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ORIGINAL PAPER

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A thermostable leucine aminopeptidase from *Bacillus kaustophilus* CCRC 11223

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Abstract Two degenerate primers established from the consensus sequences of bacterial leucine aminopeptidases (LAP) were used to amplify a 360-bp gene fragment from the chromosomal DNA of thermophilic *Bacillus kaustophilus* CCRC 11223 and the amplified fragment was successfully used as a probe to clone a leucine aminopeptidase (*lap*) gene from a genomic library of the strain. The gene consists of an open reading frame (ORF) of 1,494 bp and encodes a protein of 497 amino acid residues with a calculated molecular mass of 53.7 kDa. The complete amino acid sequence of the cloned enzyme showed greater than 30% identity with prokaryotic and eukaryotic LAPs. Phylogenetic analysis showed that *B. kaustophilus* LAP is closely related to the enzyme from *Bacillus subtilis* and is grouped with the M17 family. His_6-tagged LAP was generated in *Escherichia coli* by cloning the coding region into pQE-30 and the recombinant enzyme was purified by nickel-chelate chromatography. The pH and temperature optima for the purified enzyme were 8 and 65°C, respectively, and 50% of its activity remained after incubation at 60°C for 32 min. The enzyme preferentially hydrolyzed L-leucine-*p*-nitroanilide (L-Leu-*p*-NA) followed by Cys derivative.

Keywords *Bacillus kaustophilus* · Gene cloning · Leucine aminopeptidase · Phylogeny

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Introduction

Leucine aminopeptidases (LAPs; EC 3.4.11.1) are widely distributed cytosolic exopeptidases that selectively remove N-terminal amino acid residues from polypeptides and proteins. These enzymes are ubiquitous in nature and are of critical biological and medical importance because of their key role in protein degradation and in the metabolism of biologically active peptides (Terenius et al. 2000; Lowther and Matthews 2002; Goldberg et al. 2002). The LAP from bovine lens is a hexameric enzyme of molecular mass 324 kDa, consisting of six identical subunits (Melbye and Carpenter 1971; Carpenter and Vahl 1973). The atomic absorption spectrum has revealed that each 54-kDa subunit of the native enzyme contains two Zn^{2+} ions (Himmelhoch 1969). The macromolecular structure of the zinc-containing LAP hexamer has been determined by X-ray crystallography (Burley et al. 1990). Each monomer consists of two domains: the N-terminal (residues 1–150) and C-terminal (residues 151–482) regions. The interface between them is made up of one α-helix from the N-terminal domain packing against two α-helices of the C-terminal domain. The monomers are arranged as two layers of trimers with the active sites in the interior of the oligomer (Burley et al. 1990). This arrangement appears to restrict access to only di- and tripeptide substrates (Lowther and Matthews 2002).

Aminopeptidases (APs) are usually located intracellularly, but the extracellular enzymes are found in *Streptomyces griseus* (Vosbeck et al. 1973), *Aeromonas proteolytica* (Merkel et al. 1964), and the filamentous

fungi *Aspergillus oryzae* (Ivanova et al. 1977) and *Aspergillus sojae* (Chien et al. 2002). The molecular architecture of *S. griseus* and *A. proteolytica* aminopeptidases show that they are a monomeric metalloenzyme with a globular α/β domain and two zinc ions located closely together near the catalytic center (Chevrier et al. 1994; Greenblatt et al. 1997). Although these two enzymes exhibit little sequence homology, they have similarities to the structures of bovine lens LAP and carboxypeptidase A (Wouters and Husain 2001; Artymiuk et al. 1992).

Thermophilic *Bacillus* species with growth temperature optima between 45° and 70°C have been isolated from a wide range of environments and are important contaminants of heat-treated food products (Sharp et al. 1992). Research interest in these organisms has increased due to their biotechnological potential, especially as sources of thermostable enzymes (Vieille et al. 1996). Based on the 16S rRNA gene sequence analysis, the majority of the thermophilic *Bacillus* species belong to the genus *Bacillus* genetic groups 1 and 5 (Ash et al 1991; Rainey et al. 1994). As a member of group 5, *Bacillus kaustophilus* exhibits a close phylogenetic relationship to *Geobacillus stearothermophilus*. Recently, we have cloned and characterized a thermostable N-carbamoyl-L-amino acid amidohydrolase from *B. kaustophilus* CCRC 11223 (Hu et al. 2003). In this investigation, we report the cloning and biochemical characterization of a LAP from the same organism. These results will expand our knowledge of the exopeptidase activity of *B. kaustophilus* and serve as a solid foundation for subsequent studies on its molecular architecture.

Materials and methods

Microorganisms, vectors, and culture conditions

B. kaustophilus CCRC 11223 was obtained from the Culture Collection and Research Center (Hsinchu, Taiwan) and used as the donor of chromosomal DNA. *Escherichia coli* XL1 Blue MRF′ [*recA1 endA1 gyrA96 thi hsdR17* (r_k^- m_k^+) *supE44 relA1 lac*(F′ *proAB*$^+$ *lacI*^{q}ZΔ M15::Tn10)] (Stragene, La Jolla, Calif., USA) was used for construction of the *B. kaustophilus* genomic library. *E. coli* NovaBlue (Novagen, Madison, Wis., USA) was used for DNA manipulations and for the high-level expression of recombinant protein. Vectors used were ZAP Express vector (Stratagene) and pQE-30 (Qiagen, Valencia, Calif., USA). *B. kaustophilus* was grown on Luria-Bertani (LB) medium at 55°C. The *E. coli* cells harboring plasmids were cultivated aerobically at either 28° or 37°C in LB medium supplemented with 100 μg amipicillin ml^{-1}.

