Biosci. Biotechnol. Biochem., **70** (5), 1140–1147, 2006

Overexpression and Characterization of a Carboxypeptidase from the Hyperthermophilic Archaeon *Thermococcus* sp. NA1

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Received September 14, 2005; Accepted January 11, 2006

Genomic analysis of a hyperthermophilic archaeon, *Thermococcus* sp. NA1, revealed the presence of an 1,497 bp open reading frame, encoding a protein of 499 amino acids. The deduced amino acid sequence was similar to thermostable carboxypeptidase 1 from *Pyrococcus furiosus*, a member of peptidase family M32. Five motifs, including the HEXXH motif with two histidines coordinated with the active site metal, were conserved. The carboxypeptidase gene was cloned and overexpressed in *Escherichia coli*. Molecular masses assessed by SDS–PAGE and gel filtration were 61 kDa and 125 kDa respectively, which points to a dimeric structure for the recombinant enzyme, designated TNA1_CP. The enzyme showed optimum activity toward Z-Ala-Arg at pH 6.5 and 70–80 °C ($k_{cat}/K_m = 8.3\,mM^{-1}\,s^{-1}$). In comparison with that of *P. furiosus* CP ($k_{cat}/K_m = 667\,mM^{-1}\,s^{-1}$), TNA1_CP exhibited 80-fold lower catalytic efficiency. The enzyme showed broad substrate specificity with a preference for basic, aliphatic, and aromatic C-terminal amino acids. This broad specificity was confirmed by C-terminal ladder sequencing of porcine *N*-acetyl-renin substrate by TNA1_CP.

Key words: genomic analysis; hyperthermophile; cloning; expression; carboxypeptidase

Carboxypeptidases (CPs) act at C-terminals of the polypeptide chain and liberate a single amino acid. They can be divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes.

Metallocarboxyproteases are characterized by the requirement of a divalent metal ion for their activity. Thermostable carboxypeptidase 1 (EC 3.4.17.19), belonging to peptidase family M32, is distinct from other metallocarboxypeptidases, since it contains a HEXXH metal-binding motif.[1] Several thermostable carboxypeptidase 1 genes that span all three kingdoms of life, namely archaea, bacteria, and eucarya, have been identified. Among them, the only three thermostable carboxypeptidase 1 from *Thermus aquaticus* (carboxypeptidase Taq),[2] *T. thermophilus*,[3] and the hyperthermophilic archaeon *Pyrococcus furiosus*[4] have been purified and characterized so far. Thermostable carboxypeptidase 1 is known to be very thermostable, with a temperature optimum of 80–100 °C.[2,4] High optimum temperatures for activity have been reported for other carboxypeptidases purified from the bacterium *Thermoactinomyces vulgaris*[5] and the archaeon *Sulfolobus solfataricus*,[6] with temperature optima of 60 and 85 °C respectively.

Thermostable carboxypeptidase 1 exhibits broad substrate specificities. For instance, *P. furiosus* CP releases basic, aromatic, neutral, and polar amino acids from the C-terminus while it is unable to digest peptides with C-terminal Gly, Pro, or acidic residues (Asp and Glu).[4] In the case of carboxypeptidase Taq, it cleaves C-terminal neutral, basic, and acidic amino acids most readily, with the exception of Pro and hydrolyses amino acids with long side chains.[2]

The biological role of microbial metallocarboxypeptidases has not been established, while mammalian metallocarboxypeptidases play key roles in major biological processes, ranging from digestive-protein degradation, as effected by carboxypeptidase A and B,[7,8] to specific proteolytic processing, as in the maturation of biologically active peptides, as effected by carboxypeptidase N and enkephalin convertase.[9,10] It has been suggested that the broad specificities of *P. furiosus* CP against synthetic and natural peptide substrates might play a role in digestion and protein turnover as opposed to specific post-translational modification as seen in higher organisms.[4]

There is growing interest in thermostable enzymes because of their broad range of biotechnological applications and as useful models to understand better the structural basis of thermostability.[11,12] The broad substrate specificities of thermostable carboxypeptidase 1 allow their use in C-terminal sequencing of proteins.[2,4] Using *N*-acetyl-renin substrate, *P. furiosus* CP was able to sequence up to seven residues from the C-terminus releasing Leu, Val, Phe, Tyr, Ser, and His residues.

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Recently, we isolated a hyperthermophilic archaeon, *Thermococcus* sp. NA1, and the whole genome sequence was determined in order to search for valuable, extremely thermostable enzymes. Analysis of the genome information of *Thermococcus* sp. NA1 showed a carboxypeptidase gene belonging to thermostable carboxypeptidase 1. In this study, we describe the cloning of the gene and characterization of the recombinant enzyme with regard to its enzymatic properties.

