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Cloning and Expression of the Aminopeptidase Gene from the *Bacillus licheniformis* in *Bacillus subtilis*

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Abstract A gene (*bap*) encoding aminopeptidase from the chromosomal DNA of *Bacillus licheniformis* was cloned. The gene is 1,347 bp long and encodes a 449 amino acid preproprotein with a major mature region of 401 amino acids (calculated molecular mass 43,241 Da). N-Terminal sequence of the purified protein revealed a potential presence of N-terminal propeptide. The deduced primary amino acid sequence and the mass analysis of the purified protein suggested that a C-terminal peptide YSSVAQ was also cleaved off by a possible endogeneous protease. The amino acid sequence displayed 58% identity with that of the aminopeptidase from alkaliphilic *Bacillus halodurans*. This bacterial enzyme was overexpressed in recombinant *Escherichia coli* and *Bacillus subtilis* cells. Clones containing the intact *bap* gene, including its own promoter and signal sequence, gave rise to the synthesis of extracellular and thermostable enzyme by *B. subtilis* transformants. The secreted protein exhibited the same biochemical properties and the similar apparent molecular mass as the *B. licheniformis* original enzyme.

Key words: Aminopeptidase, gene expression, *Bacillus licheniformis*

Aminopeptidases (E.C. 3.4.11) are enzymes that catalyze the cleavage of amino acid residues at the N-terminal of peptides and proteins. These enzymes are found widely distributed amongst both prokaryotic and eukaryotic organisms. Their speeificities differ widely in respect to their ability to hydrolyze acidic, basic, or neutral N-terminal residues. Extracellular aminopeptidases derived from bacteria usually show broad substrate specificity. These aminopeptidases are classified as nonspecific enzyme or sometimes as leucine aminopeptidase, because they often show higher selectivity towards leucine [3, 7]. Some of bacterial nonspecific aminopeptidases can not hydrolyze X-Pro bonds, where X can be any amino acid except proline. This type of specificity plays an interesting role in the production of recombinant protein for N-terminal methionine processing [2].

Basic studies on bacterial aminopeptidases have allowed to characterize a number of aminopeptidases at the biochemical and molecular levels [7], while microbial aminopeptidases have been of considerable interest to many industrial applications. An extracellular aminopeptidase derived from *Bacillus licheniformis* has been initially isolated and its properties have been studied by Rodriguez-Absi and Prescott [14]. They reported that the aminopeptidase isolated from *Bacillus licheniformis* ATCC12759 have a broad specificity towards artificial substrates such as *p*-nitroanilide analogues and dipeptides. LGCI (Daejeon, Korea) later found that extracellular aminopeptidase derived from *Bacillus licheniformis* strain could selectively cleave off N-terminal methionine residues from Met-Phe-Pro-(peptide), thus suggesting that it could be used for processing of methionyl hGH (human growth hormone) to authentic hGH. The aminopeptidase purified from *Bacillus licheniformis* has also been found to be a monomeric metaloenzyme containing zinc ion with molecular weight of about 48–49 kDa by SDS-PAGE (unpublished data). In this study, the isolation of the aminopeptidase gene from *Bacillus licheniformis* and its expression in *E. coli* along with heterologous *Bacillus subtilis* are presented.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Bacillus licheniformis KCTC3058 was used as DNA and enzyme sources. *E. coli* DH5α [15] was used as a recipient for the gene cloning experiments, *E. coli* BL21(DE3)

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(Novagen, MA, U.S.A.) and *Bacillus subtilis* LKS87 [*nprR2 nprE18 ΔaprA3 amyE*] [9] as host strains for the expression of the aminopeptidase gene. pRB373 [4], a shuttle vector for *E. coli* and *B. subtilis*, was used for the expression in *B. subtilis*, and pET11a (Novagen, U.S.A.) for the expression in *E. coli*. Plasmid pHNT [13] was used for the cloning of PCR product. *E. coli, B. subtilis*, and *B. licheniformis* were incubated in an LB medium at 37°C. Agar plates containing the same medium were prepared. Kanamycin (10 μg/ml) was added as required.