DNA techniques

Chromosomal DNA of *B. kaustophilus* CCRC 11223 was isolated according to the method of Doi et al. (1983). Conventional techniques for DNA manipulations such as restriction enzyme digests, ligation, and transformations were performed as described by Sambrook and Russel (2001). DNA fragments were recovered from the agarose gel by a DNA extraction kit (Viogene, Taipei, Taiwan). The primers used were synthesized under the instructions of Oligo analysis software (National Biosciences, Mich., USA). DNA sequencing was performed by the chain-termination method using a SequiTherm ExcelII DNA sequencing kit (Epicentre Technologies, Madison, Wis., USA). Nucleotide and amino-acid sequences were analyzed with the programs BLASTX from the National Center for Biotechnology Information (National Library of Medicine, National Institute of Health, USA) and Alignment from the ExPASy molecular biology server (Swiss Institute of Bioinformatics).

Preparation of a DNA probe and cloning of the *B. kaustophilus lap* gene

Two degenerate primers, 5′- GA(T/C)GC(A/T/G/C)GA(A/G)GG(A/T/G/C) (C/A)G(A/T/G/C)T and 5′-CA(T/C)(T/C)T(A/G/T/C)GA(T/C)AT(A/T/C)GC(A/G/T/C)GG, were designed for PCR-mediated amplification of a 360-bp *lap* gene fragment. The PCR amplification was initiated at 95°C for 3 min followed by 30 cycles of denaturation at 94°C for 1.5 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. DNA labeling of the amplified fragment with a Megaprime DNA-labeling system (Amersham, Piscataway, N.J., USA) and [α-^{32}P]dCTP was performed according to the manufacturer's instructions.

The isolated chromosomal DNA of *B. kaustophilus* CCRC 11223 was subjected to partial digestion with *Sau*3A1 and size-fractionated on a 1% (w/v) agarose gel. DNA fragments of 2–7 kb were eluted from the gel and ligated into *Bam*HI/CIAP-treated ZAP Express vector to construct a genomic phage λ-ZAP library. The amplified library was transferred to positively charged nylon membranes (Hybond, Uppsala, Sweden) and hybridized with the [α-^{32}P]dCTP-labeled probe. Membranes were incubated in Rapid-hyb buffer (Amersham) at 65°C and washed under high-stringency conditions. One plaque designated *E. coli* (pBK-CMV6) was selected and subjected to DNA sequencing.

Construction of the expression plasmid and purification of the recombinant enzyme

A DNA fragment encoding the entire LAP was obtained by amplification of pBK-CMV6 with the following primers: BKLapf (5′CGCGGATCCATGTTTACGGTAAAACCTTTG-3′), which introduces sequences for a unique *Bam*HI site and in frame fusion with the 3′ end of the histidine tag leader sequence encoded in pQE-30, and Bklapr (5′-CGGGGTACCTTATTCAAACCGCTCGAC-3′, which incorporates sequences for a unique *Kpn*I site within the 3′ end (18 bp upstream of the TAA termination codon) of the *B. kaustophilus lap* gene. The PCR conditions were as described above. The PCR products were analyzed on 1% agarose gel and purified using the DNA extraction kit (Viogene). The recovered products were cloned as a *Bam*HI-*Kpn*I fragment (1,516 bp) into the respective sites of pQE-30 and transformed into competent cells of *E. coli* NovaBlue. After the selected clones had been verified by restriction enzyme analysis, one recombinant plasmid in which the *lap* gene is transcribed by a T5 promoter was obtained and designated pQE-LAP.

For the purification of the overexpressed LAP, a single colony of *E. coli* NovaBlue (pQE-LAP) was grown overnight with agitation at 37°C in 10 ml of LB medium containing 100 μg ampicillin/ml. Cultures were diluted (1:100) with 100 ml of the above medium and incubated at 28°C. Gene expression was induced in exponentially growing cells ($OD_{600\ nm}$ = 1.0) by the addition of 0.5 mM isopropyl-β-D- thiogalactopyranoside (IPTG). After a further 6 h cultivation, cells were harvested by centrifugation at 3,000 *g* for 15 min. The cells were washed with 20 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication (Sonicator XL-2020, Microsonic, Farmingdale, N.Y., USA). The cell lysate was then centrifuged at 12,000 *g* for 30 min to remove the insoluble cell debris. The resulting supernatant was mixed with Ni^{2+}-NTA resin (Qiagen) pre-equilibrated binding buffer (5 mM imidazole, 0.5 mM

NaCl, and 20 mM Tris-HCl, pH 7.9). The adherent protein was eluted from the column with a buffer containing 0.5 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9).

Protein analyses

A sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) assay was performed using a Protean III mini gel system (Bio-Rad Laboratories, Beverly, Mass., USA) according to the method of Laemmli (1970). The marker proteins were phosphorylase *b* (97.4 kDa), serum albumin (66.3 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa). The samples and marker proteins were denatured in the presence of 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol at 100°C for 5 min. Electrophoreses were carried out at room temperature and at 100 V constant voltage. The protein bands were visualized with Coomassie Brilliant Blue R-250.

Protein concentration was measured with a protein assay kit (Bio-Rad Laboratories), with serum albumin as the standard. For protein sequencing, the purified LAP was run on a 10% SDS-PAGE gel and transferred to a polyvinyldene difluoride membrane using a Mini Trans-Blot cell (Bio-Rad Laboratories). The filter was then rinsed several times in distilled water and stained with 0.2% Coomassie Brilliant Blue in 50% methanol for 10 min. The target band was cut off from the membrane and destained with 50% methanol. After being air-dried, the resulting material was wrapped in plastic, and stored at −20°C. The N-terminal sequence was determined by the Edman degradation method with an Applied Biosystems 475A gas phase sequencer (PE–Applied Biosystems).