Materials and Methods

Strains and growth conditions. *E. coli* DH5α was used for plasmid propagation and nucleotide sequencing. *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene, LaJolla, CA) and plasmid pET-24a(+) (Novagen, Madison, WI) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani medium at 37 °C, and kanamycin was added to the medium at a final concentration of 50 µg/ml.

DNA manipulation and sequencing. DNA manipulations were performed by standard procedures, as described by Sambrook and Russell.[13] Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI). Small-scale preparation of plasmid DNA from *E. coli* cells was performed with a plasmid mini kit (Qiagen, Hilden, Germany). DNA sequencing was performed with the automated sequencer (ABI3100) using a BigDye terminator kit (PE Applied Biosystems, Foster City, CA).

Cloning and expression of the carboxypeptidase-encoding gene. Based on the whole genome sequence of *Thermococcus* sp. NA1, the full length of a carboxypeptidase gene (TNA1_CP) flanked by the *Nde*I and *Hind*III sites was amplified by PCR with genomic DNA and three primers (sense [5′-CGACCCGG*CATATG*GAGGAAGTTTTCCAGAACGAAACC-3′], antisense 1 [5′-CTCCACAT*AAGCTT*GAGGTACCTCTCCTTCACCCAGCG-3′], and antisense 2 [5′-CTCCACAT*AAGCTT*TCAGAGGTACCTCTCCTTCACCCAGCG-3′]; the italicized sequences indicate the *Nde*I site in the sense primer and the *Hind*III site in the antisense primers). The amplified DNA fragments were digested with *Nde*I and *Hind*III. The fragments were then ligated with *Nde*I/*Hind*III digested pET-24a(+), and the resulting recombinant plasmids were used to transform *E. coli* DH5α. The recombinant plasmids were introduced into BL21-CodonPlus(DE3)-RIL for expression after sequence confirmation. Overexpression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase and incubation for 3 h at 37 °C. The cells were harvested by centrifugation (6,000 × *g* for 20 min at 4 °C) and resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol. The cells were disrupted by sonication and centrifuged (20,000 × *g* for 1 h at 4 °C). To purify TNA1_CP with the His_6-Tag, the resulting supernatant was applied to a column of TALON™ metal affinity resin (BD Biosciences Clontech, Palo Alto, CA) and washed with 10 mM imidazole (Sigma, St. Louis, MO) in 50 mM Tris–HCl buffer (pH 8.0) containing 0.1 M KCl and 10% glycerol, TNA1_CP was eluted with 300 mM imidazole in the buffer. To purify TNA1_CP (no tag), the supernatant was applied to a column of HiTrap Q (5 ml, Amersham Biosciences) equilibrated with 50 mM Tris–HCl (pH 7.5). A gradient from 0 to 1 M KCl in the same buffer was applied to the column. The active fractions were pooled and concentrated using Centricon YM-10 (Millipore, Bedford, MA). The sample was applied to a Superdex 200 10/300 GL column (Amersham Biosciences) equilibrated with 50 mM Tris–HCl (pH 8.0) and 0.15 M NaCl. The active fractions of TNA1_CP and TNA1_CP (no tag) were then buffer exchanged to 50 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol using Centricon YM-10.

The protein concentration was estimated from the absorbance at 280 nm using an extinction coefficient of 90,750 $M^{-1}\,cm^{-1}$. The protein purity was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis performed according to the standard procedure. Gel filtration of the pure enzyme was performed on a Superdex 200 10/300 GL column using fast protein liquid chromatography (Äkta FPLC System, Amersham Biosciences), equilibrated with 50 mM Tris–HCl (pH 8.0) and 0.2 M KCl. Thirty µg protein was loaded and eluted at a flow rate of 0.4 ml/min.

Enzyme assays. TNA1_CP activity was assayed by measuring the hydrolysis of *N*-Cbz-Alanyl-Arginine (Z-Ala-Arg) (Bachem AG, Bubendorf, Switzerland). The assay mixture (250 µl) contained 50 mM KMES (potassium 2-[*N*-morpholino]ethanesulfonic acid) buffer (pH 6.5), 0.4 mM $CoCl_2$, and 8 mM substrate. The reaction was initiated by addition of the enzyme and was incubated at 80 °C for 10 min, followed by quenching on ice. Subsequently, 600 µl of cadmium-ninhydrin reagent was added, and the samples were incubated for an additional 5 min at 80 °C for color development, followed by quenching on ice.[14] The absorbance was read at 500 nm and the enzyme activity was calculated using the arginine standard curve. One unit of activity was defined as the amount of enzyme that liberates 1 µmol of arginine from Z-Ala-Arg per min at 80 °C. Activity assays for other Z-Ala-X amino acid substrates (X = Ala, Asp, His, Leu, Met, Asn, Pro, Val, and Tyr) were performed under otherwise identical assay conditions. Appropriate amino acids were used to prepare standard curves.