Purification of Aminopeptidase

Bacillus licheniformis was cultivated in the medium containing glucose, peptone, yeast extract, and trace metals. The supernatant was recovered by centrifugation and concentrated with Centriprep-10 (Amicon, Beverly, U.S.A.) ultrafiltration. The enzyme preparation was loaded onto an SP-Sepharose equilibrated with 20 mM phosphate and eluted with a linear gradient from 0.3 M NaCl to 1 M NaCl. The active fractions were pooled and concentrated by ultrafiltration, and the concentrate was applied to Sephacryl S-200 equilibrated with 20 mM phosphate buffer (pH 6.8) and 2 M urea. The enzyme preparation from the previous step was loaded onto a DEAE-Sepharose FF equilibrated with 20 mM phosphate buffer (pH 6.8) and eluted with a linear gradient of NaCl from 0 to 0.3 M in 20 mM phosphate buffer. The purified aminopeptidase was filtered through a 0.22 μm filter, and stored at −70°C until use.

Amino Acid Sequencing of Tryptic Peptides

The purified aminopeptidase was digested with trypsin according to the method of Stone *et al.* [16]. The tryptic peptides were separated by RP-HPLC using Vydac C_{18} reverse phase column (Grace Vydac, Hesperia, U.S.A.). The peptides were eluted with a linear gradient of acetonitrile 0.05% TFA (trifluoroacetic acid) at 0.5 ml/min at 214 nm detection. The sequence of the purified peptides was determined by N-terminal amino acid sequencing.

Synthesis of Oligonucleotide Primers

Oligonucleotide primers for PCR cloning of the aminopeptidase gene were designed on the basis of peptide sequences obtained by trypsin digestion of purified enzyme from *B. licheniformis*. The 5'-upstream (LAP-5) and 3'-downstream (LAP-3) primers were 5'-AA(T/C) CCN GA(T/C) AT(T/C/A) GTN TA(T/C)-3' and 5'-(A/G)AA NAG NAC (A/G)TC (T/C)TT NCC-3', respectively, which were derived from possible DNA sequences corresponding to the amino acid sequences of two parts of aminopeptidase, Asn-Pro-Asp-Ile-Val-Tyr and Gly-Lys-Asp-Val-Leu-Phe-Leu.

Amplification of DNA Fragment Encoding Aminopeptidase

The PCR was performed with *B. licheniformis* chromosomal DNA as a template for 32 cycles on a DNA thermal cycler (Perkin-Elmer, Foster City, U.S.A.). The thermal cycle of the PCR procedures included a denaturation at 94°C for 30 s, annealing at 40°C for 45 s, and extension at 72°C for 1 min. The PCR product was cloned into the pHNT vector and sequenced. This PCR product was also used as a hybridization probe to isolate the entire *bap* gene in the *B. licheniformis* genomic library.

Construction and Screening of *B. licheniformis* Genomic Library

Bacillus licheniformis genomic DNA was prepared from the cells according to the method described by Murray and Thompson [10]. The *B. licheniformis* genomic DNA was partially digested with *Sau*3A, and fragments ranging between 2 and 3 kb were selected on 0.8% agarose gel. The size-fractionated fragments were ligated into *Bam*HI digested-λ ZAP Express vector (Stratagene, La Jolla, U.S.A.), and added to a lambda packaging extract. *E. coli* XL1-Blue MRF (Stratagene, U.S.A.) was transduced with the packaging mixture. Duplicate filters of the library were screened with ^{32}P-labeled PCR fragment. A positive clone was isolated through 2nd and 3rd screenings, and its DNA was sequenced on both strands using an ABI377B DNA sequencer (Perkin-Elmer, U.S.A.).

Cloning of Aminopeptidase Gene in *E. coli* and *B. subtilis*

The initial isolate of genomic library contained a 2.6-kb insert. The insert was subcloned in pBK-CMV (Stratagene, U.S.A.) and named pLAP32, which was subsequently used as a source for the aminopeptidase gene (*bap*). Open reading frame of *bap* gene was amplified from genomic DNA by PCR with a forward primer (5'-ACG CGG ATC CGA AAA ATT CAG CAA AAA ATT CAA T-3') and reverse primer (5'-CAT GAG ATC TTT GGG CGA CTG AGC TGT AAG-3'). The amplified 1.2-kb PCR DNA was digested with *Bam*HI and *Bgl*II, and then inserted into the *Bam*HI site of pET11a, thereby producing a plasmid (pELAP113) harboring the ORF of the *bap* gene. For the expression of the *bap* gene in *B. subtilis*, a 2.6 kb of the original clone including the promoter, 3'-untranslated region, and the coding sequence of the aminopeptidase gene was recovered from the pLAP32 by digestion with *Hin*dIII and *Sac*I, and cloned into *Hin*dIII and *Sac*I-digested pRB373, with the product designated as pLAP373.

