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Characterization of a novel metallocarboxypeptidase from *Streptomyces cinnamoneus* TH-2

Kun Wan[1], Misugi Uraji[1], Jiro Arima[2] and Tadashi Hatanaka[1*]

Abstract

Background: Carboxypeptidases are exopeptidases that catalyze the release of amino acids from the C-terminus of peptides or proteins. The peptides consisting of hydrophobic amino acids taste bitter. Therefore, the hydrolytic capability of carboxypeptidase toward hydrophobic amino acids at the C-terminus of peptides is useful for the degradation of bitter peptides.

Results: Using the genome data of *Streptomyces cinnamoneus* TH-2, we expressed and characterized a novel metallocarboxypeptidase (TH2-CP) in *Streptomyces lividans*. TH2-CP had a molecular mass of 37.7 kDa. As TH2-CP possesses a zinc-binding consensus motif (HXXE……H) and N-terminal prosegment residues, we suggest that TH2-CP could be classified into the M14A subfamily. In the presence of Z-Gly-Leu as the substrate, TH2-CP showed optimum activity at pHs 7 and 8 in potassium phosphate and Tris–HCl buffers, respectively. The optimum temperature for activity was 51 °C. Furthermore, 50 % activity was conserved after incubation at 38 °C for 30 min. TH2-CP showed broad substrate specificity, with a preference for hydrophobic amino acids, as demonstrated by casein hydrolysate breakdown.

Conclusions: A novel metallocarboxypeptidase, TH2-CP, from *S. cinnamoneus* TH-2 was characterized. TH2-CP preferred substrates with hydrophobic amino acids at the C-terminal position for casein peptides. This property indicates that TH2-CP can be used to decrease the bitterness of peptides in food industries.

Keywords: Casein, Metallocarboxypeptidase, *Streptomyces*

Background

Streptomycetes produce a wide variety of enzymes, such as peptidases, lipases, and glucohydrolases. Such enzymes have industrial applications. To date, we have discovered several enzymes from *Streptomyces cinnamoneus* TH-2 (formerly named *S. septatus*) by screening enzymatic activity. This strain produces extracellular enzymes including phospholipase D, aminopeptidases, and metalloendopeptidases (Arima et al. 2004; Hatanaka et al. 2002, 2005). For these reasons, this strain has the potential to be a resource of useful enzymes.

Carboxypeptidases are exopeptidases that hydrolyze peptide bonds from the C-terminus of peptides or proteins. They function in a variety of physiological processes such as fibrinolysis, inflammation, blood coagulation, and prohormone and neuropeptide processing (Arolas et al. 2007; Vendrell et al. 2000). They can also be used to decrease the bitterness of peptides in food industries (Fu et al. 2011; Ge and Zhang 1996; Komai et al. 2007; Umetsu et al. 1983). According to the presence of particular active site residues, carboxypeptidases are classified as metallocarboxypeptidases, serine carboxypeptidases, or cysteine carboxypeptidases. Among them, metallocarboxypeptidases possess a conserved active site motif, (HXXE……H) or (HEXXH), which requires their binding to divalent metal ions for activity (Lee et al. 1996). To date, there are few reports concerning the characterization of metallocarboxypeptidase from the genus *Streptomyces*. Using

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genome data from *S. cinnamoneus* TH-2, we succeed in expressing and characterizing a novel metallocarboxypeptidase, TH2-CP. TH2-CP shows broad substrate specificity, with a preference for hydrophobic amino acids.