Enzyme assay

LAP activity was assayed by monitoring the hydrolysis of L-leucine *p*-nitroanilide (L-Leu-*p*-NA). The reaction mixture contained 10 mM L-Leu-*p*-NA, 20 mM potassium phosphate buffer (pH 8.0), 1 mM $NiCl_2$, and an appropriate amount of the purified enzyme in a final volume of 1 ml. The mixture was incubated at 55°C for 10 min and the reaction was terminated by the addition of 100 µl of acetic acid. The absorbance at 405 nm was then measured. One unit (U) of LAP activity is defined as the amount of the enzyme that releases 1 µmol of *p*-nitroanilide per minute under the assay conditions.

Biochemical studies

The effect of pH on LAP activity was investigated at 55°C in 20 mM Tris-maleate buffer (pH 4.0–6.0), 20 mM potassium phosphate buffer (pH 6.0–8.0), 20 mM Tris-HCl buffer (pH 8.0–9.0), and 20 mM glycine-NaOH buffer (pH 9–12). To determine the effect of temperature on the recombinant LAP (rLAP), activity assays were performed in 20 mM potassium phosphate buffer (pH 8.0) at 30°–90°C. To determine the thermostability of the purified rLAP, the enzyme was adjusted to 50 mg/ml of the protein concentration with 20 mM potassium phosphate buffer (pH 8.0). The enzyme solution was incubated at 30°, 60°, 70°, and 80°C, respectively, for designated time periods. After incubation, 100 µl of the enzyme solution was withdrawn to determine the residual activity under the standard assay conditions.

A steady-state kinetics study of the purified rLAP was performed at 55°C in 20 mM potassium phosphate buffer (pH 8.0) with L-Leu-*p*-NA concentrations in the range of 0.1–10 mM. The K_m and k_{cat} values were determined by fitting the initial rates as a function of substrate concentration to the Michaelis-Menten equation using Grafit software (Sigma-Aldrich Chemical C, Mo., USA).

The hydrolytic activity of the purified enzyme against several *p*-nitroanilide derivatives was determined. The reaction mixture contained 50 µl of purified enzyme, 100 µl of 10 mM substrate, 20 µl of 50 mM $NiCl_2$, and 820 µl of 20 mM potassium phosphate buffer (pH 8.0).

Sequence accession number

The nucleotide sequence of the *lap* gene from *B. kaustophilus* CCRC 11223 and the encoded amino acid sequence have been deposited in the Genbank nucleotide database under accession number AY308074.

Results and discussion

Cloning and sequencing of a LAP-encoding gene from *B. kaustophilus*

A comparison of the amino-acid sequences of prokaryotic and eukaryotic LAPs identified at least five highly conserved motifs, which are located at the C-terminal portion of these enzymes. Based on the conserved DAEGRL and HLDIAG motifs, two degenerate primers were synthesized and used for PCR amplification of the respective DNA fragment. It is worth noting that the deduced amino-acid sequence of the amplified fragment had considerable similarity to known LAPs. To clone the genomic DNA encoding *B. kaustophilus* LAP, the PCR product was labeled with [α-^{32}P]dCTP and used as the hybridization probe. After screening the λc 1857 *Sam*7 genomic library by plaque hybridization, seven positive clones were obtained. One clone with a 2.3-kb insert was selected for DNA sequencing. The nucleotide sequence of the DNA fragment contained an open reading frame (ORF) encoding 497 amino acids with a calculated molecular mass of 53,700 Da. A possible ribosome-binding site (GAAAGGG) is situated 8 bp upstream of the ATG putative initiation codon and the stem-loop area located at downstream region might be a putative transcription terminator. To provide direct experimental proof for the expression of the *lapA* gene in *B. kaustophilus*, we mapped its transcription initiation site(s) by primer extension. The results revealed that there are two *lapA*-specific mRNA species in *B. kaustophilus*. The longer mRNA species initiates at a G residue at 30 bp upstream of the *lapA* ATG start codon (data not shown). Inspection of the DNA sequence upstream of this initiation site revealed the presence of putative –10 (TATAAT) and –35 (TTAGAT) sequences that closely resemble the consensus sequence of promoters recognized by the main vegetative sigma factor (σ^A) of *Bacillus subtilis* (Helmann 1995). In addition to the above mRNA, a smaller transcript located within the coding region (12 bp downstream of the start codon) was also detected (data not shown). We are currently unable to say whether this mRNA species is a degradation product of the main *lapA* mRNA or whether it represents a transcript initiating from another promoter.

Comparison of amino acid sequences

BLAST searching of Genbank using the translated ORF yielded 30–57% sequence identities with other LAP sequences from a variety of organisms. The closest match was with the LAP sequence of *B. subtilis* (57% amino-

acid identity; 72% similarity). Multiple sequence alignment among *B. kaustophilus* LAP, *B. subtilis* LAP, bovine lens LAP, and *E. coli* aminopeptidase A revealed several significantly conserved domains (Fig. 1). These domains are located at the C-terminal regions of the aligned enzymes. The crystal structure of bovine lens LAP shows that the core of the C-terminal domain has a triple-layered structure consisting of a central eight-stranded *β*-sheet sandwich between five *α*-helices on each side (Burley et al. 1990). A pair of metal ions is located near the edge of an eight-stranded, saddle-shaped *β*-sheet and the six active sites are situated in the interior of the hexamer, where they line a disk-shaped cavity (Burley et al. 1990). Interestingly, the molecular structure of *E. coli* aminopeptidase A exhibits very little change in the geometry of the C-terminal domain (Sträter et al. 1999). The aligned data showed that the amino-acid residues essential for the metal binding sites (Asp-255, Asp-273, Asp-332, and Glu-334) and the catalytic sites (Lys-250 and Arg-336) of the bovine lens LAP are conserved in *B. kaustophilus* LAP (Fig. 1), suggesting that the cloned enzyme is a zinc-coordinated metalloprotease.