C-Terminal ladder sequencing of N-acetyl rennin substrate with TNA1_CP. The sequencing reaction was carried out with the following reaction mixture in a total volume of 20 µl: 5 mM KMES buffer (pH 6.5), 50 µM

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T. NA1              MEEVFQNETIKQILAKYRRIWAISHARSVLGWDMEVNMPREGIFERSVAQGELSVLSQEF 60
P. furiosus         MEEVFQNETIKQILAKYRRIWAIGHAQSVLGWDLEVNMPKEGILERSVAQGELSVLSHEL 60
T. aquaticus        ---MTPEAAYQNLLEFQRETAYLGSLGALAAWDQRTMIPRKGHGHRARQMAALARLLHER 57
T. thermophilus     ---MTPEAAYQNLLEFQRETAYLASLGALAAWDQRTMIPKKGHEHRARQMAALARLLHQR 57
                       :  : : :::*   *.   :.   :: .** .. :*::*  .*:   . *: * ::

T. NA1              LLKPEFVELVEKAKG---IEDLNEYERGVVRVLDRSIRISKSFPPEFLREMSEVTSQATK 117
P. furiosus         LLHPEFVNLVEKAKG---LENLNEYERGIVRVLDRSIRIARAFPPEFIREVSETTSLATK 117
T. aquaticus        ATDPRIGEWLEKVEGSSLVEDPLSDAAVNVRAWRRAYERARAIPERLAVELAQARSEGET 117
T. thermophilus     MTDPRIGEWLEKVEGSPLVQDPLSDAAVNVREWRQAYERARAIPERLAVELAQAESEAES 117
                    .*.: : :**.:*   :::  .     **   :: . ::::* .:  *::::. * . .

T. NA1              AWEEAKRTNDYSKFEPWLDRIIDLAKRAADYLGYE---------DEPYDALLDLFEEGTT 168
P. furiosus         AWEEAKAKDDFSKFEPWLDKIISLAKRAAEYLGYE---------EEPYDALLDLYEEGLR 168
T. aquaticus        AWEALRPRDDWQGFLPYLKRLFALAKEEAEILMAVGPDPLDPPYGELYDALLDGYEPGAR 177
T. thermophilus     FWEEARPRDDWRGFLPYLKRVYALTKEKAEVLFALPPAPGDPPYGELYDALLDGYEPGMR 177
                     **  :  :*:  * *:*.::  *:*. *: *             * ****** :* *

T. NA1              TRDVERMFKKLEKELKPLLEKIMDEGKVPQSHPLEKEKYKREQMERVNLWILEKFGFPLG 228
P. furiosus         TRDVEKMFEVLEKKLKPLLDKILEEGKVPREHPLEKEKYEREWMERVNLWILQKFGFPLG 228
T. aquaticus        ARDLEPLFRELSSGLKGLLDRILGSGRRPDVGVLHR-HYPKEAQRAFALELLQACGYDLE 236
T. thermophilus     ARELLPLFAELKEGLKGLLDRILGSGKRPDTSILHR-PYPVEAQRRFALELLSACGYDLE 236
                    :*::  :*  *.. ** **::*: .*: *    *.:  *  *  . . * :*.  *: *

T. NA1              VRSRLDVSA HPF TTEFGIR DVRIT TRYEGYDFRRTILSTV HEFGH ALYELQQDERFMFSP 288
P. furiosus         TRARLDVSA HPF TTEFGIR DVRIT TRYEGYDFRRTILSTV HEFGH ALYELQQDERFMFTP 288
T. aquaticus        AG-RLDPTA HPF EIAIGPG DVRIT TRYYEDFFNAGIFGTL HEMGH ALYEQGLPEAHWGTP 295
T. thermophilus     AG-RLDPTA HPF EIAIGPG DVRIT TRYYEDFFNAGIFGTL HEMGH ALYEQGLPKEHWGTP 295
                    .  *** :****   :*  ********    *.  *:.*:**:******    : .  :*

T. NA1              IAGGVSLGI HESQ SRFWENVIGRSREFAELIHPVLKENLPFMANYTPEDVYLYFNMVRPD 348
P. furiosus         IAGGVSLGI HESQ SRFWENIIGRSKEFVELIYPVLKENLPFMSNYTPEDVYLYFNIVRPD 348
T. aquaticus        RGEAASLGV HESQ SRTWENLVGRSLGFWERFFPRAKEVFSSLADVRLEDFHFAVNAVEPS 355
T. thermophilus     RGDAVSLGV HESQ SRTWENLVGRSLGFWERFFPRAREVFASLGDVSLEDFHFAVNAVEPS 355
                     . ..***:****** ***::***  * * :.*  :* :. :.:   **.:: .* *.*.

