. J Biol Chem 267:8390–8395
7. Hermes HFM, Sonk T, Peters PJH, Van Balken JAM, Kamphuis J, Dijkhuizen L (1993) Purification and characterization of an L-aminopeptidase from *Pseudomonas putida* ATCC12633. Appl Environ Microbiol 59:4330–4334
8. Ishibashi N, Arita Y, Kaneshisa H, Kogura K, Okai H, Fukui S (1987) Bitterness of leucine-containing peptides. Agric Biol Chem 51:2389–2394
9. Ishibashi N, Sadamori K, Yamamoto O, Kaneshisa H, Kouge K, Okai H, Fukui S (1987) Bitterness of phenylalanine- and tyrosine-containing peptides. Agric Biol Chem 51:3309–3313
10. Ishibashi N, Ono I, Kato K, Shigenaga T, Shinoda I, Oka H, Fukui S (1988) Role of the hydrophobic amino acid residue in the bitterness of peptides. Agric Biol Chem 52:91–94
11. Kuo LY, Hwang GY, Lai YJ, Yang SL, Lin LL (2003) Overexpression, purification, and characterization of the recombinant leucine aminopeptidase II of *Bacillus stearothermophilus*. Curr Microbiol 47:40–45
12. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
13. Lai WL, Chou LY, Ting CY, Kirby R, Tsai YC, Wang AH, Liaw SH (2004) The functional role of the binuclear metal center in D-aminoacylase: one-metal activation and second-metal attenuation. J Biol Chem 279:13962–13967
14. Lin LL, Hsu WH, Wu CP, Chi MC, Chou WM, Hu HY (2004) A thermostable leucine aminopeptidase from *Bacillus kaustophilus* CCRC 11223. Extremophiles 8:79–87
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
16. Merkel JR, Lee CC, Freund TS (1981) A dimeric, extracellular, heatstable aminopeptidase produced by a marine pseudomonad. Biochim Biophys Acta 661:32–38
17. Myrin PA, Hofsten BV (1974) Purification and metal ion activation of an aminopeptidase (aminopeptidase II) from *Bacillus stearothermophilus*. Biochim Biophys Acta 350:13–25
18. Nakadai T, Nasuno S, Iguchi N (1973) Purification and properties of leucine aminopeptidase I from *Aspergillus oryzae*. Agric Biol Chem 37:757–765
19. Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV (2001) Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, G. th. Int J Syst Evol Microbiol 51:433–446
20. Ney KH (1979) Bitterness of peptides: amino acid composition and chain length. In: Bondreau JC (ed) Food taste chemistry. American Chemical Society, Washington, D.C., p. 149
21. Pawlett D, Bruce G (1996) Debittering of protein hydrolysates. In: Godfrey T, West S (eds) Industrial enzymology. Stockton Press, New York, p. 181

22. Ratnayake S, Selvarkumar P, Hayashi K (2003) A putative proline iminopeptidase of *Thermotoga maritima* is a leucine aminopeptidase with lysine-*p*-nitroanilide hydrolyzing activity. Enzyme Microb Technol 32:414–421
23. Stoll E, Weder HG, Coulson AR (1976) Aminopeptidase II from *Bacillus stearothermophilus*. Biochim Biophys Acta 438:212–220
24. Uwajima T, Yoshikawa N, Terada O (1973) A crystalline aminopeptidase from *Streptomyces peptidofaciens*: physicochemical properties and characteristics as a Cd-metalloprotease. Agric Biol Chem 37:2727–2733
25. Vitale L, Renko M, Lenaric B, Turk V, Pokorny M (1986) *Streptomyces rimosus* extracellular proteases: isolation and characterization of leucine aminopeptidase. Appl Microbiol Biotechnol 23:449–455
26. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703