Methods

Materials

A protein assay kit and gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories Inc. (USA). All peptides were purchased from Bachem AG (Bubendorf, Switzerland). PD-10 desalting columns were purchased from GE Healthcare (USA). Snake venom L-amino acid oxidase (LAAO) was purchased from Sigma Chemical (USA) and used without further purification. Horseradish peroxidase was purchased from Wako Chemicals (Osaka, Japan). Endoprotease from a *Bacillus* sp. (Bioprase SP) was a gift from Nagase ChemteX (Fukuchiyama, Japan). EZ:faast™ kits (Phenomenex, USA) for amino acid analysis of protein hydrolysates were purchased from SHIMADZU GLC Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

Analysis of genome sequence of *S. cinnamoneus* TH-2

Genomic DNA was prepared from the strain TH-2 by the method of Hopwood et al. (1985). Sequencing of the genomic DNA was performed using a Hiseq next-generation sequencer (illumina, USA) and an Ion PGM™ next-generation sequencer (Thermo Fisher Scientific, USA). Sequence reads were assembled into a draft genome sequence (ca. 6.3 Mbp) using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). The draft sequence was sent to the Microbial Genome Annotation Pipeline (MiGAP, Database Center for Life Science, Japan) for annotation.

Cloning, expression and preparation of TH2-CP for enzymatic assays

TH2-CP was amplified by PCR using the sense primer 5′-CATATGTCCCGAAAACGCCTGGCAGGGC-3′ (the underlined region represents the *Nde*I site and includes the initiation codon) and the antisense primer 5′-AAGCTTCAGTCGGCCGACCG-3′ (the underlined region represents the *Hin*dIII site and is adjacent to the stop codon). PCR was performed using the Tks Gflex DNA Polymerase system (Takara, Shiga, Japan). The PCR product (1.4 kb) was cloned into the pCR-Blunt II-TOPO Vector system (Invitrogen, USA) and correct cloning was confirmed by sequencing. The inserted fragment was subcloned into the *Nde*I–*Hin*dIII gap of pTONA5a (Hatanaka et al. 2008). The constructed pTONA5a-TH2-CP was then transformed in *S. lividans* 1326 by conjugation, as described by Hatanaka et al. (2008). The resultant *S. lividans* 1326 transformants were incubated and grown in 50-ml medium (8 g of KH_2PO_4/l, 20 g of glucose/l, 0.5 g of $MgSO_4 \cdot 7H_2O$/l, 5 g of polypeptone/l, and 5 g of yeast extract/l) in a 500-ml baffled flask at 30 °C for 3 days with rotary shaking at 150 rpm. After centrifugation of the culture, the supernatant was loaded onto PD-10 columns (GE Healthcare) for buffer exchange with 20 mM phosphate buffer (pH 7) or distilled water. The enzyme solution obtained was used for the characterization of TH2-CP2. Simultaneously, the supernatant was concentrated by ammonium sulfate precipitation at 70 % (w/v) saturation at 4 °C. The precipitates were collected by centrifugation at 20,000*g* for 1 h at 4 °C, and then dissolved in 1 ml of 20 mM Tris–HCl (pH 8) buffer containing 0.15 M NaCl. After centrifugation of the dissolved solution, the resultant supernatant was filtered and subsequently loaded onto a HiLoad™ 16/60 Superdex™ 75 pg column (GE Healthcare). The active fraction eluted with 20 mM Tris–HCl (pH 8) containing 0.25 M NaCl was used for the determination of N-terminal sequences of TH2-CP.

Kinetics

The optimum pH was determined as follows. A mixture of 5 µl of 200 mM NaOAc (pH 4–5.6) or 200 mM potassium phosphate buffer (pH 5.5–8) or 200 mM Tris–HCl (pH 7–8.5) buffer, 2.5 µl of substrate solution (100 mM Z-Gly-Leu), and 37.5 µl of distilled water was added to 5 µl of supernatant enzyme solution from a culture at 30 °C. The enzymatic reaction mixture was incubated at 40 °C for 30 min, and then the reaction was stopped by heat treatment (95 °C, 5 min). The free amino acids released by the hydrolysis of peptides were detected by the 4-aminoantipyrine-phenol method coupled with the reaction of LAAO (Arima et al. 2006). After heat treatment, 20 µl of the reaction liquid was added to 180 µl of a premix solution containing LAAO (0.2 mg/ml). After incubation for 30 min at 37 °C, the absorbance of the solution was determined at 505 nm. This assay was performed in triplicate, and the mean of absorbance at each pH is shown as the percentage of the mean in Tris–HCl buffer (pH 8). Note that the method of evaluating the free amino acids was used for all subsequent procedures.