The zinc hydrolyase superfamily consists of a group of divergently related proteins, including aminopeptidases, carboxypeptidases, and non-peptidase enzymes such as succinyl-diaminopimelate desuccinylases. Members of this superfamily include families M17 (LAPs), M20 (carboxypeptidases G2, deacetylases, and succinyl-diaminopimelate desuccinylases), and M28 (APs and transferring receptor homologues) (Barrett et al. 1998). Homology between these proteins has been inferred based on a shared structural scaffold comprising eight *β*-strands and six *α*-helices with the active site at the C-terminal end of the central four parallel *β*-strands (Artymiuk et al. 1992; Rowsell et al. 1997). Comparison of the deduced amino acid sequence of *B. kaustophilus* LAP with those from zinc hydrolyase superfamily was performed using the CLUSTAL-W program (http://clustalw.genome.ad.jp). These results were reiterated by phylogenetic analysis of the amino-acid sequences (Fig. 2). Based on the analysis, *B. kaustophilus* LAP is grouped to the M17 family and clustered together with the enzymes from *B. subtilis* and *Bacillus halodurans*. The sequence identity was 13% between the cloned LAP and its closest neighbor, paCPG2 (Minton et al. 1984),

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         1                                                                              80
BKLAP    -MFTVKPLPS-AKERDEALVVGLFEGANS------WSGLAGEYDARLGGRLSELQKEGDISAKRGRIATVHPFLPMGAKR
BSLAP    -----MFYAVQQSEHTETLVVGLFQ-KSQ------LTGKALEIDEMLEGHLTQLLKEGDVSAKPNQVSKVFPPSSAGMKR
ECLAP    MEFSVKSGSP-EKQRSACIVVGVFE-PRR------LSPIAEQLDKISDGYISALLRRGELEGKPGQTLLLHHVPNVLSER
BLLAP    --------------MTKGLVLGIYS-KEKEEDEPQFTSAGENFNKLVSGKLREILNISGPPLKAGKTRTFYG-LHEDFPS

         81                                                                             160
BKLAP    LYFVGLGKKEELTFERLREVFGKLFRTLKQAKRTKAAVALDTFTTEAVDGNEAAHALAEAYHLATYEFPGYKQKKSQPDY
BSLAP    IYFVGLGREANYSFEQAKERFAHVFQAIHKDRKQETAVLLDTFISEDVPPADAAHALAESCLLASYEVQDYKHKSNEPDK
ECLAP    ILLIGCGKERELDERQYKQVIQKTINTLNDTGSMEAVCFLTELHVKGRNNYWKVRQAVETAKETLYSFDQLKTNKSEPRR
BLLAP    VVVVGLGKKTAGIDEQENWHEGKENIRAAVAAGCRQIQDL-EIPSVEVDPCGDAQAAAEGAVLGLYEYDDLKQK------

         161                                                                            240
BKLAP    QLESLI--VYTAAEAAEIEASLFVGSVYGKATNSARTLVNTPGNLLTASDLADYAVKLANRYDFDY--EVLEKEDMERLG
BSLAP    QIEAVY--VVTDEDTQEVQAGLRVGQAYGQGTNSARTLVNMPGNMLTATDLASYAEELAAKYDFEC--EILEKSEMEELG
ECLAP    PLRKMVFNVPTRRELTSGERAIQHGLAIAAGIKAAKDLGNMPPNICNAAYLASQARQLADSYSKNVITRVIGEQQMKELG
BLLAP    --RKVV--VSAKLHGSEDQEAWQRGVLFASGQNLARRLMETPANEMTPTKFAEIVEENLKSASIKTDVFIRPKSWIEEQE

         241                                                                            320
                                              *    *      •          *
BKLAP    MGALLAVNQGSKQPPKLIVLKYQGK-DEWEDVIALVGKGVTFDTGGYCLKPRDSMVDMKTDMAGAAAVLGAMEAIGELRP
BSLAP    MGGILAVNQGSTEPPKMIVLKYQGK-KEWEDVVGLVGKGITFDTGGYSIKTKSGIVGMKSDMGGAAAVLGAMETIGELRP
ECLAP    MHSYLAVGQGSQNESLMSVIEYKGNASEDARPIVLVGKGLTFDSGGISIKPSEGMDEMKYDMCGAAAVYGVMRMVAELQL
BLLAP    MGSFLSVAKGSEEPPVFLEIHYKGSPNASEPPLVFVGKGITFDSGGISIKAAANMDLMRADMGGAATICSAIVSAAKLDL

         321                                                                            400
                                                * * •
BKLAP    EQNVLAVIPATDNMISGEAFKPDDVITSLSGKTIEVKNTDAEGRLILADAVTYAKQHGASYIIDVATLTGGVIVALGTDK
BSLAP    EQNVLCVIPSTDNMISGGAMKPDDVIVSLSGKTIEILNTDAEGRLVLADGITYAKQHGASVLVDVATLTGGVVVALGTET
ECLAP    PINVIGVLAGCENMPGGRAYRPGDVLTTMSGQTVEVLNTDAEGRLVLCDVLTYVERFEPEAVIDVATLTGACVIALGHHI
BLLAP    PINIVGLAPLCENMPSGKANKPGDVVRARNGKTIQVDNTDAEGRLILADALCYAHTFNPKVIINAATLTGAMDIALGSGA