T. NA1              F IRTESD VVTYNFHILLRFKLERMMLNEGVKAKDLPELWNEEMERLLGIRPKTYAE GILQ 408
P. furiosus         F IRTEAD VVTYNFHILLRFKLERLMVSEEIKAKDLPEMWNDEMERLLGIRPRKYSE GILQ 408
T. aquaticus        L IRVEAD EVTYNLHILVRLELELALFRGELFLEDLPEAWREKYRAYLGVAPRDYKD GVMQ 415
T. thermophilus     L IRVEAD EVTYNLHILVRLELELALFRGELSPEDLPEAWAEKYRDHLGVAPKDYKD GVMQ 415
                    :**.*:* ****:***:*::**  :.   :  :**** * :: .  **: *: * :*::*

T. NA1              DIHW AHGTVGYFPTYSIGTLLSAQIYYHMKRDIPDFEEKVARAEFEPIKAWLREKIHRWG 468
P. furiosus         DIHW AHGSIGYFPTYTIGTLLSAQLYYHIKKDIPDFEEKVAKAEFDPIKAWLREKIHRWG 468
T. aquaticus        DVHW SGGMFGYFPTYTLGNLYAAQFFAKAQEELGPLEPLFARGEFTPFLDWTRRKIHAEG 475
T. thermophilus     DVHW AGGLFGYFPTYTLGNLYAAQFFQKAEAELGPLEPRFARGEFQPFLDWTRARIHAEG 475
                    *:**: * .******::*.* :**:: : : ::  :*  .*:.** *:  * * :**  *

T. NA1              SIYPPKDLLKKAIGEELNPEYFVRWVKERYL----- 499
P. furiosus         SIYPPKELLKKAIGEDMDAEYFVRWVKEKYL----- 499
T. aquaticus        SRFRPRALVERVTGSPPGAQAFLRYLEAKYGALYGF 511
T. thermophilus     SRFRPRVLVERVTGEAPSARPFLAYLEKKYAALYG- 510
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Fig. 1. Sequence Comparison of Peptidase Family M32.

Dashes indicate gaps and numbers on the right represent the position of the last residue in the original sequence. Identical residues among the four enzymes are marked with *****, and residues with conserved substitutions and semi-conserved substitutions are marked with **:** and, **.** respectively. Conserved motifs appear at 238–240 (HPF), DXRXT (248–252), HESQ (298–301), HEXXH (269–273), IRXXAD (350–355), and GXXQDXHW (405–412). Accession numbers with percent identity to carboxypeptidase from *Thermococcus* sp. NA1 (*T.* NA1) are as follows: *P. furiosus* DSM 3638 (NP_578185), 84%, *T. aquaticus* YT-1 (P42663), 35% and *T. thermophilus* HB27 (YP_005684), 35%.

porcine *N*-acetyl-renin substrate (Sigma, St. Louis, MO), 0.2 mM $CoCl_2$, and 150 nM TNA1_CP. The sample was incubated at 80 °C for 3 min, followed by quenching on ice and the addition of 0.1% TFA. It was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Ultraflex, Bruker Daltonik, Bremen, Germany), and the peaks were assigned to within 0.3 Da of their monoisotopic masses. The amino acid sequence of porcine *N*-acetyl-renin substrate (MW 1801.3) was Ac-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser. The matrix used was α-cyanohydroxycinnamic acid and the acceleration voltage was 25 kV.

Nucleotide sequence accession number. The reported nucleotide sequence has been submitted to the GenBank/EMBL Data Bank under accession no. DQ144135.

Results and Discussion

Primary structure of the TNA1_CP gene and expression of the recombinant enzyme

Recently, we isolated a hyperthermophilic archaeon, *Thermococcus* sp. NA1, growing at high temperatures (70–90 °C), and, by analyzing the genome sequence, we found that the open reading frame, composed of 1,497 bp, encodes a protein homologous to thermostable carboxypeptidase 1, which were purified and characterized from *P. furiosus* DSM 3638 (84% identity),[15,16] *T. aquaticus* YT-1 (35% identity),[17] and *T. thermophilus* HB27 (35% identity)[3] (Fig. 1). The encoded protein consists of 499 amino acids with a predicted molecular mass of 59,198 Da and a pI of 5.61. It appeared that it is an intracellular protein due to a lack of signal peptide, which is consistent with other peptidase family M32. Similarity analysis has shown the presence of CP-homologous genes in genome sequences of other archaea whose deduced amino acid sequences showed fairly high identities to TNA1_CP (33 to 92%). In particular, those from Thermococcales, including *Thermococcus kodakaraensis*,[18] *Pyrococcus horikoshii*,[19] and *Pyrococcus abyssi*,[20] were quite similar to TNA1_CP (83 to 92%). On the other hand, similarities were lower between TNA1_CP and bacterial orthologs (29 to 37% identity). The sequence alignment of TNA1_CP and its homologs revealed that five motifs in addition to the HEXXH motif, based on the data obtained from *P. furiosus* CP,[16] are well conserved, except for one amino acid, A354, from the IRXXAD exchanged with S354. The protein ligands (H269, H273, and E299) and the proton shuttle (E270) forming the active site of TNA1_CP are also conserved, hence the protein might adopt a conformation similar to those found in other peptidases containing the HEXXH motif.