The optimum temperature was determined as follows. A mixture of 5 µl of 200 mM potassium phosphate buffer (pH 7), 2.5 µl of substrate solution (100 mM Z-Gly-Leu), and 37.5 µl of distilled water was added to 5 µl of enzyme solution (20 mM potassium phosphate buffer, pH 7). Then, the enzymatic reaction mixture was incubated at 40–60 °C for 30 min. Finally, the free amino acids released were detected. This assay was performed in triplicate, and the mean absorbance at each temperature is shown as the percentage of the mean at 51 °C.

Thermostability was determined as follows. A mixture of 5 µl of 200 mM potassium phosphate buffer (pH 7), 5 µl of

enzyme solution (20 mM potassium phosphate buffer, pH 7), and 37.5 µl of distilled water was prepared and incubated at 5–45 °C for 30 min. Then, 2.5 µl of substrate solution (100 mM Z-Gly-Leu) was added to the mixture to start the enzymatic reaction at 40 °C for 30 min. Finally, the free amino acids released were detected. This assay was performed in triplicate, and the mean absorbance at each temperature is shown as the percentage of the mean at 5 °C.

pH stability was determined as follows. An enzyme mixture of 0.56 µl of 200 mM potassium phosphate buffer (pH 5.5–8), and 5 µl of enzyme solution (distilled water), was prepared and incubated at 5 °C overnight. Then, a substrate mixture of 2.5 µl of substrate solution (100 mM Z-Gly-Leu), 5 µl of 200 mM potassium phosphate buffer (pH 7), and 36.94 µl distilled water was prepared and added to the enzyme mixture to start the enzymatic reaction at 40 °C for 30 min. Finally, the free amino acids released were detected. This assay was performed in triplicate, and the mean absorbance at each pH is shown as the percentage of the mean in potassium phosphate buffer (pH 7).

Except for two tripeptides, Phe-Phe-Phe and Tyr-Tyr-Tyr, the specific activity and kinetics of other substrates were evaluated as follows. A mixture of 5 µl of substrate solution (specific activity evaluation, 50 mM; kinetics evaluation, 3–50 mM), 5 µl of 200 mM sodium phosphate buffer (pH 7), and 35 µl distilled water was added to 5 µl of enzyme solution (20 mM sodium phosphate buffer, pH 7). Then, the enzymatic reaction mixture was incubated at 40 °C for 30 min. For Phe-Phe-Phe and Tyr-Tyr-Tyr, the enzymatic reaction mixture consisted of 40 µl of substrate solution (1 mM), 5 µl of 200 mM sodium phosphate buffer (pH 7), and 5 µl of 160-fold diluted enzyme solution (20 mM sodium phosphate buffer, pH 7). Finally, the free amino acids released were detected. This assay was performed in triplicate and the free amino acids were quantified by a standard curve of 0, 0.5, and 1 mM free L-leucine. K_m and V_{max} were calculated from the Hanes–Woolf plots obtained using the Michaelis–Menten equation.

Determination of N-terminal amino acid sequence

The purified TH2-CP was blotted onto a PVDF membrane after 12 % SDS-PAGE under denaturing conditions. The protein blotted on the PVDF membrane was used for determining the N-terminal amino acid sequence using a protein sequencer (Shimadzu PPSQ-31A).

Assay of TH2-CP activity inhibition

The assay of TH2-CP activity inhibition was performed using phenylmethylsulfonyl fluoride, iodoacetic acid, 2-iodoacetamide, *N*-ethylmaleimide, and 1,10-phenanthroline, respectively. An enzyme mixture of 0.56 µl of 10 mM inhibitor or distilled water, and 5 µl of enzyme solution (20 mM potassium phosphate buffer, pH 7), was prepared and incubated overnight at 5 °C. Then, a substrate mixture of 2.5 µl of substrate solution (100 mM Z-Gly-Leu), 5 µl of 200 mM potassium phosphate buffer (pH 7), and 36.94 µl distilled water was prepared and added to the enzyme mixture to start the enzymatic reaction at 40 °C for 30 min. Finally, the free amino acids released were detected. This assay was performed in triplicate, and the mean absorbance for each inhibitor is shown as the percentage of the mean in distilled water.