         401                                                                            480
BKLAP    TGAMTNNEELFQQLLEASMETGEFIWRLPITENDRERVRSSKIADLNN-SPGREGHAIMGGAFIGEFAEDTPWVHLDIAG
BSLAP    TGAMTNDQSFYQQVADAAQECGEAIWQLPITEKDKKRVKSSQMADLSN-SPGREGHAIMAGTFLGEFAESTPWVHLDIAG
ECLAP    TGLMANHNPLAHELIAASEQSGDRAWRLPLGDEYQEQLESN-FADMAN-IGGRPGGAITAGCFLSRFTRKYNWAHLDIAG
BLLAP    TGVFTNSSWLWNKLFEASIETGDRVWRMPLFEHYTRQVIDCQLADVNNIGKYRSAGACTAAAFLKEFVTHPKWAHLDIAG

         481                                 515
BKLAP    TAATKKDGDLGPAGATGVMVRTLTAFVERFE---------
BSLAP    TATANKATCFGPAGATGVMARTLATLAERFTLEEDKNE--
ECLAP    TAWRSGKA----KGATGRPVALLAQFLLNRAG---FNGEE
BLLAP    VMTNKDEVPYLRKGMAGRPTRTLIEFLFRFSQ--------
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Fig. 1 Alignment of the deduced amino acid with some leucine aminopeptidases. Abbreviations are: *BKLAP B. kaustophilus* LAP, *BSLAP B. subtilis* LAP (TrEMBL O32106), *ECLAP E. coli* LAP (TrEMBL P11648), *BLLAP* bovine lens LAP (TrEMBL P00727). The conserved residues are *shaded*. Gaps are represented by *dashes*. The essential zinc-binding and active-site residues are marked by *stars* and *closed circles*, respectively. The N-terminal amino acid sequence of *B. kaustophilus* enzyme determined by Edman degradation is *underlined*

