Journal of Bioscience and Bioengineering
VOL. 111 No. 2, 134–139, 2011

www.elsevier.com/locate/jbiosc

Family M42 aminopeptidase from the syntrophic bacterium *Symbiobacterium thermophilum*: Characterization using recombinant protein

Yasuko Kumaki,[1] Masahiro Ogawa,[1] Takako Hirano,[2] Keiko Yoshikawa,[1] Naoya Iwasawa,[1] Takurou Yagi,[1] Wataru Hakamata,[1] Tadatake Oku,[1] and Toshiyuki Nishio[1,*]

Department of Chemistry and Life Science, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan[1] *and Life Science Research Center, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan*[2]

Received 27 July 2010; accepted 22 September 2010
Available online 20 October 2010

The chromosomal DNA of the syntrophic thermophile *Symbiobacterium thermophilum* contains open reading frames of the genes encoding family M42 aminopeptidases, Pep1079, Pep1080, and Pep1081. To characterize these peptidases, the genes were cloned into *Escherichia coli* and overexpressed. Our experiments using the recombinant proteins confirmed that Pep1079, Pep1080, and Pep1081 are components of arginyl or lysinyl aminopeptidases that require Co^{2+} for enzymatic activity. Coexistence of Pep1079 and Pep1080 is necessary for expressing high peptidase activity. Pep1081 enhances the activity of Pep1079 and Pep1080.

[**Key words:** *Symbiobacterium thermophilum*; Family M42 aminopeptidase; Gene cloning; Recombinant protein; Enzyme characterization]

Symbiobacterium thermophilum, a rod-shaped syntrophic thermophile, was isolated while screening compost for heat-stable enzymes such as tryptophanase and β-tyrosinase (1–5). Molecular analysis of the 16S rRNA gene sequence revealed that this bacterium represents a novel phylogenic branch in the Gram-positive bacterial group that does not cluster to any other genus (6,7). *S. thermophilum* depends upon the presence of another thermophilic bacterium, *Bacillus* sp. strain S, for growth (7–10). In 2004, the whole *S. thermophilum* genome sequence was published (GenBank accession number AP006840) (11).

We are interested in the glycolytic and the proteolytic enzymes involved in *S. thermophilum* nutriment acquisition. However, since independent cultivation of syntrophic organisms such as *S. thermophilum* is difficult, it is necessary to use recombinant strategies to produce proteins of interest. Previously, we cloned the gene encoding β-*N*-acetylhexosaminidase, an *exo*-type β-glycosidase involved in chitin oligosaccharide hydrolysis, based on the *S. thermophilum* genome sequence and overproduced, purified, and characterized the recombinant enzyme (12).

We then turned our attention to the products of three consecutive genes (locus tag: STH1079, STH1080, and STH1081) that BLASTP homology searching suggested encode family M42 aminopeptidases or endo-1,4-β-glucanases because of our interest in the function of these proteins. These genes of interest overlap by several nucleotides on the chromosomal DNA of *S. thermophilum*, and putative ribosome binding sequences exist on the upstream of each gene (Fig. 1A). The MEROPS peptidase database (http://merops.sanger.ac.uk/), which groups peptidases with significant sequence similarity into families and assigns families of common origin to a clan (13–15), classifies the proteins deduced from above three genes into the M42 family of clan MH. In this database, the protein encoded by gene *STH1079* is an unassigned peptidase, while the proteins encoded by genes *STH1080* and *STH1081* are classified as non-peptidase homologues.

Aminopeptidases are *exo*-type proteases that cleave amino acids from the N-termini of oligo- or polypeptides, and they are found in every cell. These enzymes break down unneeded or abnormal polypeptides and peptide-based nutrients within or outside the cell and are thus critical to the maintenance of cellular function. The enzymes belonging to family M42 are co-catalytic metallopeptidases, which typically bind two atoms of cobalt or zinc. Various enzymes showing different substrate specificities are classified into this family. All of the M42 aminopeptidases that have been characterized have multimeric structures. If proteins encoded by three genes, *STH1079*, *STH1080*, and *STH1081*, of *S. thermophilum* are aminopeptidases, it is important to investigate what characteristics they have.

Here, we report on the characterization of the substrate specificity and the physiological properties of abovementioned three *S. thermophilum* proteins overproduced in *Escherichia coli*.

MATERIALS AND METHODS

Reagents and instrumentation *p*-Nitroanilide (*p*NA) derivatives of amino acids, Ala-*p*NA, Leu-*p*NA, Glu-*p*NA, and His-*p*NA, were purchased from the Peptide Institute (Osaka, Japan). Arg-*p*NA, Lys-*p*NA, Phe-*p*NA, and Pro-*p*NA were obtained from Kokusan Chemicals (Tokyo, Japan). Gly-*p*NA, Ile-*p*NA, Met-*p*NA, Val-*p*NA, and Ac-Arg-

* Corresponding author. Tel./fax: +81 466 84 3951.
E-mail address: nishio.toshiyuki@nihon-u.ac.jp (T. Nishio).

1389-1723/$ - see front matter © 2010, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2010.09.012