The TNA1_CP gene was amplified with PCR and the expressed enzyme was purified from a soluble cell extract. Analysis by SDS/PAGE showed that the 61-kDa protein (Fig. 2), which was the expected size of the fusion product comprising the 59.2-kDa CP protein and a 1.5-kDa peptide corresponding to the -KLAAALEH$_6$-(His_6-Tag) at the C-terminal region of protein, was the major component of the purified sample. Gel filtration under non-denaturing conditions showed a protein peak corresponding to a molecular mass of 125 kDa. These data point to a dimeric structure for the enzyme (data not shown).

Biochemical characterization of purified TNA1_CP

The functionality of TNA1_CP was confirmed by its ability to hydrolyze Z-Ala-Arg, and this substrate was used in all routine assays. Carboxypeptidase activity was strongly stimulated at high temperatures and showed a temperature optimum at 70–80 °C, but the activity decreased rapidly above 80 °C (Fig. 3A). The influence of pH on TNA1_CP activity was evaluated by using different buffers, sodium acetate (pH 4.0–5.0), KMES (pH 5.5–6.5), HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (pH 7–7.5), Tris–HCl (pH 8–10), and the optimum values were pH 6.5 (Fig. 3B). It is worth noting that carboxypeptidase Taq from eubacteria *T. aquaticus* has an optimum pH of 8, but archaeal *P. furiosus* CP and TNA1_CP have optimum pHs of 6.5.

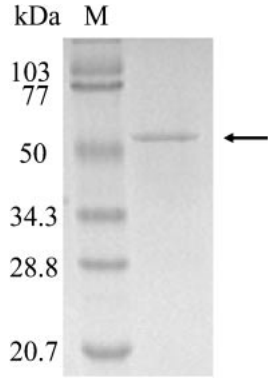

Fig. 2. SDS–PAGE (12%) of the Purified Enzyme.

The molecular mass standards (lane M) were phosphorylase *b* (103 kDa), bovine serum albumin (77 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.3 kDa), soybean trypsin inhibitor (28.8 kDa), and lysozyme (20.7 kDa). The band corresponding to the enzyme is indicated by the arrow.

As shown in Fig. 1, protein ligands for metal binding were conserved in the HEXXH motif and the HESQ sequence, implying that the activity of TNA1_CP is influenced by the addition of metal ions. The addition of Co^{2+} ions to the enzyme solution stimulated activity 3.7-fold, but Co^{2+} ions could not be replaced with other divalent cations (Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) at a concentration of 0.2 mM (Fig. 4). The control exhibited residual activity prior to the addition of Co^{2+}, probably caused by the remaining metal ions after buffer exchange or tight binding of Co^{2+}. No CP activity was observed in the presence of a metal chelating agent, EDTA (1 mM). Carboxypeptidase Taq was activated by metal ions in the order $Co^{2+} > Ca^{2+} > Mg^{2+} > Cu^{2+} > Zn^{2+}$,[2] and *T. thermophilus* CP required a divalent metal ion such as Zn^{2+} or Co^{2+} for its activity.[3] On the other hand, *P. furiosus* CP were activated by Co^{2+} but not by Zn^{2+},[4] and the metal requirement of TNA1_CP (data not shown) was similar to that of *P. furiosus* CP.[4] It has been reported that *P. furiosus* CP and *T. thermophilus* CP are associated with a variety of metals, such as Pb^{2+}, Yb^{2+}, Mg^{2+} and Zn^{2+} in the crystal structures,[3,16] and further investigation is needed to determine what is the biologically relevant metal for those CPs, including TNA1_CP.

Based on the result that Cu^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} lowered the residual activity below 50% (Fig. 4), TNA1_CP was treated with metal ions, and then Co^{2+}