Aminopeptidase Assay

Recombinant *B. subtilis* LKS87 harboring pLAP373 was grown in a 20 ml LB medium supplemented with kanamycin (10 μg/ml) at 37°C. The culture supernatant was collected at the stationary phase by centrifugation at 4,000 ×*g* for 5 min. The aminopeptidase activity was measured spectrophotometrically at 405 nm using a synthetic substrate, L-leucine-*p*-nitroanilide (leu-pNA). Briefly, 50 μl

of culture supernatant and 20 µl of 0.1 M leu-pNA in DMSO were added to 950 µl of 1 M Tris, pH 8.5, and the mixture was incubated at 60°C for 3 min. One unit of aminopeptidase was defined as the amount of the enzyme per milliliter required to produce 10 micromoles of p-nitroaniline per minute under the described conditions.

N-Terminal Amino Acid Sequencing

N-Terminal sequencing was performed using Applied Biosystems 476A Protein Sequencer (Applied Biosystems, Foster City, U.S.A.) according to the manufacturer's instruction. Data were collected and analyzed using Applied Biosystems 610 Data Analysis software.

Mass Spectrometry

An aminopeptidase sample at about 1 mg/ml was dialyzed in 100 mM acetic acid and its mass was measured by Q-TOF (Micromass, Manchester, U.K.). The sample was injected at a flow rate of 0.5 µl/min to ESI source of Q-TOF MS. MS spectra were obtained with the capillary voltage and collision energy was set at 3.5 kV and 10 eV, and collision gas pressure was adjusted at 10–15 psi.

Miscellaneous Procedures

The DNA sequence analysis was performed using the primer walking method with an automated sequencer. DNA manipulation, plasmid isolation, and agarose gel electrophoresis were all carried out according to Sambrook *et al.* [15]. *E. coli* and *B. subtilis* were transformed by electroporation using a Gene Pulser (Bio-Rad, Richmond, U.S.A.) as described previously [6, 8, 19].

Nucleotide Sequence Number

The GenBank accession number for the *bap* genomic DNA sequence is AF358651.

Results and Discussion

Cloning of the Aminopeptidase Gene from *B. licheniformis*

B. licheniformis aminopeptidase was purified from the culture media and digested with trypsin to produce a series of peptides, as described in Materials and Methods. The amino acid sequencing of a few selected peptides was performed to design the degenerate oligonucleotide primers. The amino acid sequences of two of those trypsin-digested fragments were determined to be NPDIVY and GKDVLFLHQ, respectively. Computer search on the SwissProt database showed that these peptide sequences were homologous with those of aminopeptidases from other species. In a PCR with degenerate LAP-5 and LAP-3 primers, a DNA fragment of about 390 bp was weakly amplified (Fig. 1) from chromosomal DNA of *B. licheniformis*. Sequence

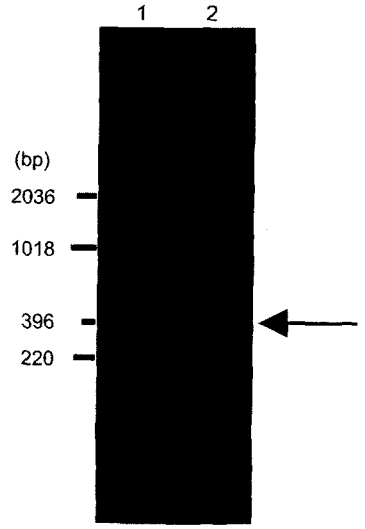

Fig. 1. PCR ampilification of *B. licheniformis* DNA.
The chromosomal DNA was amplified with LAP-5 and LAP-3 primers (lane 2). The arrow indicates the amplified 390-bp fragment. Lane 1: 1 kb size marker.

analysis of the 390-bp PCR product revealed that its deduced amino acid sequence also contained other peptide fragment of trypsin digests as well as two fragments used to design PCR primers, suggesting that the amplified DNA fragment was derived from the aminopeptidase gene of *B. licheniformis* of interest. Since the PCR fragment was identified to be a part of the aminopeptidase gene, the 390-bp fragment thus obtained was used as a hybridization probe for screening the full-length aminopeptidase gene from a genomic library of *B. licheniformis*. Approximately 30,000 individual plaques from the genomic library were screened by plaque hybridization with the ^{32}P-labeled PCR product described above. A clone (named LAP132) with a 2.6-kb DNA insert was isolated and characterized to obtain the nucleotide sequence and location of the aminopeptidase gene.