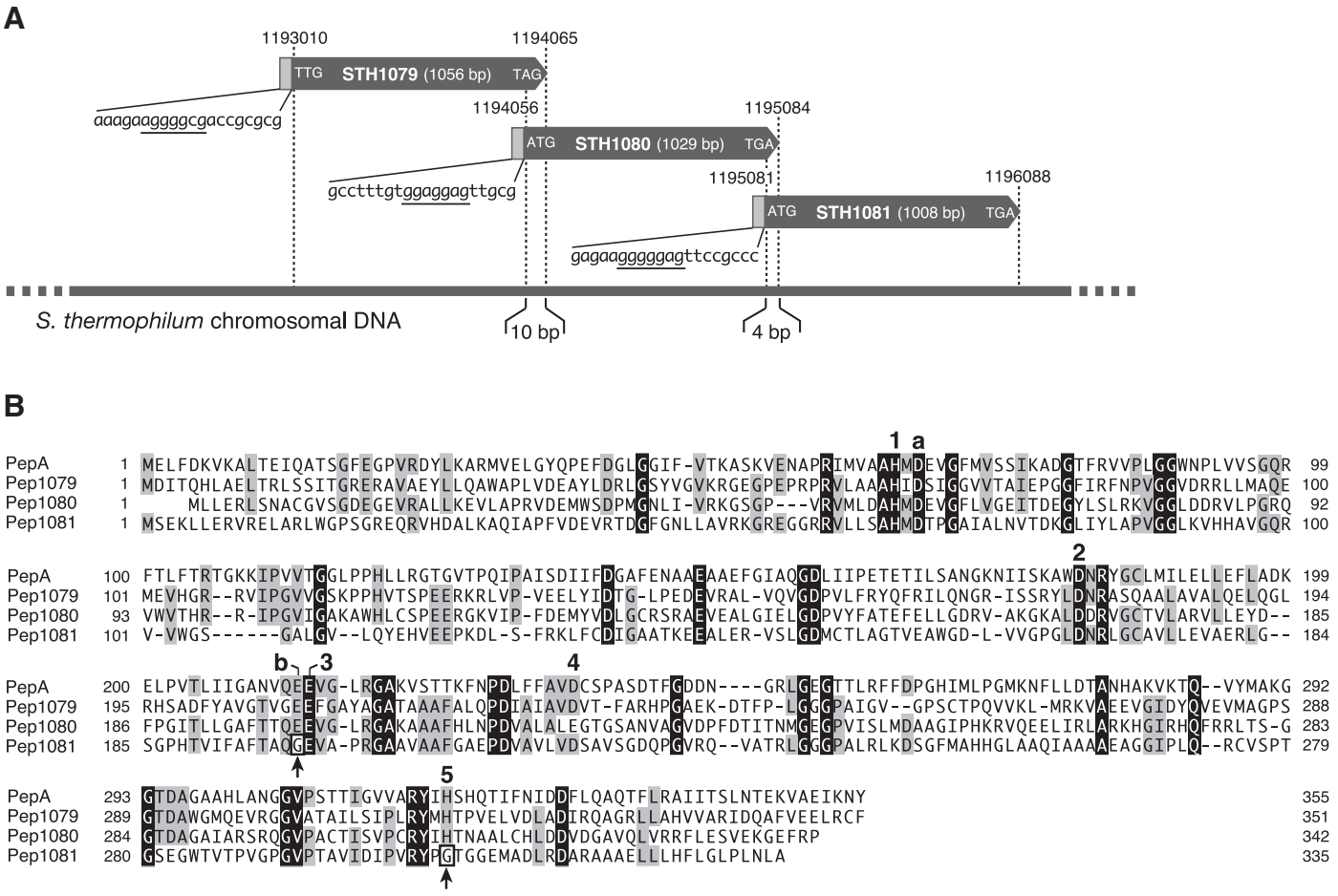

FIG. 1. Cluster of genes *STH1079*, *STH1080*, and *STH1081* and amino acid sequence alignment of their products (Pep1079, Pep1080, and Pep1081) and PepA. (A) Cluster of genes *STH1079*, *STH1080*, and *STH1081*. Underlined letters indicate the nucleotides of putative ribosome binding sequences. (B) Amino acid sequence alignment of PepA, Pep1079, Pep1080, and Pep1081. Amino acids highlighted in black and gray indicate identical and highly homologous amino acids, respectively. PepA catalytic amino acids are represented by a and b; PepA amino acids involved in binding metal ions are indicated by 1, 2, 3, 4, and 5. Arrows indicate Pep1081 amino acids in the positions corresponding to PepA b and 5.

*p*NA were obtained from Bachem (Bubendorf, Switzerland). Ala-Ala, Leu-Ala, Phe-Ala, Asp-Ala, and Tyr-Ala were purchased from Sigma-Aldrich (St. Louis, MO, USA). Arg-Phe, Glu-Ala, His-Ala, Lys-Ala, and Met-Ala were obtained from Kokusan Chemicals. Arg-Ala and Gly-Ala were purchased from Tokyo Chemical Ind. (Tokyo, Japan) and Bachem, respectively. Arg-Asp, Arg-Leu, Arg-Met, Arg-Thr, and Arg-Ala-Ala-Arg-Ala were synthesized by GenScript (Piscataway, NJ, USA). All other chemicals were of analytical grade. Thin layer chromatography (TLC) plates (Silica Gel 60, 0.25 mm) were obtained from Merck (Darmstadt, Germany). Nucleotide sequence analysis was performed using a Thermo Sequenase Fluorescence-labeled Primer Cycle Sequencing kit (GE Healthcare Bioscience, Uppsala, Sweden) and an automated DNA sequencer DSQ2000L (Shimadzu, Kyoto, Japan). The N-terminal amino acid sequence of purified proteins was determined using a Model 492 Procise protein sequencer (Applied Biosystems, Foster City, CA, USA). Interactions between purified proteins were investigated using a Biacore X biomolecular interaction analyzer (GE Healthcare Bioscience).

Microbial strains and plasmids *S. thermophilum* IAM14863 served as the source for gene cloning. Chromosomal DNA was donated by the Life Science Research Center (College of Bioresource Sciences, Nihon University, Japan). *E. coli* DH5α (Takara, Siga, Japan) served as the host strain for gene cloning and propagation of the genetic construction. *E. coli* Rosetta-gami2(DE3) (Merck) was the host strain for protein production. pGEM-T Easy plasmid (Promega, Madison, WI, USA) served as the PCR product cloning vector for DNA sequence analysis, and pET-25b(+) (Merck) was used for construction of the plasmids for protein expression.

Gene cloning and expression plasmid construction Following *Hin*dIII (Toyobo, Osaka, Japan) and *Xho*I (Nippon gene, Tokyo, Japan) digestion of *S. thermophilum* chromosomal DNA, STH1079, STH1080, and STH1081 were amplified by PCR using 12 ng of DNA fragment mixture and 12 pmol of the following synthetic oligonucleotide primers (non-complimentary nucleotides added are shown in italics; *Nde*I site of forward primers and *Xho*I site of reverse primers are underlined): 5′-*CCATAT*GGACATCACCCAACACCTGGCAG-3′ (STH1079 forward primer) and 5′-*GCTCGAG*AAGCATCGCAACTCCTCCAC-3′ (STH1079 reverse primer); 5′-*CCATAT*GGTTCTAGAAACGGCTGTCCAACG-3′ (STH1080 forward primer) and 5′-*GCTCGAG*TGGGCGGAACTCCCCCTTCTC-3′ (STH1080 reverse primer); 5′-*CGCATATG*AGCGAGAAGCTGCTGGAGCGC-3′ (STH1081 forward primer) and 5′-*GCTCGAG*GGCCAGATTAAGCGGAAGGCC-3′ (STH1081 reverse primer).