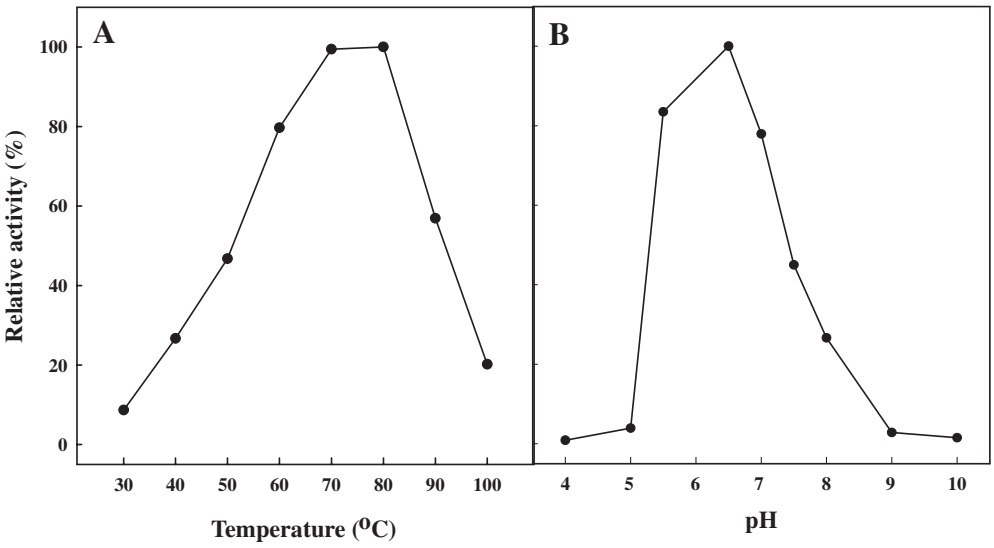

Fig. 3. The Effects of Temperature (A) and pH (B) on the Activities of TNA1_CP.

A, Activity assays were performed under standard conditions as the sample temperature was increased from 30 to 100 °C. B, Activity assays were performed under standard conditions with the following buffers (each at 50 mM): sodium acetate, pH 4.0–5.0; KMES, pH 5.5–6.5; HEPES, pH 7.0–7.5; Tris–HCl, pH 8.0–10.0.

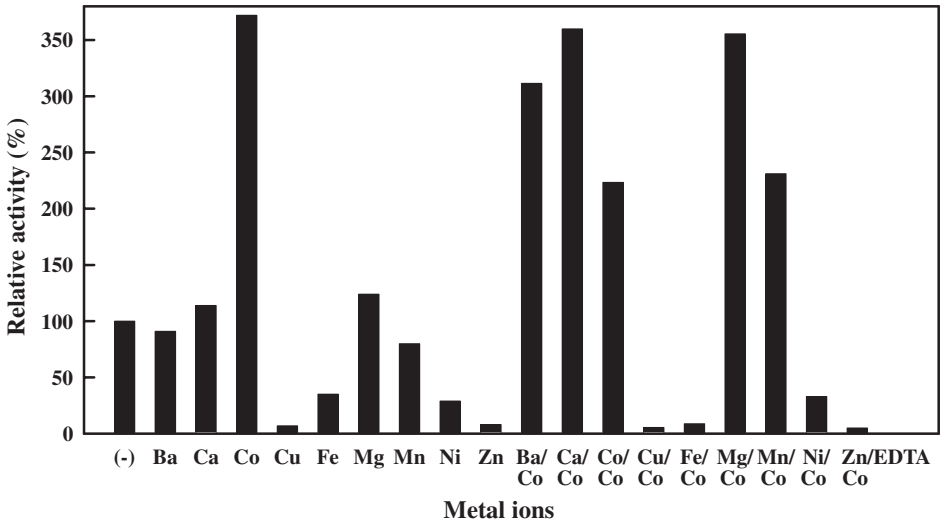

Fig. 4. Effects of Metal Ions on the Activities of TNA1_CP.

Activity assays were performed under standard conditions, while various divalent metal ions (0.2 mM) and EDTA (1 mM) alone or in combination with Co^{2+} (0.2 mM) were added to the diafiltered enzyme solution. (−) means control without any metal ions.

was added to assess their inhibitory effect. As shown in Fig. 4, the inhibitory activity of Cu^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+} was not overcome by the presence of Co^{2+}, suggesting that the inhibitory metal ions bind TNA1_CP tightly in place of Co^{2+} and perhaps accompany a conformational change. On the other hand, Ba^{2+} and Mn^{2+} had little effect, and Ca^{2+} and Mg^{2+} exhibited no effect on TNA1_CP activity.

The thermostability of TNA1_CP was evaluated by incubating the enzyme in 50 mM KMES buffer (pH 6.5) containing 0.2 mM $CoCl_2$ at 80 °C and 90 °C for up to 100 min. TNA1_CP lost enzymatic activity with a half-life ($t_{1/2}$) of 84 min at 80 °C (Fig. 5), and thus exhibits better thermostability than *P. furiosus* CP (40 min).[4] The inactivation of TNA1_CP at 90 °C was quite fast, 50% of the activity remaining after only 8 min. The thermostability of TNA1_CP was highly affected by the presence of Co^{2+}. In the absence of Co^{2+} in the reaction during heating, the half-life values of TNA1_CP decreased to 7.4 min and 5.0 min at 80 °C and 90 °C respectively, which means that the conformation of TNA1_CP-Co^{2+} might be more stable (data not shown). Without Co^{2+}, no activation of TNA1_CP by heat treatment (at 80 °C up to 20 min, data not shown) was observed, which might explain the rapid inactivation of the enzyme at 80 °C. Cheng *et al.*[4] suggested that