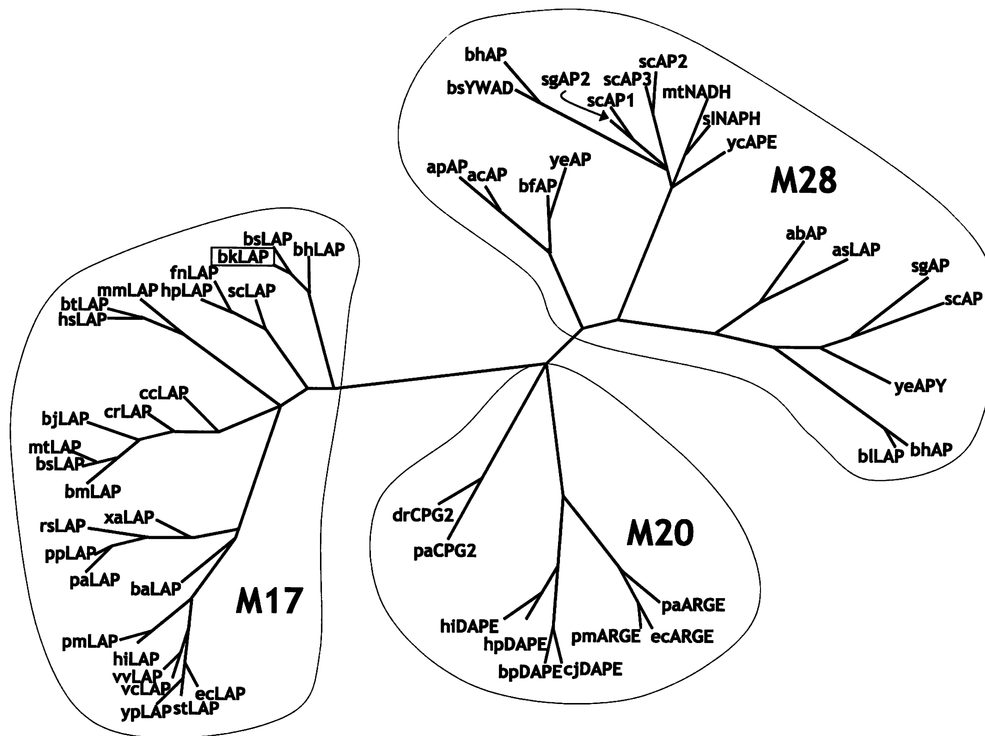

Fig. 2 Unrooted phylogenetic tree created by sequence homology. **M17**: bkLAP, *Bacillus kaustophilus* LAP; bsLAP, *Bacillus subtilis* LAP (TrEMBL O32106); bhLAP, *Bacillus halodurans* LAP (TrEMBL Q9K7G0); hpLAP, *Helicobacter pylori* LAP (TrEMBL O25294); fnLAP, *Fusobacterium nucleatum* LAP (TrEMBL Q8RHT8); scLAP, *Streptomyces coelicolor* LAP (TrEMBL Q9S2Q7); mmLAP, *Mus musculus* LAP (TrEMBL Q9CPY7); btLAP, *Bos taurus* LAP (TrEMBL P00727); hsLAP, *Homo sapiens* LAP (TrEMBL AAH06199); ccLAP, *Caulobacter crescentus* LAP (TrEMBL Q9A7M9); crLAP, *Cowdria ruminantium* LAP (TrEMBL Q93FS7); bjLAP, *Bradyrhizobium japonicum* LAP (TrEMBL BAC49373); mtLAP, *Mycobacterium tuberculosis* LAP (TrEMBL Q10401); bsLAP, *Brucella suis* LAP (TrEMBL Q8G1M4); bmLAP, *Brucella melitensis* LAP (TrEMBL Q8YG99); xaLAP, *Xanthomonas axonopodis* LAP (TrEMBL Q8PGR0); rsLAP, *Ralstonia solanacearum* LAP (TrEMBL Q8XWQ8); ppLAP, *Pseudomonas putida* LAP (TrEMBL AAN66605); paLAP, *Pseudomonas aeruginosa* LAP (TrEMBL O68822); baLAP, *Buchnera aphidicola* LAP (TrEMBL AAO27053); pmLAP, *Pasteurella multocida* LAP (TrEMBL P57823); hiLAP, *Haemophilus influenzae* LAP (P45334); vvLAP, *Vibrio vulnificus* LAP (TrEMBL Q8DCE5); vcLAP, *Vibrio cholerae* LAP (TrEMBL Q9K2W5); ypLAP, *Yersinia pestis* LAP (TrEMBL Q8ZBH3); stLAP, *Salmonella typhimurium* LAP (TrEMBL Q8ZK29); ecLAP, *Escherichia coli* LAP (TrEMBL P11648). **M20**: drCPG2, *Deinococcus radiodurans* CPG2 (pir C75268); paCPG2, *P. aeruginosa* CPG2 (TrEMBL Q9I056); hiDAPE, *H. influenzae* DAPE (SWISS-PROT DAPE_HAEIN); hpDAPE, *Helicobacter jejuni* DAPE (TrEMBL O25002); bpDAPE, *Bordetella pertussis* DAPE (TrEMBL Q9ZEX1); cjDAPE, *C. jejuni* DAPE (trEMBL O25002); pmARGE, *P. multocida* acetylornithine deacetylase (ARGE) (TrEMBL Q9CLT9); ecARGE, *E. coli* ARGE (SWISS-PROT ARGE_ECOLI); paARGE, *P. aeruginosa* ARGE (GenPept AAG08591). **M28**: blLAP, *Bacillus licheniformis* LAP (TrEMBL Q93EJ5); bhAP, *B. halodurans* aminopeptidase (AP) (TrEMBL Q9K671); yeAPY, *Saccharomyces cerevisiae* AP (SWISS-PROT APE3_YEAST); scAP, *S. coelocolor* AP (TrEMBL Q9F2X2); sgAP, *Streptomyces griseus* AP (TrEMBL P80561); asLAP, *Aspergillus sojae* LAP (TrEMBL Q8J2N2); abAP, *Agaricus bisporus* AP (TrEMBL Q8WZH8); ycAPE, *S. cerevisiae* AP (TrEMBL P37302); slNAPH, *Streptomyces lipmanii* N-acetylpuromycin N-acetylhydrolase ((TrEMBL Q53737); mtNAPH, *M. tuberculosis* hypothetical protein (TrEMBL P96264); scAP2, *S. coelicolor* AP2 (GenPept CAC05888); scAP3, *S. coelicolor* AP3 (GenPept CAC01508); scAP1, *S. coelicolor* AP1 (GenPept CAC08290); sgAP2, *S. griseus* AP2 (TrEMBL P50561); bhAP, *B. halodurans* AP (TrEMBL Q9K671); bsYWAD, *B. subtilis* hypothetical peptidase ywaD (SWISS-PROT YWAD_BACSU); yeAP, *S. cerevisiae* AP (MEROPS MER05131); bfAP, *Botryotinia fuckeliana* AP (MEROPS MER13609); acAP, *Aeromonas caviae* AP (TrEMBL O82996); apAP, *Aeromonas proteolytica* AP (TrEMBL Q01693)

in the tree with a prepeptide structure. The relative locations of *B. kaustophilus* LAP, acAP, and paCPG2 in the tree suggested that the common ancestor of these enzymes contains a prepeptide domain. Since the AP enzymes, such as acAP (Izawa and Hayashi 1996), have N-terminal prepeptide sequences, it is possible that the prepeptide domains of LAPs were lost after the M17 ancestor diverged from the other AP enzymes. Loss of the prepeptide domain may have been concomitant with the acquisition of the M17 family's unique N-terminal domain to reroute its ancestor from the secretory pathway to the cytosol.

Expression and purification of the recombinant enzyme

For high-level expression of *B. kaustophilus lap* gene, the PCR-amplified fragment containing the entire coding sequence was digested with *Bam*HI and *Kpn*I, and inserted into the expression vector under the control of the T5 promoter. The rLAP has ten additional amino acids, MRGSHHHHHH, at its N-terminus. *E. coli* NovaBlue cells harboring pQE-LAP were induced with 0.5 mM IPTG. After 1-, 3-, 6-, and 20-h inductions, an aliquot of the bacterial culture was centrifuged and resuspended in loading buffer, and the total cellular proteins were