The forward and reverse primers were designed based on the nucleotide sequences of the 3′- and 5′-terminal regions of STH1079, STH1080, and STH1081, with integrated restriction enzyme recognition sequences. In the construction of the forward primer for STH1080 amplification, nucleotide (C) to the right of the initiation codon (ATG) was changed to a G in order to obtain the L2V mutant protein. After 30 amplification cycles (each cycle consisting of denaturation at 98°C for 20 s, annealing at 58°C for 1 min, elongation at 72°C for 3 min), PCR products were incubated at 72°C for 1 h, separated by agarose gel electrophoresis, and subcloned into pGEM-T Easy by TA-cloning to yield the plasmids pGEM-STH1079, pGEM-STH1080, and pGEM-STH1081. These plasmids were then used to confirm the nucleotide sequence of the cloned genes.

DNA sequence analysis followed the dideoxynucleotide method. After *Nde*I and *Xho*I digestion of pGEM-STH1079, pGEM-STH1080, and pGEM-STH1081, each DNA fragment produced was separated by agarose gel electrophoresis and inserted into the *Nde*I and *Xho*I sites of pET-25b(+) to yield the expression plasmids: pET-STH1079, pET-STH1080, and pET-STH1081. *E. coli* Rosetta-gami2(DE3) was transformed with each expression plasmid, and the transformants were selected on LB-agar plates supplemented with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol.

Production and purification of recombinant proteins *E. coli* Rosetta-gami2 (DE3) cells harboring each expression plasmid were grown at 30°C with shaking in 600 ml of LB broth supplemented with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol until the OD_{600} reached 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the culture was incubated for an additional 4 h. The *E. coli* cells were harvested by centrifugation (6500 × *g*, 10 min, 4°C), resuspended in 20 ml of 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 500 mM NaCl and 20 mM imidazole, and then treated with lysozyme at 30°C for 2 h. Cells were disrupted by sonication, and cell debris and remaining intact cells were removed by centrifugation (9500 × *g*, 15 min, 4°C). The resulting cell-free extract was filtered using 0.45-μm cellulose acetate disposable syringe filters (Advantec, Tokyo, Japan) and loaded on a HisTrap HP affinity

column (5 ml, GE Healthcare Bioscience) equilibrated with 20 mM HEPES buffer (pH 7.4) containing 500 mM NaCl and 20 mM imidazole. The chelating resin was washed with the same buffer, and protein was eluted with a linear gradient of 20–300 mM imidazole in 20 mM HEPES buffer (pH 7.4) containing 500 mM NaCl. Protein-containing fractions were dialyzed against 50 mM Tris–HCl buffer (pH 7.4) and concentrated by filtration using a diaflow filtration device installed on an ultrafiltration membrane RC YM10 (ϕ44.5 mm, Millipore, Billerica, MA, USA). The homogeneity of purified proteins was confirmed by SDS–PAGE gels stained with Coomassie Brilliant Blue R250 (Tokyo Chemical Ind.). Protein concentration was determined by the Lowry method using bovine serum albumin (Sigma-Aldrich) as a standard.

Enzyme activity assay Aminopeptidase activity was assayed using aminoacyl-*p*NA derivatives and dipeptides as the substrates. Peptidase activity against *p*NA derivatives was assessed by measuring *p*-nitroaniline release. Briefly, recombinant proteins were incubated at 37°C in 100 µl 50 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM substrate, 0.1 mM $CoCl_2$, and 100 mM NaCl. The amount of *p*-nitroaniline liberated was determined by measuring the absorbance at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of *p*-nitroaniline/min under the assay conditions. Kinetic studies of Arg-*p*NA hydrolysis were performed at concentrations ranging from 0.2 to 1.0 mM, and K_m and V_{max} values were calculated from reciprocal plots of the reaction curves.

Peptidase activity against peptides was monitored by analyzing the amino acids released. Recombinant proteins were incubated at 37°C for 12 h in 90 µl of 50 mM Tris–HCl buffer (pH 7.4) containing 50 mM substrate, 0.1 mM $CoCl_2$, and 100 mM NaCl. The identity of amino acids produced was determined by silica gel TLC. After development of TLC plates, amino acids were visualized by spraying the plates with 0.7% (w/v) ninhydrine in iPrOH followed by heating.

The effect of pH, temperature, and metal ions on aminopeptidase activity was investigated using Arg-*p*NA as a substrate. The following buffer solutions were used to investigate the effect of pH: 100 mM 4-morpholineethansulfonic acid (MES) buffer (pH 5.5–6.5), 100 mM 4-morpholinepropanesulfonic acid (MOPS) buffer (pH 6.5–7.5), and 100 mM Tris–HCl buffer (pH 7.0–8.5). To investigate the effect of metal ions, various bivalent metal chloride salts were added to the reaction mixture at a final concentration of 0.1 mM. For this experiment, each recombinant protein was dialyzed before use against 50 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C, followed by dialysis at 4°C against the same buffer minus EDTA.

Analysis of interactions between recombinant proteins Real-time interactions between recombinant proteins were determined by surface plasmon resonance (SPR) analysis carried out at 25°C. Each purified recombinant protein was immobilized on the surface of a CM5 sensor chip (research grade, GE Healthcare Bioscience) by the amine coupling method using *N*-ethyl-*N*′-(3-diethylaminopropyl)-carbodiimide hydrochloride and *N*-hydroxysuccinimide. The immobilization was carried out by injecting 35 µl of 50 mM Tris–HCl buffer (pH 7.4) containing 250 nM purified protein at flow rate of 5 µl/min. The strength of binding affinity between proteins was demonstrated by the response unit (RU). Ten mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 0.005% (w/v) Tween 20, and 0.5 mM $CoCl_2$ was used as a running buffer. Aqueous $MgCl_2$ (3 M) was used to dissociate proteins that adhered to the sensor chip.