Nucleotide Sequence of the Aminopeptidase Gene

The result described above suggested that the 2.6-kb long DNA insert from a LAP132 clone contained the aminopeptidase gene (*bap*). Therefore, the nucleotide sequence of the *bap* gene involved in this clone was determined, and Fig. 2 shows the nucleotide sequence of the open reading frame capable of encoding the aminopeptidase gene and its flanking regions together with the derived amino acid sequence. Starting from the initiation codon ATG at nucleotide position 192 and terminating in the TAA codon at position 1,539, a single open reading frame was identified to be composed of 1,347 bp, corresponding to 449 amino acid residues, which included all the amino acid sequences of the trypsin-digested peptide fragments. A putative Shine-Dalgarno sequence

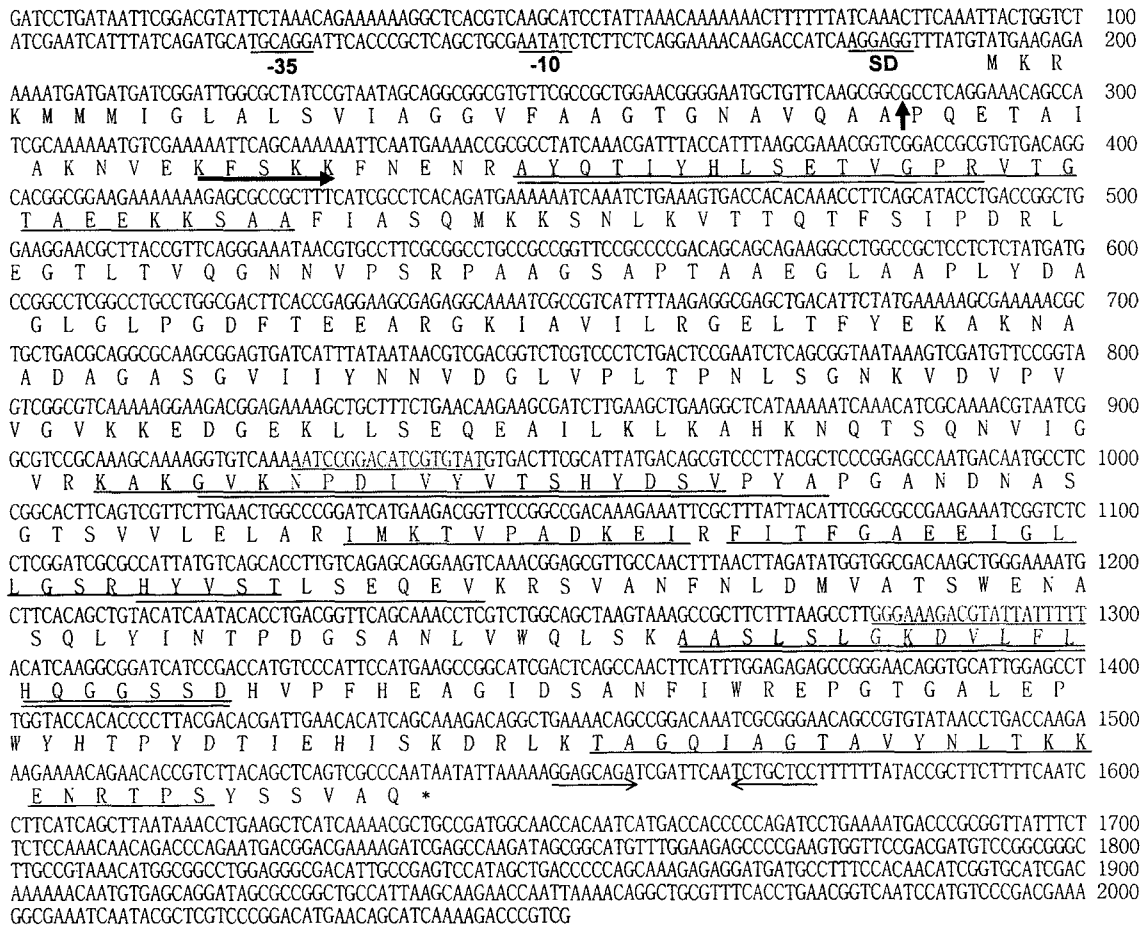

Fig. 2. Nucleotide and deduced amino acid sequences of the aminopeptidase gene from *Bacillus licheniformis*.
The deduced amino acid residues are shown below the first letter of the respective codons. A probable Shine-Dalgarno sequence an d putative promoter regions (-35 and -10 regions) are underlined. The transcription termination sequence is shown by horizontal inverted arrows. The putative processing site for the signal peptide sequence is indicated by a vertical arrow. The amino acid sequence of the underlined peptides has been confir med through amino acid sequencing of the enzyme purified from natural host. The region marked by a horizontal arrow was identified as the major N-termi nal region in the enzyme derived from both natural *B. licheniformis* host and recombinant *B. subtilis* host.

AGGAGG (-13 to -8) is located eight nucleotides upstream from the initiation codon ATG. The sequence TGCAGG and AATAT with a 20-bp spacing, resembling the -10 and -35 regions for *E. coli* promoters, are observed 69 bp upstream from the presumptive translation start. The ORF is followed by a potential bidirectional rho-independent terminator, ending in a run of T-residues.