separated with 10% SDS-PAGE. The protein patterns of the total cell extracts with or without IPTG induction are shown in Fig. 3A. Analysis of the total proteins from IPTG-induced *E. coli* NovaBlue (pQE-LAP) exhibited a predominant protein band with apparent M_r of approximately 54 kDa, comparing well with the calculated mass of the affinity-tagged translational product of the *lap* gene (54.7 kDa). To date, many efforts have been made towards heterologous gene expression in recombinant *E. coli* in order to enhance the quality of recombinant proteins (Olins and Lee 1993). In this study, several different induction temperatures were tried for overexpression of the rLAP. It is worthwhile to note that the optimal temperature for the production of active rLAP is around 28°C. In fact, the results obtained at 20°, 28°, and 32°C were very similar, except that the time point for maximal expression was delayed at 20°C, and was earlier at 32°C than that at 28°C. Additionally, the optimum IPTG concentrations for the expression of rLAP were in the range 0.1–1 mM. There was a significant reduction in the level of active enzyme when IPTG concentration exceeded 3 mM. For maximal production of active rLAP, IPTG at a final concentration of 0.5 mM, and an induction temperature and time of 28°C and 6 h, respectively, were used in the subsequent experiments. Under these conditions, the expressed protein comprised up to 24% of the total cell proteins of IPTG-induced *E. coli* NovaBlue (pQE-LAP) when the SDS-PAGE gels were analyzed by a densitometric gel scanning system. The overexpressed LAP in the cell-free extract had a specific activity of 7.4 U/mg protein and the activity for the purified enzyme was 127.5 U/mg protein, indicating that the protein was purified approximately 17.2-fold by nickel-chelate chromatography (Fig. 3B).

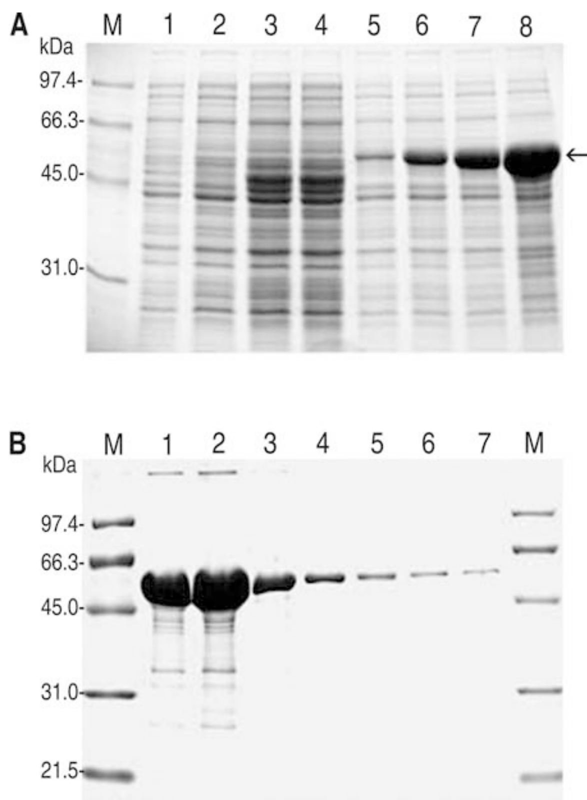

Fig. 3A, B SDS-PAGE analysis of total cell proteins from *E. coli* NovaBlue (pQE-LAP) and the eluted fractions from nickel-chelate chromatography. **A** Cell free extracts from the recombinant *E. coli*. Lanes: M molecular mass marker, *1* 1 h cultivation without IPTG induction, *2* 3 h cultivation without IPTG induction, *3* 6 h cultivation without IPTG induction, *4* 20 h cultivation without IPTG induction, *5* 1 h cultivation with IPTG induction, *6* 3 h cultivation with IPTG induction, *7* 6 h cultivation with IPTG induction, *8* 20 h cultivation with IPTG induction. *Arrow* indicates the position of rLAP. **B** Nickel-chelate column chromatography. Lanes: *M* molecular mass marker, *1–7* fractions 1–7

Enzymatic properties of rLAP

Amidolytic activity against L-Leu-*p*-NA was optimal at pH 8.0 (Fig. 4), and strong activity was still detectable at pH 9. However, activity rapidly declined under neutral conditions (pH 7.0). The apparent temperature dependence of the purified rLAP in a 10 min assay at pH 8.0 is shown in Fig. 5A. The enzyme was most active at 65°C and more than 70% of maximal activity was found from 40° to 70°C. The enzyme decay obeyed first-order kinetics and showed half-lives of 17 and 10 min when incubated at 70° and 80°C, respectively (Fig. 5B). The thermostability of a protein is determined by many factors, which include hydrogen bonding networks, hydrophobic interactions, optimized core packing, salt bridges, and the reduction of the entropy of unfolding (Vieille and Zeikus 1996). While most natural proteins seem to achieve their individual stability by accumulating a large number of weekly stabilizing interactions that result in a large net effect, some have acquired specialized structural features that cannot easily be transferred in a general way into other proteins (Demirjian et al. 2001). Generally, thermostable enzymes are isolated from organisms growing near the range of maximum protein stability (Vieille et al. 1996). However, the

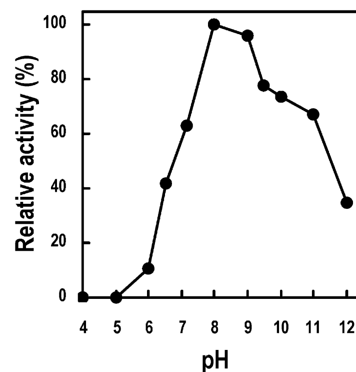

Fig. 4 Effect of pH on activity of the purified rLAP. Enzyme activities were measured at various pH values under standard assay conditions