Effects of divalent metal ions on TH2-CP activity

The effects of divalent metal ions on TH2-CP activity was investigated using cobalt chloride ($CoCl_2$), nickel chloride ($NiCl_2$), zinc chloride ($ZnCl_2$), magnesium chloride ($MgCl_2$), manganese chloride ($MnCl_2$), and calcium chloride ($CaCl_2$), respectively. An enzyme mixture of 0.56 µl of 10 mM divalent metal ion solution or distilled water, and 5 µl of enzyme solution (20 mM potassium phosphate buffer, pH 7), was prepared and incubated overnight at 5 °C. A substrate mixture of 2.5 µl of substrate solution (100 mM Z-Gly-Leu), 5 µl of 200 mM potassium phosphate buffer (pH 7), and 36.94 µl distilled water was prepared and added to the enzyme mixture to start the enzymatic reaction at 40 °C for 30 min. Finally, the free amino acids released were detected. This assay was performed in triplicate, and the mean absorbance for each metal ion is shown as the percentage of the mean in distilled water.

Assay of TH2-CP-catalyzed casein hydrolysate

A mixture of 33 µl of Bioprase SP (100 proteolytic units/mg, 10 mg/ml) and 10 ml of 0.6 % (w/v) aqueous dispersion of casein (40 mM NaH_2PO_4, pH 7.5) was prepared. It was incubated at 40 °C overnight and then at 80 °C for 30 min to inactivate Bioprase SP. After centrifugation, 250 µl of the resultant supernatant was added to 100 µl of enzyme solution (20 mM NaH_2PO_4, pH 7), or 100 µl of 20 mM NaH_2PO_4 (pH 7) buffer. The reaction solution was incubated at 40 °C for 6 h with rotary shaking at 1400 rpm, and then at 80 °C for 30 min to inactivate TH2–CP2. Both of these reactions were performed in triplicate. For the amino acid analysis of casein hydrolysate, a 100-µl aliquot of each of the triplicate reaction solutions was obtained to be acidulated with 0.2 M HCl, and then analyzed by gas chromatography (GC-2025, SHIMADZU GLC Ltd.) using an EZ:faast™ kit. The released free amino acids were quantified using a 250 µM standard mixture of free amino acids. To calculate the rate of casein hydrolysis by TH2-CP, the hydrolysis of casein hydrolysate by 6 M HCl was also assayed. In this assay, 2 ml of aqueous dispersion of casein (10 mg/ml, 6 M HCl) was heated at 110 °C for 15 h, and then 100 µl of the solution was taken for analysis using an EZ:faast™ kit. This assay was also performed in triplicate.

Results and discussion

Expression of carboxypeptidase (CP) genes

A good number of candidate genes encoding various enzymes were predicted by MiGAP analysis. Among them, there are six candidate genes encoding CP enzymes. Sequence analysis using the Signal P 4.1 Server (http://www.chs.dtu.dk/services/SignalP/) indicated that three of the six were predicted to possess signal peptides. We cloned these three genes into the pTONA5a vector and expressed them in *S. lividans* 1326. Only the culture supernatant from TH2-CP expression proved to possess carboxypeptidase activity (data not shown).

The TH2-CP encoded by 1368 nucleotides has a theoretical molecular weight of 45.8 kDa (excluding the predicted signal peptide) and is predicted to belong to the metallocarboxypeptidase M14 family by MiGAP analysis. The conserved, predicted motif HXXE and the third zinc ligand H of M14 for TH2-CP are shown in Fig. 1, but further studies are necessary to demonstrate the presence of this motif. SDS-PAGE (Fig. 2) showed that TH2-CP has an actual molecular weight of approximately 37 kDa. This result indicated that there is a prosegment of residues at the N terminus of TH2-CP beside the signal peptide; thus, TH2-CP can be assigned to the M14A subfamily.