The nucleotide and amino acid sequence analyses suggested that the enzyme was initially synthesized as a 449-residue precursor protein, followed by processing to a mature enzyme consisting of 407 amino acid residues. The amino terminal domain (position 1-30) showed a pattern resembling a signal peptide sequence with a positively charged extremity and a hydrophobic stretch of amino acids [18]. Analysis of the region immediately preceding the amino terminus of purified *B. licheniformis* aminopeptidase revealed a potential cleavage site with the sequence Ala-Val-Gln-Ala ↓ Ala (position 30, 31) and a potential NH_2 terminus (Lys-Phe-Ser-; position 43) in the mature protein after the removal of 12 amino acids by an endogeneous protease. The amino acid sequence was compared with those of other aminopeptidases in the protein databases, and a high overall identity (58%) was found only with the aminopeptidase from *Bacillus halodurans*, an alkaliphilic extremophile [17].

Expression of the Recombinant Aminopeptidase in *E. coli*

In order to investigate the properties of the mature 43-kDa protein, the coding sequence of the mature aminopeptidase gene was cloned under the T7 promoter in an expression vector, pET11a. The resulting recombinant plasmid pELAP113 was used to express a fusion protein with T7-tag peptide in *E. coli* strain BL21(DE3). After induction with 1 mM IPTG, the overexpression of aminopeptidase was monitored on SDS-PAGE (Fig. 3). The size of the aminopeptidase

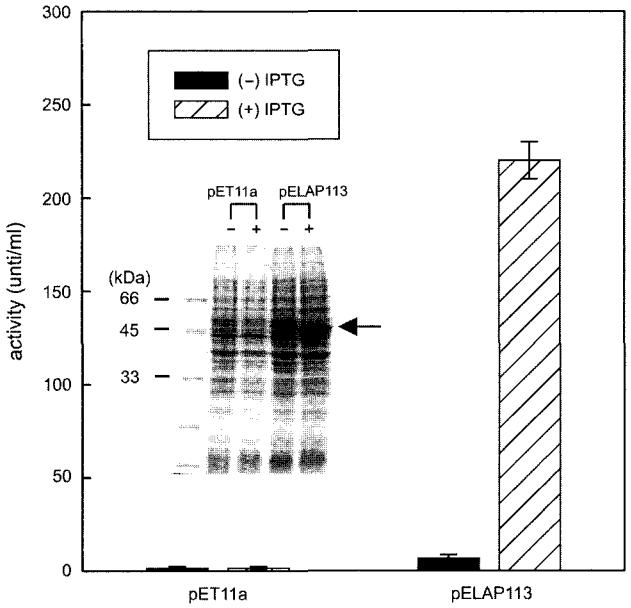

Fig. 3. Expression and enzyme activities of AP from *E. coli* BL21(DE3) harboring plasmid pELAP113.
Soluble proteins were extracted from cultures uninduced (-) or induced (+) with IPTG for 3 h. The aminopeptidase activities of the cell extracts were determined at 70°C as described in Materials and Methods. The arrow indicates the position of LAP fusion protein on SDS-PAGE.