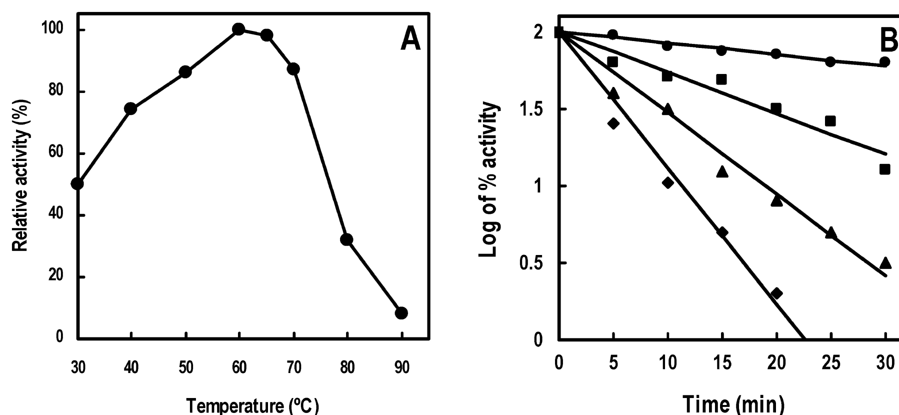

Fig. 5 Effect of temperature on activity (**A**) and stability (**B**) of the purified rLAP. For the determination of thermostability, enzyme activities were assayed after the buffered enzyme was incubated at 30° (*circles*), 60° (*squares*), 70° (*triangles*), and 80°C (*diamonds*), respectively

Table 1 Effect of divalent metal ions on the activity of the purified rLAP

Metal ions	Concentration (mM)	Relative activity (%)
None	–	100
Mg^{2+}	1	156
Cu^{2+}	1	161
Zn^{2+}	1	104
Ca^{2+}	1	97
Co^{2+}	1	349
Mn^{2+}	0.1	218
	1	376
	10	297
	100	132
Ni^{2+}	0.1	1,565
	1	2,014
	10	1,600
	100	498

Table 2 Substrate specificity of the purified rLAP

Substrate	Specific activity (U/mg)	Relative activity (%)
Leu-*p*-NA	69.3	100
Cys-*p*-NA	6.7	9.7
Ala-*p*-NA	1.2	1.7
Pro-*p*-NA	1.0	1.4
Cbz-Gly-Gly-Leu-*p*-NA	–[a]	–
Cbz-Leu-Leu-Glu-β-naphthylamide	–	–

[a] – not detected

aminopeptidases from *E. coli* and *Rickettsia prowazekii* fully retained their activities after heating the enzymes at 70°C for 5 min (Vogt 1970; Wood et al. 1993). Since *E. coli* and *R. prowazekii* are mesophiles, it is possible that aminopeptidases of both microorganisms have evolved their structural features to become thermally adapted proteins.

The affinity for L-Leu-*p*-NA (K_m), turnover rate (k_{cat}), and catalytic efficiency (k_{cat}/K_m) of the purified rLAP were estimated to be 290 μM, 0.08 min^{-1}, and 0.27 $min^{-1}{\cdot}mM^{-1}$, respectively. The effect of chemicals on enzyme activity is shown in Table 1. In the absence of metal ions, the specific activity of the purified rLAP was 3.6 U/mg protein. Zn^{2+} and Ca^{2+} ions had no significantly stimulatory effect on the amidolytic activity. Like *Leishmania* LAP (Morty and Morehead 2002), the amidolytic activity was enhanced by Mn^{2+} and Ni^{2+} ions. Metalloaminopeptidases exhibit a broad range of metal-ion dependence. *S. griseus* aminopeptidase needs Zn^{2+} (Greenblatt et al. 1997), while other aminopeptidases are reported to be activated by Mn^{2+} (Cottrell et al. 2000), Co^{2+} (Herrera-Camacho et al. 2000), and Ca^{2+} (Ando et al. 1999). The zinc-binding sites for bovine lens (Kim and Lipscomb 1993) and tomato (Gu and Walling 2002) LAPs have been identified and consist of site 1, which readily exchanges Zn^{2+} for other divalent metal cations including Mn^{2+} and Co^{2+}, and site 2, which binds Zn^{2+} much more strongly and retains its Zn^{2+} under conditions that allow exchange of the Zn^{2+} in site 1. In this study, the activation of *B. kaustophilus* LAP by Ni^{2+} and Mn^{2+} could be due to the substitution of the site 1 Zn^{2+} with these metal ions. Indeed, substitution of the site 1 Zn^{2+} of porcine kidney LAP with Mn^{2+} and Co^{2+} has been shown to activate that enzyme by elevating the k_{cat} (van Wart et al. 1981).

Optimal amidolytic activity was observed against L-Leu-*p*-NA (Table 2). L-Cys-*p*-NA was hydrolyzed considerably less efficiently and very poor activity was detected against L-Ala-*p*-NA and L-Pro-*p*-NA. No activity was observed against other L-amino acid amides, Cbz-L-Leu-β-naphthylamine or Cbz-Gly-Gly-Leu-*p*-NA, indicating that the rLAP is a strict aminopeptidase lacking endopeptidase activity. It is worth mentioning that *B. kaustophilus* LAP possessed activity against L-Cys-*p*-NA. The cysteinyl aminopeptidase activity is a novel and unique feature for the cloned enzyme, since it is a usual property of cysteinyl aminopeptidase/oxytocinase (EC 3.4.11.3), which belongs to the M1 family of zinc metallopeptidases (Rogi et al. 1996).

In conclusion, the acquisition of extra domains by gene shuffling is a well-documented method of evolution (Saier 1996). The comparatively lower sequence identity between the structural scaffolds in the LAP proteins suggests that change has been more rapid in the noncatalytic domain of the enzymes. The residues essential for catalytic activity and metal ion coordination of the cloned enzyme are currently being investigated. To our

knowledge, this is the first report on the characterization of a thermostable LAP from the genus *Bacillus*. The resultant information will contribute to our understanding the exopeptidase activity of this genus.

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