N-terminal amino acid sequence of TH2-CP

The N-terminal amino acid sequence of TH2-CP of *S. cinnamoneus* TH-2 was determined to be VTDDA (Fig. 1). Excluding the signal peptide and pro-segment, the theoretical molecular weight of TH2-CP2 was 37.7 kDa. This value corresponds to the observed result of SDS-PAGE (Fig. 2).

Effects of pH and temperature on TH2-CP activity

As shown in Fig. 3a, the optimum pHs for TH2-CP activity were approximately pH 7 and 8 in potassium phosphate and Tris–HCl buffers at 40 °C, respectively. However, the stability of TH2-CP in potassium

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       10        20        30        40        50        60        70        80        90       100       110       120
ATGTCCCGAAAACGCCTGGCAGGGCTGGCGGTCGGAGTGACGGTCATGGCCGCGGCGGCCATGCTCCCGCAGGCCACGACGGCCCTCGCCGGTGCCTCGCAGAGCCGTGACACCGCGGTC
M  S  R  K  R  L  A  G  L  A  V  G  V  T  V  M  A  A  A  A  M  L  P  Q  A  T  T  A  L  A  G  A  S  Q  S  R  D  T  A  V

      130       140       150       160       170       180       190       200       210       220       230       240
CTCTACGAGGTCCCCACGCACCACAAGCAGGACGCGCAGACCCTGATCGCCGCCGGGTACGACCTCCTGGAGCGCCGCCAGGGCGACCGCCTCTTCGTCCTCGGCGACCCGGCGACCGGC
L  Y  E  V  P  T  H  H  K  Q  D  A  Q  T  L  I  A  A  G  Y  D  L  L  E  R  R  Q  G  D  R  L  F  V  L  G  D  P  A  T  G

      250       260       270       280       290       300       310       320       330       340       350       360
GACGGCCTGCGCCGCCTCGGCTTCTCCTCCACCGCCGTCCGCACGATGCCGAGACCGGTCCCGAAGAAGTCGGTGACGGACGACGCGGGCGACACGTACGACGGCGGCTATCACACCGTC
D  G  L  R  R  L  G  F  S  S  T  A  V  R  T  M  P  R  P  V  P  K  K  S  V  T  D  D  A  G  D  T  Y  D  G  G  Y  H  T  V

      370       380       390       400       410       420       430       440       450       460       470       480
GGCGCCCACTACGCGCACCTGGACCAGCTCGCCGCCCAGCACCCGGACCTGGCCACGGTCGTCACCTACGGCAAGTCCTGGCTGAAGCAGACCGGTTCGGGCGGCCATGACCTCAAGGCC
G  A  H  Y  A  H  L  D  Q  L  A  A  Q  H  P  D  L  A  T  V  V  T  Y  G  K  S  W  L  K  Q  T  G  S  G  G  H  D  L  K  A

      490       500       510       520       530       540       550       560       570       580       590       600
GTCTGCATCACCAAGATGAAGCCCGGCGACTGCTCGCTCGACCCGAACCCGGCCAAGCCCCGCTTCTTCCTGATGAGCCAGATCCACGCGCGCGAGCTCACCACCGGCGAGATGTCCTGG
V  C  I  T  K  M  K  P  G  D  C  S  L  D  P  N  P  A  K  P  R  F  F  L  M  S  Q  I  H  A  R  E  L  T  T  G  E  M  S  W

      610       620       630       640       650       660       670       680       690       700       710       720
CGCTGGACCGATGAGCTGACGGGGCAGTACGGCAAGGACCCGGACATCACCAAGCTCATGGACACCACCGAGATGTGGGTCGTGCCGATAGCCAACCCCGACGGCGTCGACATCGTCGCC
R  W  T  D  E  L  T  G  Q  Y  G  K  D  P  D  I  T  K  L  M  D  T  T  E  M  W  V  V  P  I  A  N  P  D  G  V  D  I  V  A