RESULTS

Sequence analysis of gene products Aminopeptidases of Gram-positive bacteria belonging to family M42 of clan MH contain two catalytic amino acids (Asp and Glu) and five amino acids (commonly Glu, 2 His, and 2 Asp) involved in metal ion binding (16). The predicted amino acid sequences of the proteins (Pep1079, Pep1080, and Pep1081) derived from STH1079 (MEROPS accession number MER041553), STH1080 (MEROPS accession number MER041550), and STH1081 (MEROPS accession number MER041551), respectively, were compared to the sequence of a glutamyl aminopeptidase (PepA) from *Lactococcus lactice* (MEROPS accession number MER002040, UniProt accession number Q48677) (17–21), which is considered a typical family M42 aminopeptidase (Fig. 1B). Aligning the sequence of PepA with those of Pep1079, Pep1080, and Pep1081 indicated only minimal (25–34%) sequence homology, but the sequences around the two catalytic and five metal ion-binding amino acids characteristic of family M42 aminopeptidases are comparatively conserved. Amino acids corresponding to the seven catalytic and metal-binding residues exist in Pep1079 and Pep1080. Although amino acids corresponding to catalytic residue Asp67 and metal-binding residues His65, Asp181, Glu214, and Asp236 are present in Pep1081, the residues in positions corresponding to catalytic residue Glu213 and metal-binding residue His319 are Gly. It is possible therefore that both Pep1079 and Pep1080 function as aminopeptidases, while Pep1081 may have a different function.

Production and purification of recombinant proteins Because syntrophic microorganisms such as *S. thermophilum* will not propagate in pure cultures, we used recombinant strategies to overexpress the products of genes *STH1079*, *STH1080*, and *STH1081* in *E. coli*. The recombinant proteins (rPep1079, rPep1080L2V, and rPep1081) were produced as 6×His-tag fusion proteins in transformed *E. coli* cells induced by IPTG and were purified from cell-free extracts using Ni-chelating column chromatography. In the case of the recombinant protein from gene *STH1080*, an L2V mutant was produced to avoid the degradation by methionyl aminopeptidase of *E. coli*.

Each purified protein produced a single band with a molecular mass of about 40 kDa on SDS–PAGE (Fig. 2). The N-terminal amino acid sequence of each recombinant protein was as follows: rPep1079, MDITQHLAELTRLSS; rPep1080L2V, MVLERLSNACGVSGD; rPep1081, MSEKLLERVRELARL, confirming that the purified proteins were products from genes *STH1079*, *STH1080*, and *STH1081*. From 600 ml *E. coli* cultures, approximately 4.36, 3.90, and 5.09 mg of electrophoretically homogeneous rPep1079, rPep1080L2V, and rPep1081 protein were obtained, respectively.

Aminopeptidase activity of the recombinant proteins The BLASTP homology searching showed the possibility that Pep1079, Pep1080, and Pep1081 are b-glucanase or aminopeptidase. When we examined the function of these proteins, any proteins showed no b-glucanase activity under various conditions, while each protein slightly showed the aminopeptidase activity. Therefore, we decided to handle these proteins as the aminopeptidase. The aminopeptidase activity of each recombinant protein was assessed using *p*NA derivatives of twelve amino acids as substrates. Since all of the characterized family M42 aminopeptidases exist in multimeric forms (22–26), each recombinant protein was assayed for aminopeptidase activity individually and in combination with one or two other proteins. As shown in Table 1, although each recombinant protein did not show high hydrolytic activity against all substrates used when assayed alone, markedly higher activity against *p*NA derivatives of two basic amino acids (Arg and Lys) was observed when

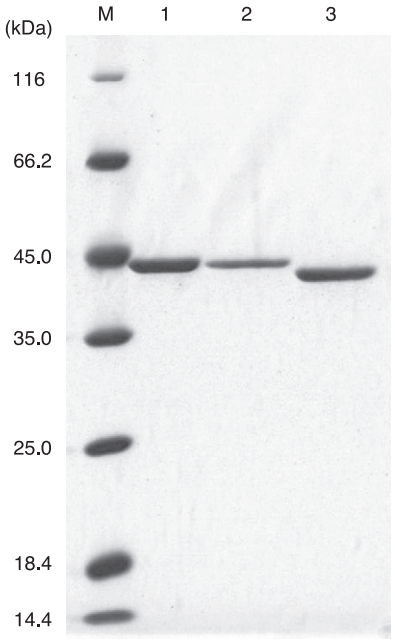

FIG. 2. SDS–PAGE analysis of purified recombinant proteins. Lane M contains molecular mass standards (Fermentas, Burlington, Ontario, Canada). Lanes 1, 2, and 3 contain rPep1079, rPep1080L2V, and rPep1081.

TABLE 1. Hydrolysis of aminoacyl-*p*NA derivatives by recombinant proteins.

Amino acids of *p*NA derivatives	Specific activity (mU/mg of protein)												
	Arg	Lys	His	Glu	Gly	Ala	Leu	Ile	Val	Met	Pro	Phe	Ac-Arg
Recombinant proteins													
rPep1079[a]	17 ± 0.33	14 ± 0.11	n.d.	2.4 ± 0.07	n.d.	3.3 ± 0.01	2.0 ± 0.02	2.8 ± 0.04	2.7 ± 0.05	n.d.	n.d.	n.d.	n.d.
rPep1080L2V[a]	32 ± 0.94	30 ± 0.93	n.d.	5.9 ± 0.07	5.6 ± 0.17	5.9 ± 0.18	4.6 ± 0.22	6.5 ± 0.15	5.2 ± 0.12	5.5 ± 0.19	4.5 ± 0.24	7.1 ± 0.17	n.d.
rPep1081[a]	10 ± 0.22	13 ± 0.19	n.d.	1.6 ± 0.07	1.7 ± 0.02	2.3 ± 0.09	1.5 ± 0.08	3.1 ± 0.03	n.d.	n.d.	2.1 ± 0.12	5.1 ± 0.05	n.d.
(rPep1079, rPep1080L2V)[b]	311 ± 11.3	205 ± 13.7	n.d.	2.1 ± 0.05	2.3 ± 0.02	4.0 ± 0.07	3.1 ± 0.18	3.7 ± 0.03	1.9 ± 0.01	3.5 ± 0.06	2.7 ± 0.12	4.8 ± 0.12	n.d.
(rPep1079, rPep1081)[b]	23 ± 0.27	13 ± 0.65	n.d.	2.2 ± 0.02	3.7 ± 0.09	2.4 ± 0.09	2.7 ± 0.13	10 ± 0.70	2.2 ± 0.10	3.7 ± 0.09	3.0 ± 0.08	4.5 ± 0.13	n.d.
(rPep1080L2V, rPep1081)[b]	18 ± 0.35	11 ± 0.16	n.d.	1.6 ± 0.04	n.d.	2.7 ± 0.09	2.6 ± 0.15	3.7 ± 0.08	2.4 ± 0.08	n.d.	2.3 ± 0.04	5.2 ± 0.06	n.d.
(rPep1079, rPep1080L2V, rPep1081)[c]	742 ± 28.5	714 ± 37.4	n.d.	2.3 ± 0.02	2.2 ± 0.03	9.3 ± 0.25	2.0 ± 0.10	8.0 ± 0.32	3.0 ± 0.06	11 ± 0.43	2.5 ± 0.10	7.7 ± 0.19	n.d.

n.d., No detectable activity. The values (mean ± SEM) were determined by three independent experiments. In each experiment, the assay was conducted using three reaction mixtures per one substrate.