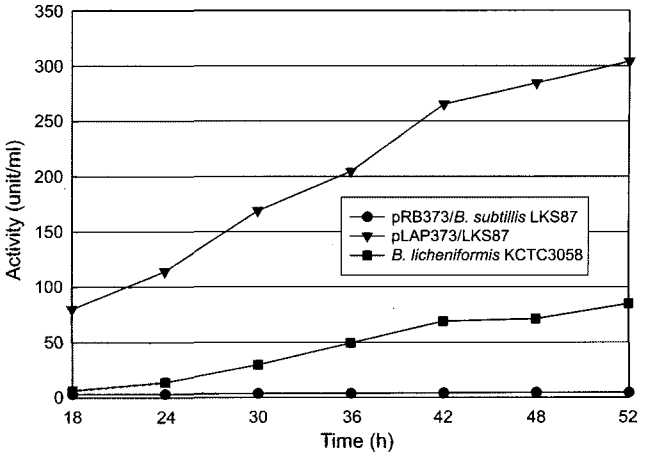

Fig. 5. Extracellular aminopeptidase activities of *B. subtilis* carrying the plasmid pLAP373 (▼), control plasmid pRB373 (●), and *B. licheniformis* KCTC3058 (■).
The AP activities secreted into the supernatant were measured at different culture times.

estimated by SDS-PAGE (45 kDa) was in agreement with its deduced molecular mass. The aminopeptidase activity was detected in the soluble protein fraction of IPTG-induced cell. The enzyme exhibited the highest activity at 70°C, thereby indicating that it was originated from *B. licheniformis*.

Expression of the Recombinant Aminopeptidase in *Bacillus subtilis*

The aminopeptidase was identified as one of the extracellular proteases synthesized and secreted by *B. licheniformis*. But the amount released was barely detected in the culture media. To express as an authentic enzyme in heterologous *Bacillus*, the aminopeptidase gene of *B. licheniformis* was cloned into the *Bacillus subtilis* vector pRB373. Therefore, the 2.6 kb-long *Hin*dIII-*Sac*I fragment containing its own promoter, 3' untranslated regions, and the structural gene were inserted into pRB373. The *B. subtilis* transformant was cultured into the stationary phase, and after centrifugation of growth medium, the supernatant was directly analyzed on SDS-PAGE (Fig. 4). A substantial amount of the enzyme was released into the culture medium, and the size of recombinant AP secreted in *B. subtilis* was indistinguishable from that of the *B. licheniformis* enzyme, suggesting that proper processing also occurred in *B. subtilis*. The aminopeptidase activity was detected almost exclusively in the culture medium in *B. subtilis* transformant (Fig. 5). To examine the correct processing of the signal peptide, the N-terminal amino acid sequence of aminopeptidase isolated from the culture medium was analyzed through N-terminal sequencing. Although the N-terminal sequence appeared to be heterogeneous, the major form, started with Lys-Phe-Ser-Lys-Lys- (KFSKK-), as in *B. licheniformis*. These results indicated that the expression and processing of the aminopeptidase in *B. subtilis* occurred in a similar manner as in *B. licheniformis*. However, the total mass analysis of the major aminopeptidase form, starting with KFSKK-, identified that the C-terminal peptide sequence YSSVAQ in the sequence deduced from the gene was not present at the C-terminus of the purified enzyme (Fig. 6). Judging from the N-terminal sequence and mass data, the YSSVAQ sequence of C-terminal region seemed to be cleaved off in *B. subtilis* as well as in the natural host. Although the processing of C-terminal is currently unknown,

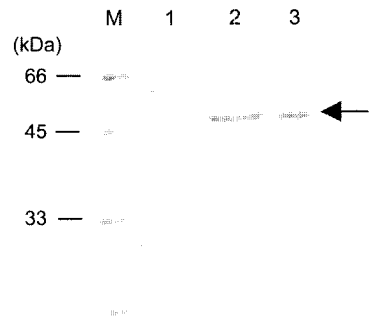

Fig. 4. Expression of *lap* in *Bacillus subtilis*.
After cultivation for 48 h, the cultures were centrifuged and 20 μl of the supernatant culture media were directly resolved on SDS-PAGE. Lane M, molecular marker; lane 1, culture medium of pRB373 transformant; lane 2, culture medium of pLAP373 transformant; lane 3, purified AP from *B. licheniformis* KCTC3058.