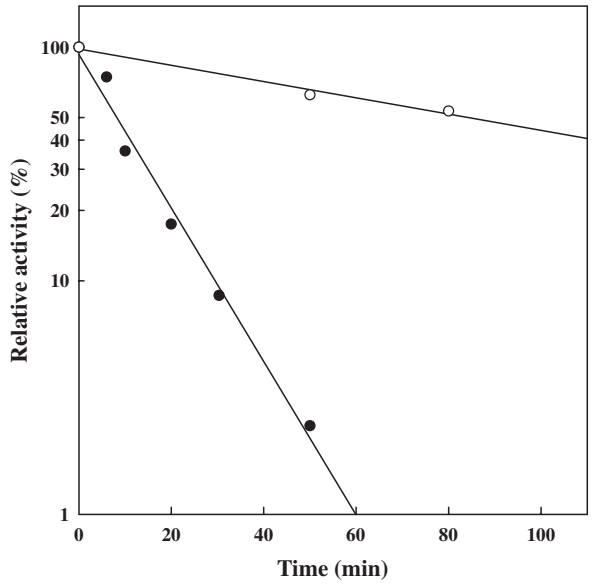

Fig. 5. Effect of Temperature on TNA1_CP Stability.

TNA1_CP (0.8 μg/ml) was incubated at 80 °C (○) and 90 °C (●) in 50 mM KMES buffer, pH 6.5, containing 0.2 mM $CoCl_2$. At the times shown, aliquots were taken out, and the activities were measured in the same buffer at 80 °C using Z-Ala-Arg as substrate.

Table 1. Substrate Specificities of TNA1_CP and *P. furiosus* CP as Determined with Z-Ala-X Substrates

Substrate	Rate of hydrolysis (%)* with	
	TNA1_CP	*P. furiosus* CP#
Z-Ala-Arg	100	100
Z-Ala-Leu	62	27
Z-Ala-Met	49	61
Z-Ala-Tyr	24	12
Z-Ala-Ala	16	14
Z-Ala-Val	12	6

*All values are percentages expressed relative to the rate of hydrolysis of Z-Ala-Arg (100%) for each protein.
#Data from reference 4.

Table 2. Kinetic Parameters of TNA1_CPs and *P. furiosus* CP as Determined with Z-Ala-Arg Substrate*

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
TNA1_CP	1.4	11.6	8.3
TNA1_CP (no tag)	1.6	12.8	8.0
P. furiosus CP#	0.9	600	667

*The typical error in K_m and k_{cat} determinations did not exceed 13%.
#Data from reference 4.

P. furiosus CP might be stabilized *in vivo* by solutes such as *cyclic*-2,3-diphosphoglycerate, di-*myo*-inositol-1,1′-phosphate, and mannosyl glycerate. However, *in vitro* supplementation of 12.5% glycerol did not protect TNA1_CP from inactivation at either temperature (data not shown).

TNA1_CP exhibited relatively broad specificity with a preference toward basic, aliphatic, and aromatic C-terminal amino acids (Table 1). No activity was detected under standard conditions against Z-Ala-Asp, Z-Ala-Pro, Z-Ala-His, or Z-Ala-Asn among the substrates tested. In comparison with *P. furiosus* CP,[4] the preference was in a similar pattern, but not identical, *viz.*, Arg > Met > Leu > Ala > Tyr > Val for *P. furiosus* CP, and Arg > Leu > Met > Tyr > Ala > Val for TNA1_CP1. According to Umetsu *et al.*,[21] the hydrolysis rates were strikingly affected by amino acids in the penultimate position from the C-termini of the substrates. Therefore, other amino acids than Ala in the Z-Ala-X (X = Asp, Pro, His, and Asn) substrate might enhance the cleavage of substrates. Many carboxypeptidases exhibiting broad substrate specificities have been reported.[22] On the basis of preferences for amino acids at the C-terminus, *Penicillium janthinellum* CP,[23] a member of the serine carboxypeptidases, and carboxypeptidase T from *Thermoactinomyces* sp.[24] cleaved the peptide bonds formed by basic and hydrophobic C-terminal amino acid residues, whereas carboxypeptidase A and carboxypeptidase C, and carboxypeptidase B and carboxypeptidase D exhibited strong preferences for hydrophobic and basic residues respectively.[1,25]