[a] About 128 pmol of protein was used.

[b] About 64 pmol of each protein was mixed.

[c] About 43 pmol of each protein was mixed.

rPep1079 and rPep1080L2V were mixed. On the other hand, mixing rPep1079 and rPep1081 or rPep1080L2V and rPep1081 did not result in any increase in aminopeptidase activity. The addition of rPep1081 to the mixture of rPep1079 and rPep1080L2V enhanced the activity of these two proteins against Arg-*p*NA and Lys-*p*NA even further. These results suggest that coexistence of rPep1079 and rPep1080L2V is indispensable for high arginyl or lysinyl aminopeptidase activity and that rPep1081 enhances the activity of rPep1079 and rPep1080L2V. Ac-Arg-*p*NA was not hydrolyzed at all in any case, indicating that rPep1079, rPep1080L2V, and rPep1081 do not have aminoacylase activity. Reciprocal plots of reaction rate ($1/v$) against substrate concentration ($1/S$) for kinetic analysis of Arg-*p*NA hydrolysis by a mixture of the recombinant proteins produced straight lines. The K_m values obtained from these plots for a mixture of rPep1079 and rPep1080L2V and a mixture of rPep1079, rPep1080L2V, and rPep1081 were 0.164 mM and 0.130 mM, respectively, indicating that rPep1081 does not influence the affinity of a mixture of rPep1079 and rPep1080L2V for Arg-*p*NA. The V_{max}/K_m values obtained using a mixture of rPep1079 and rPep1080L2V and a mixture of rPep1079, rPep1080L2V, and rPep1081 were 0.414 and 2.38, respectively, indicating that rPep1081 enhances the enzymatic activity of a mixture of rPep1079 and rPep1080L2V. When rPep1079 and rPep1080L2V were mixed in the molar ratio of 1:0.5, Arg-*p*NA, hydrolyzing activity decreased about 40% than the activity when these proteins were mixed in the molar ratio of 1:1. On the other hand, when rPep1079 and rPep1080L2V were mixed in the molar ratio of 1:2, the activity increased only about 10%. These data indicate that these proteins should exist in the molar ration of at least 1:1 to express high aminopeptidase activity. The Arg-*p*NA hydrolyzing activity of a mixture of all three recombinant proteins was highest at a pH of around 7.0 and at a temperature of around 55°C. Among the metal salts examined, protein mixtures required Co^{2+} ions for maximum Arg-*p*NA hydrolyzing activity, which is consistent with the characteristics of many metalloaminopeptidases belonging to family M42 (Table 2).

TABLE 2. Effect of metal cations on activity of mixture of rPep1079, rPep1080L2V, and rPep1081.

Metal ion	Relative activity (%)[a]	Metal ion	Relative activity (%)[a]
None	11.0	Fe^{2+}	21.2
Ca^{2+}	10.8	Mg^{2+}	11.0
Cd^{2+}	24.6	Mn^{2+}	16.7
Co^{2+}	100	Ni^{2+}	12.5
Cu^{2+}	13.2	Zn^{2+}	19.3

The values are representative of three independent experiments. In each experiment, the assay was conducted using three reaction mixtures per one metal ion.

[a] Enzyme activity was expressed as the percentage of the activity in the presence of Co^{2+}, which was defined as 100%.

Hydrolysis of four aminoacyl-Ala dipeptides (Arg-Ala, Glu-Ala, Leu-Ala, and Phe-Ala) by isolated and paired recombinant proteins was examined using silica gel TLC (Fig. 3A). Conspicuous hydrolysis product spots for Arg-Ala were observed when rPep1079 and rPep1080L2V were combined. These analyses confirmed that coexistence of these two proteins is indispensable for dipeptide hydrolysis. The substrate specificity of a mixture of the three recombinant proteins was elucidated using eleven aminoacyl-Ala dipeptides (Fig. 3B). Conspicuous hydrolysis product spots were observed when both Arg-Ala and Lys-Ala were used as substrates. Although Phe-Ala was hydrolyzed with lower activity, other dipeptides examined were scarcely hydrolyzed by the mixture of all three enzymes, results that were identical to those obtained when *p*NA derivatives were used as substrates. Next, we examined the hydrolytic activity of a mixture of all three recombinant proteins against six dipeptides containing Arg at N-terminal ends (Fig. 3C). Conspicuous hydrolysis spots were observed for all of the dipeptides examined, indicating that the C-terminal dipeptide amino acid does not significantly influence the activity of the enzymes. Moreover, we investigated the hydrolytic activity of a mixture of all three recombinant proteins against a pentapeptide Arg-Ala-Ala-Arg-Ala. As the result, hydrolysis products were not observed at all (data not shown), indicating that this protein mixture is hard to react to long peptide.

Analysis of interactions between recombinant proteins To confirm that rPep1079, rPep1080L2V, and rPep1081 assemble into a complex to produce enzymatic activity, interactions between them were analyzed by SPR. When rPep1079 was immobilized as a ligand on sensor chips, the strongest interaction was observed with rPep1080L2V (Fig. 4A). A strong response was also observed when rPep1080L2V was used as the ligand and rPep1079 served as the analyte (Fig. 4B). Although the binding affinity was lower than that between rPep1079 and rPep1080L2V, affinity was also observed between rPep1079 and rPep1081, and between rPep1080L2V and rPep1081 (Fig. 4A, B, and C). Moreover, although weak, each protein showed a self-assembling property. Binding affinity and activity assay data together suggest that Pep1079, Pep1080, and Pep1081 form a complex that functions as an aminopeptidase.