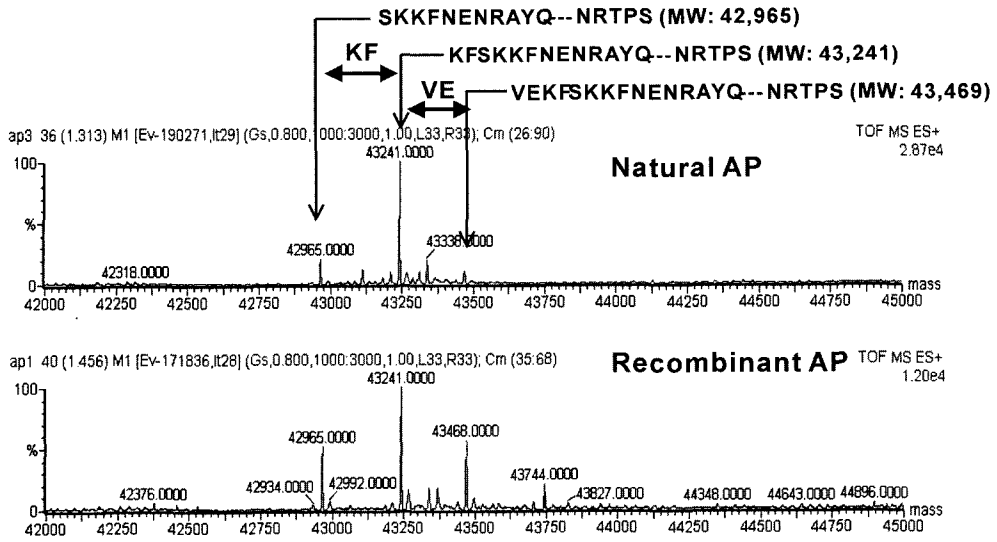

Fig. 6. Mass analysis of aminopeptidases and their estimated primary sequence.
The aminopeptidases purified from natural *B. licheniformis* or recombinant *B. subtilis* were analyzed by Q-TOF mass spectrometry. The primary sequences were estimated by combination of N-terminal amino acid sequence and the mass data.

one possibility is that such a C-terminal processing can occur by a sequence-specific endoprotease. C-terminal propeptide has also been reported in the aminopeptidases from *Vibrio proteolyticus* [20] and *Aeromonas caviae* [11]. Claveau and Riendeau [5] previously identified that C-terminal amino acids are essential for the secretion and maturation of human protease cathepsin. A similar C-terminal signal was also identified in the polygalacturonase of Gram-negative bacteria *Erwinia carotovora* [12]. It would be of particular interest to study whether the C-terminal peptide contains information related to processing of the aminopeptidase protein. To characterize their possible roles, further studies on site-directed mutagenesis of C-terminal amino acids are currently in progress.

Regarding the aminopeptidase activity, the recombinant enzyme expressed in *B. subtilis* was as thermostable as the aminopeptidase of *B. licheniformis* (Fig. 7). The optimum temperature of the aminopeptidase was between 70 and 80°C (data not shown). After exposure at 70°C and 80°C for 1 h, the enzyme retained 80% and 15% of its original activity, respectively. No loss of enzyme activity occurred after incubation for 2 h at 50°C. These results suggest *Bacillus subtilis* to be efficiently used in the production of the aminopeptidase of *B. licheniformis*.

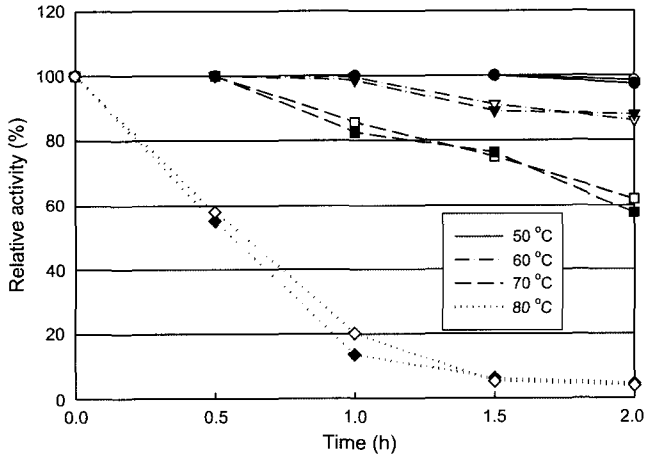

Fig. 7. Effect of temperature on aminopeptidase activity of purified enzymes from *B. licheniformis* (open symbols), and *B. subtilis* (closed symbols) carrying the plasmid pLAP373.
Thermostabilities were determined after incubation of the supernatant at various temperatures. The activities were expressed relative to the maximum value at various temperatures.

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