Kinetic analysis was conducted using Z-Ala-Arg, and kinetic parameters such as K_m and k_{cat} values were calculated from the measured activity for TNA1_CP (Table 2). In comparison with those of *P. furiosus* CP ($K_m = 0.9$ mM and $k_{cat} = 600\,s^{-1}$), TNA1_CP exhibited 80-fold lower catalytic efficiency toward Z-Ala-Arg. To investigate the possibility of the His_6-Tag at the C-terminus of TNA1_CP affecting affinity or activity toward the substrate, TNA1_CP protein having no His_6-Tag, TNA1_CP (no tag), was prepared, and kinetic constants were obtained (Table 2). TNA1_CP (no tag) gave a virtually identical result, indicating that the decrease in catalytic efficiency of TNA1_CP was not caused by the presence of a His_6-Tag at the C-terminus. It is not yet understood why the two enzymes showing high identity (84%) were different in catalytic efficiency. The oligomeric status of TNA1_CP was not different from that of *P. furiosus* CP. Amino acids for dimer formation in *P. furiosus* CP[16] were also present in TNA1_CP, and the dimeric structure was confirmed by gel filtration column chromatography (125 kDa on a Superdex 200, data not shown). In addition, as discussed above, most of the critical residues for carboxypeptidase activity were found in TNA1_CP: an HPF sequence, a DXRXT sequence, an HESQ sequence, and a GXXQ-DXHW sequence in addition to the characteristic metalloprotease HEXXH motif. One possible candidate to explain the decrease in catalytic efficiency is S354, since an A354 in the IRXXAD sequence was changed to S354 in TNA1_CP. Arndt *et al.* (2002)[16] proposed that the IRXXAD and GXXQDXHW signature sequences might work in concert to bind the substrate C-terminal carboxylate and promote release of the cleaved amino acid

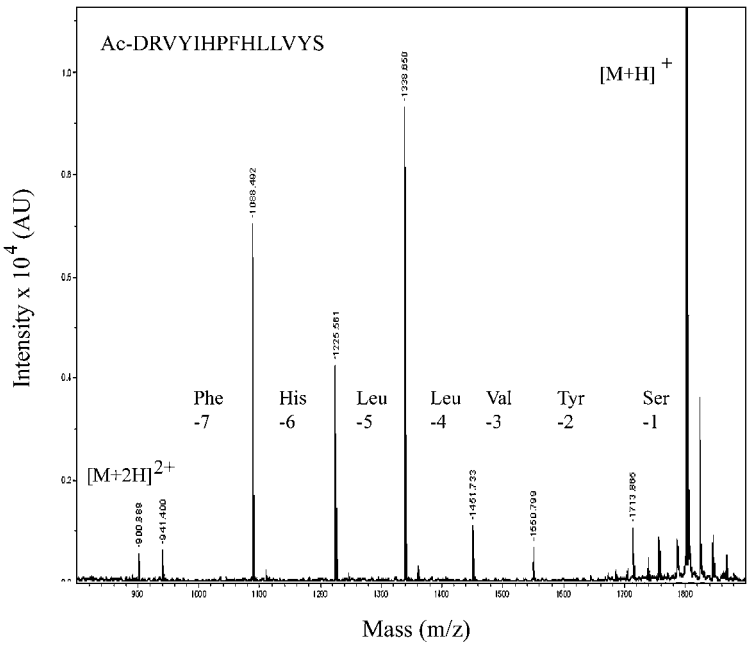

Fig. 6. MALDI-TOF Spectrum of C-Terminal Ladder Sequencing of *N*-Acetyl-renin Substrate by TNA1_CP.
Fifty μM *N*-acetyl-renin substrate was digested in 5 mM KMES buffer (pH 6.5) containing 0.2 mM $CoCl_2$ and 150 nM TNA1_CP at 80 °C for 3 min, and the sample was analyzed by MALDI-TOF mass spectrometry.

product. Nevertheless, it is not certain how the change of A354 to S354 can effect the decrease in kinetic value, and this should be investigated further. In addition, further experiments are required to determine whether the enzyme was folded properly during purification from *E. coli* or exhibited non-ideal behavior.

C-Terminal ladder sequencing of the peptide

Sequential C-terminal hydrolysis of the peptide *N*-acetyl-renin substrate by TNA1_CP, followed by MALDI-TOF mass detection, offered unambiguous proof of carboxypeptidase activity (Fig. 6). Under the reaction conditions, TNA1_CP sequenced up to seven residues from the C-terminus of substrate, releasing Ser, Tyr, Val, Leu, His, and Phe residues. These sequencing experiments confirmed the broad specificity of TNA1_CP demonstrated in the experiments with ZAX dipeptides.

In this study, we characterized a thermostable carboxypeptidase from a hyperthermophilic archaeon, *Thermococcus* sp. NA1. The TNA1_CP can be applied to C-terminal sequencing of peptides, and practical application is in progress.

Acknowledgments

We would like to thank Dr. J. H. Hong and Mr. S. C. Shin of Macrogen, Inc., for valuable information. This work was been supported by the KORDI in-house program (PE97201) and the Marine and Extreme Genome Research Center Program of the Ministry of Maritime Affairs and Fisheries, of the Republic of Korea.

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