DISCUSSION

Although the products of *S. thermophilum* chromosomal genes *STH1079*, *STH1080*, and *STH1081* (Pep1079, Pep1080, and Pep1081) show minimal aminopeptidase activity individually, significant

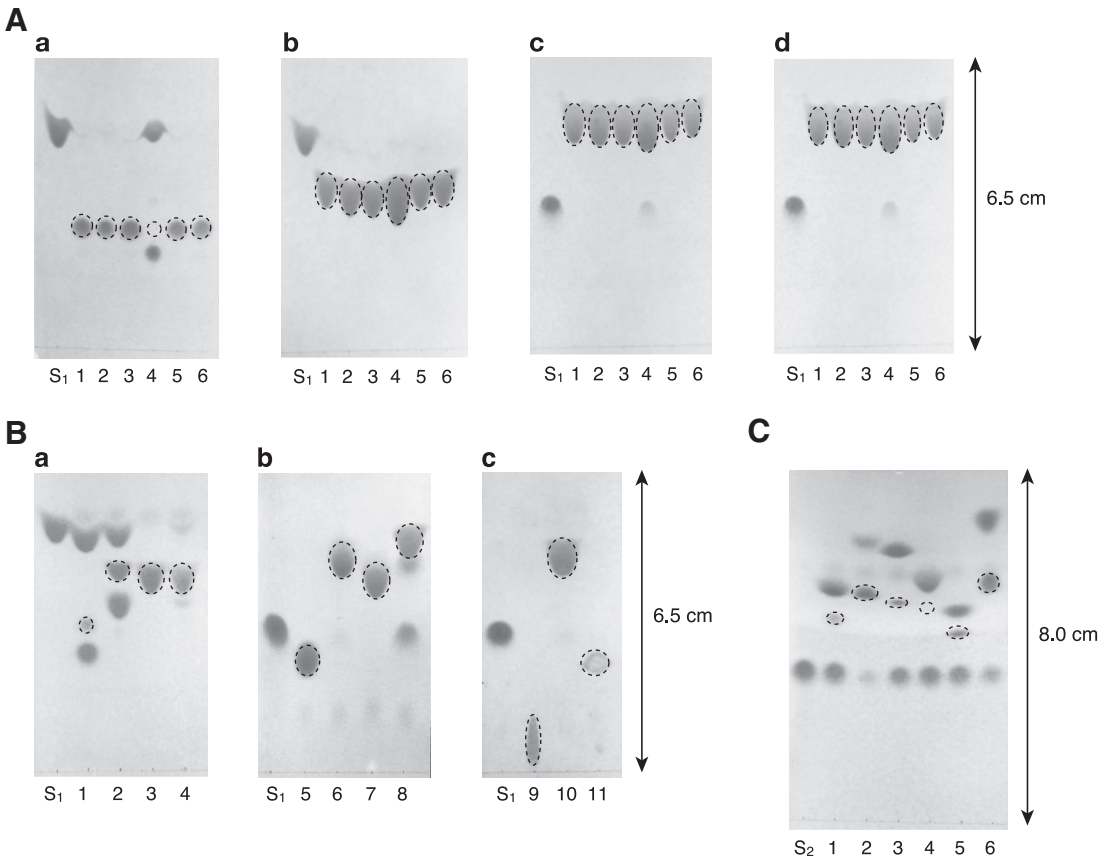

FIG. 3. TLC of dipeptide hydrolysis products. Spots encircled by dashed lines represent dipeptides used as substrates. Lanes S_1 and S_2 contain Ala and Arg, respectively. (A) Products from aminoacyl-Ala dipeptides hydrolyzed by rPep1079, rPep1080L2V, or rPep1081 (about 128 pmol of each protein) and mixtures of two recombinant proteins (about 64 pmol of each protein was mixed). TLC plates a, b, c, and d show products of the following dipeptides: Arg-Ala (a), Glu-Ala (b), Leu-Ala (c), and Phe-Ala (d). Lanes 1–6 contain hydrolysis products of the following proteins: rPep1079 (1), rPep1080L2V (2), rPep1081 (3), mixture of rPep1079 and rPep1080L2V (4), mixture of rPep1079 and rPep1081 (5), and mixture of rPep1080L2V and rPep1081 (6). The reaction products were developed twice using 3:2 iPrOH:25% aqueous NH_3 (TLC plates a and b) and 1:2.1:0.9 EtOH:PrOH:H_2O (TLC plates c and d) as the mobile phase solvent. (B) Aminoacyl-Ala dipeptide hydrolysis products produced by the mixture of rPep1079, rPep1080L2V, and rPep1081 (about 43 pmol of each protein). Lanes 1–11 contain the hydrolysis products of the following dipeptides: Arg-Ala (1), Lys-Ala (2), Glu-Ala (3), Asp-Ala (4), Ala-Ala (5), Leu-Ala (6), Met-Ala (7), Phe-Ala (8), His-Ala (9), Tyr-Ala (10), and Gly-Ala (11). The reaction products were developed twice using 3:2 iPrOH:25% aqueous NH_3 (TLC plate a), 1:2.1:0.9 EtOH:PrOH:H_2O (TLC plate b), and 10:3 PrOH:H_2O (TLC plate c) as the mobile phase solvents. (C) Arg-amino acid dipeptide hydrolysis products produced by the mixture of rPep1079, rPep1080L2V, and rPep1081 (about 43 pmol of each protein). Lanes 1–6 contain the hydrolysis products of the following dipeptides: Arg-Ala (1), Arg-Leu (2), Arg-Met (3), Arg-Thr (4), Arg-Asp (5), and Arg-Phe (6). The reaction products were developed three times using 1:1 iPrOH:5% aqueous NH_3 as the mobile phase solvent. The TLC data are representative of three independent experiments.

peptidase activity appears when the proteins coexist. These findings, combined with protein interaction data, indicate that Pep1079, Pep1080, and Pep1081 function as a peptidase by forming a complex. These proteins have the following characteristics: (i) coexistence of Pep1079 and Pep1080 is indispensable for high aminopeptidase activity, (ii) Pep1081 enhances the activity of a mixture of Pep1079 and Pep1080, (iii) Co^{2+} is required for maximum aminopeptidase activity, (iv) the protein mixture is most active against substrates that have an arginine or a lysine in the N-terminal end, and (v) the proteins do not demonstrate aminoacylase activity.

Several family M42 aminopeptidases, including PhTET2 (*Pyrococcus horikoshii* aminopeptidase) (24), DAP (*Thermococcus onnurineus* aminopeptidase) (27), and API (*Geobacillus stearothermophilus* aminopeptidase) (28), are known to be highly active in hydrolyzing Leu-*p*NA in various aminoacyl-*p*NA derivatives, while PepA (*Lactococcus lactis* aminopeptidase) (17,18,20) and PhTET1 (*Pyrococcus horikoshii* aminopeptidase) (29) are glutamyl- and methionyl aminopeptidases, respectively. The TET (*Haloarcula marismortui* aminopeptidase) enzymes show broad substrate specificity, and among the characterized members of the family M42 aminopeptidases, the only enzyme known to have high hydrolytic activity against Lys-*p*NA and Arg-*p*NA is PhTET3 (*Pyrococcus horikoshii* aminopeptidase) (30). Enzymes such as TET, PhTET1, and PhTET3 form homogenous multimeric complexes (22–26). API consists of 12 multimers composed of two types of subunits (α and β) of differing molecular mass (28). Arginyl or lysinyl metallopeptidase complexes consisting of three different subunit proteins have not been described among the family M42 peptidases, indicating that the products of *S. thermophilum* genes *STH1079*, *STH1080*, and *STH1081* are components of a new family M42 aminopeptidase type of complex. To fully investigate the reaction mechanism of this aminopeptidase complex, it will be necessary to determine the 3D structure of the complex. Using recombinant forms of these proteins, we are investigating the structure of the complex.

ACKNOWLEDGMENTS

The authors express sincere gratitude to Dr. K. Ueda and Dr. T. Beppu of Nihon University for their advice. This work was supported by a grant from the High-Tech Research Center Project of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

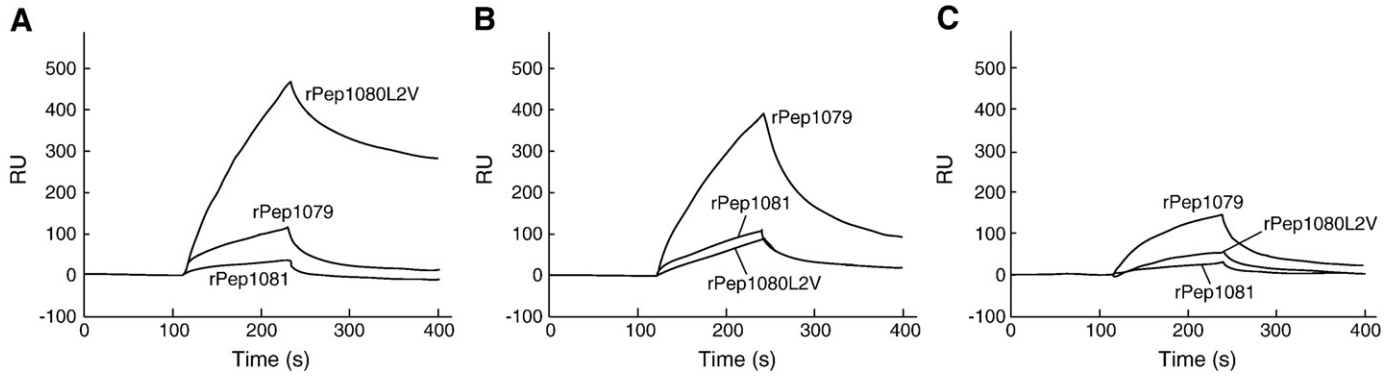

FIG. 4. SPR sensorgrams comparing the strength of interaction between rPep1079, rPep1080L2V, and rPep1081. Running buffer containing each protein (2.5 μM) was injected into one flow cell over a 2-min period at a flow rate of 20 μl/min. (A) Sensorgrams using the sensor chip on which 5308 RU of rPep1079 was immobilized. (B) Sensorgrams using the sensor chip on which 11816 RU of rPep1080L2V was immobilized. (C) Sensorgrams using the sensor chip on which 7237 RU of rPep1081 was immobilized. The SPR sensorgram data are representative of four independent experiments.

References

1. **Suzuki, S., Horinouchi, S., and Beppu, T.:** Growth of a tryptophanase-producing thermophile, *Symbiobacterium thermophilum* gen. nov., sp. nov., is dependent on co-culture with a *Bacillus* sp, J. Gen. Microbiol., **134**, 2353–2362 (1988).
2. **Suzuki, S., Hirahara, T., Horinouchi, S., and Beppu, T.:** Purification and properties of thermostable tryptophanase from an obligately symbolic thermophile, Symbiobacterium thermophilum, Agric. Biol. Chem., **55**, 3059–3066 (1991).
3. **Hirahara, T., Suzuki, S., Horinouchi, S., and Beppu, T.:** Cloning, nucleotide sequences, and overexpression in *Escherichia coli* of tandem copies of a tryptophanase gene in an obligately symbiotic thermophile, Symbiobacterium thermophilum, Appl. Env. Microbiol., **58**, 2633–2642 (1991).
4. **Suzuki, S., Hirahara, T., Shim, J. K., Horinouchi, S., and Beppu, T.:** Purification and properties of thermostable β-tyrosinase from an obligately symbiotic thermophile, Symbiobacterium thermophilum, Biosci. Biotechnol. Biochem., **56**, 84–89 (1992).
5. **Hirahara, T., Horinouchi, S., and Beppu, T.:** Cloning, nucleotide sequence, and overexpression in *Escherichia coli* of the β-tyrosinase gene from an obligately symbiotic thermophile, Symbiobacterium thermophilum, Appl. Microbiol. Biotechnol., **39**, 341–346 (1993).
6. **Ohno, M., Shiratori, H., Park, M. J., Saitoh, Y., Kumon, Y., Yamashita, N., Hirata, A., Nishida, H., Ueda, K., and Beppu, T.:** *Symbiobacterium thermophilum* gen. nov., sp. nov., a symbiotic thermophile that depends on co-culture with a *Bacillus* strain for growth, Int. J. Syst. Evol. Microbiol., **50**, 1829–1832 (2000).
7. **Ueda, K. and Beppu, T.:** Lessons from studies of *Symbiobacterium thermophilum*, a unique syntrophic bacterium, Biosci. Biotechnol. Biochem., **71**, 1115–1121 (2007).
8. **Ohno, M., Okano, I., Watsuji, T., Kakinuma, T., Ueda, K., and Beppu, T.:** Establishing the independent culture of a strictly symbiotic bacterium *Symbiobacterium thermophilum* from its supporting *Bacillus* strain, Biosci. Biotechnol. Biochem., **63**, 1083–1090 (1999).
9. **Watsuji, T., Yamada, S., Yamabe, T., Watanabe, Y., Kato, T., Saito, T., Ueda, K., and Beppu, T.:** Identification of indole derivatives as self-growth inhibitors of *Symbiobacterium thermophilum*, a unique bacterium whose growth depends on coculture with a *Bacillus* sp., Appl. Env. Microbiol., **73**, 6159–6165 (2007).
10. **Watsuji, T., Kato, T., Ueda, K., and Beppu, T.:** CO_2 supply induces the growth of *Symbiobacterium thermophilum*, a syntrophic bacterium, Biosci. Biotechnol. Biochem., **70**, 753–756 (2006).
11. **Ueda, K., Yamashita, A., Ishikawa, J., Shimada, M., Watsuji, T., Morimura, K., Ikeda, H., Hattori, M., and Beppu, T.:** Genome sequence of *Symbiobacterium thermophilum*, an uncultivable bacterium that depends on microbial commensalisms, Nucleic Acids Res., **32**, 4937–4944 (2004).
12. **Ogawa, M., Kitagawa, M., Tanaka, H., Ueda, K., Watsuji, T., Beppu, T., Kondo, A., Kawachi, R., Oku, T., and Nishio, T.:** A β-*N*-acetylhexosaminidase from *Symbiobacterium thermophilum*: gene cloning, overexpression, purification, and characterization, Enzyme Microb. Technol., **38**, 457–464 (2006).
13. **Barrett, A. J., Rawlings, N. D., and O'Brien, E. A.:** The MEROPS database as a protease information system, J. Struct. Biol., **134**, 95–102 (2001).
14. **Rawlings, N. D. and Morton, F. R.:** The MEROPS batch BLAST: a tool to detect peptidases and their non-peptidase homologues in a genome, Biochimie, **90**, 243–259 (2008).
15. **Rawlings, N. D., Morton, F. R., Kok, C. Y., Kong, J., and Barrett, A. J.:** MEROPS: the peptidase database, Nucleic Acids Res., **36**, D320–D325 (2008).
16. **Rawlings, N. D. and Barrett, A. J.:** Introduction: metallopeptidases and their clans, pp. 231–268, in: Barrett, A. J., Rawlings, N. D. and Woessner, J. F. (Eds.), Handbook of proteolytic enzymes, Elsevier, London (2004).
17. **Niven, G. W.:** Purification and characterization of aminopeptidase A from *Lactococcus lactis* subsp. *lactis* NCDO 712, J. Gen. Microbiol., **137**, 1207–1212 (1991).
18. **Bacon, C. L., Jennings, P. V., Fhaolain, I. N., and O'Cuinn, G.:** Purification and characterization of an aminopeptidase A from cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2, Int. Dairy J., **4**, 503–519 (1994).
19. **I'Anson, K. J. A., Movahedi, S., Griffin, H. G., Gasson, M. J., and Mulholland, F.:** A non-essential glutamyl aminopeptidase is required for optimal growth of *Lactococcus lactis* MG1363 in milk, Miclobiology, **141**, 2873–2881 (1995).
20. **Niven, G. W.:** Glutamyl aminopeptidase (*Lactococcus*), pp. 965–968, in: Barrett, A. J., Rawlings, N. D. and Woessner, J. F. (Eds.), Handbook of proteolytic enzymes, Elsevier, London (2004).
21. **Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M. J., Kuipers, O. P., Sinderen, D., and Kok, J.:** Complete genome sequence of prototype lactic acid bacterium *Lactococcus lactice* subsp. *cremoris* MG1363, J. Bacteriol., **189**, 3256–3270 (2007).
22. **Franzetti, B., Schoehn, G., Hernandez, J.-F., Jaquinod, M., Ruigrok, R. W. H., and Zaccai, G.:** Tetrahedral aminopeptidase: a novel large protease complex from archaea, EMBO J., **21**, 2132–2138 (2002).
23. **Russo, S. and Baumann, U.:** Crystal structure of a dodecameric tetrahedral-shaped aminopeptidase, J. Biol. Chem., **279**, 51275–51281 (2004).
24. **Durá, M. A., Receveur-Brechot, V., Andrieu, J. P., Ebel, C., Schoehn, G., Rousseland, A., and Franzetti, B.:** Characterization of a TET-like aminopeptidase complex from the hyperthermophilic archaeon *Pyrococcus horikoshii*, Biochemistry, **44**, 3477–3486 (2005).
25. **Borissenko, L. and Groll, M.:** Crystal structure of TET protease reveals complementary protein degradation pathways in prokaryotes, J. Mol. Biol., **346**, 1207–1219 (2005).
26. **Schoehn, G., Vellieux, F. M. D., Dura, M. A., Receveur-Bréchot, V., Fabry, C. M. S., Ruigrok, R. W. H., Ebel, C., Roussel, A., and Franzetti, B.:** An archaeal peptidase assembles into two different quaternary structures, J. Biol. Chem., **281**, 36327–36337 (2006).
27. **Lee, H. S., Cho, Y., Kim, Y. J., Nam, K., Lee, J. H., and Kang, S. G.:** Biochemical characterization of deblocking aminopeptidase from hyperthermophilic archaeon *Thermococcus onnurineus* NA1, J. Biosci. Bioeng., **104**, 188–194 (2007).
28. **Motoshima, H. and Kaminogawa, S.:** *Bacillus* aminopeptidase I, pp. 968–970, in: Barrett, A. J., Rawlings, N. D. and Woessner, J. F. (Eds.), Handbook of proteolytic enzymes, Elsevier, London (2004).
29. **Tsunasawa, S.:** N-terminal deblocking aminopeptidase (*Pyrococcus furiosus*), pp. 970–972, in: Barrett, A. J., Rawlings, N. D. and Woessner, J. F. (Eds.), Handbook of proteolytic enzymes, Elsevier, London (2004).
30. **Durá, M. A., Rosenbaum, E., Larabi, A., Gabel, F., Vellieux, F. M. D., and Franzetti, B.:** The structural and biochemical characterizations of a novel TET peptidase complex from *Pyrococcus horikoshii* reveal an integrated peptide degradation system in hyperthermophilic archaea, Mol. Microbiol., **72**, 26–40 (2009).