      730       740       750       760       770       780       790       800       810       820       830       840
AGCGGCGGCGACAACCCCGTCTGGCAGCGCAAGAACGCCGACGACTCCCATGGCGCGTCCTGTAGTTGGACCGGTCAGATAGGCGTCGACCTCAACCGCAACGCGGGCTCCCACTGGGGC
S  G  G  D  N  P  V  W  Q  R  K  N  A  D  D  S  H  G  A  S  C  S  W  T  G  Q  I  G  V  D  L  N  R  N  A  G  S  H  W  G

      850       860       870       880       890       900       910       920       930       940       950       960
GGCCTCGGCACCTCCACCCAGCCCTGCAACGAGGTCTACCTCGGCCCCGCCGCCGACTCCGAGCCGGAGAACTCCGCGCTGGAGGGCCTCTTCCGGCAGCTCTACCCGGCCGTCCGCACC
G  L  G  T  S  T  Q  P  C  N  E  V  Y  L  G  P  A  A  D  S  E  P  E  N  S  A  L  E  G  L  F  R  Q  L  Y  P  A  V  R  T

      970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
GGCACCGGCGACAGCGACCCCGCGCCCGCCGACGCCCGCGGCATGATGATCAGCCTCCACAGCGACGCCAGCATGGTCCTCTTCCCCTGGGAGTCCGACCACCGCGTGCACAGCGGCAAC
G  T  G  D  S  D  P  A  P  A  D  A  R  G  M  M  I  S  L  H  S  D  A  S  M  V  L  F  P  W  E  S  D  H  R  V  H  S  G  N

     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
GACGCCGCGCTGCGCGCCCTGGCCGACCGCATGGGCTCGATCACCGGCTACCAGTCCGGACAGGCGGGCGAGATCCTCTACGACGCCTCCGGCGGCACCGACGACTGGACGTACGACAAG
D  A  A  L  R  A  L  A  D  R  M  G  S  I  T  G  Y  Q  S  G  Q  A  G  E  I  L  Y  D  A  S  G  G  T  D  D  W  T  Y  D  K

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
CTCGGGCTGGCCAGCTTCACCATCGAGATCGGGGACAGCGACGGGCGCGGCTGCGACGGGTTCACCCCGGCGTACTCCTGCCAGGACAGCTACTTCTGGCCGAAGATCAAGCCCGCCCTG
L  G  L  A  S  F  T  I  E  I  G  D  S  D  G  R  G  C  D  G  F  T  P  A  Y  S  C  Q  D  S  Y  F  W  P  K  I  K  P  A  L

     1330      1340      1350      1360      1370
ATGTACGCGGCGCAGCACGCGGCGGCGCCGTACCGGTCGGCCGACTGA
M  Y  A  A  Q  H  A  A  A  P  Y  R  S  A  D  End
```

Fig. 1 Nucleic acids and amino acids sequence of TH2-CP. The accession number of TH2-CP is LCO43136. The predicted signal sequence of TH2-CP is indicated by a *dashed line*. The consensus sequence (HXXE……H) is in *bold* and the N-terminal sequence *underlined*

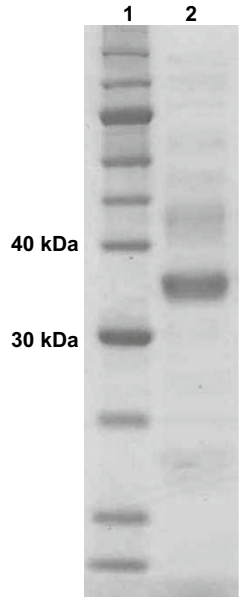

Fig. 2 SDS-PAGE of TH2-CP solution. *Lane 1* contains molecular weight protein markers. *Lane 2* contains 6 µg of TH2-CP2

phosphate buffer was much higher than that in Tris–HCl buffer (Fig. 3b and data not shown). Therefore, we used the phosphate buffer as the reaction buffer at pH 7 in the subsequent experiments. Unlike the carboxypeptidases from several genera of fungus, such as *Aspergillus* (Ichishima 1972), *Penicillium* (Yokoyama et al. 1974), and *Monascus* (Liu et al. 2004a, b), which have an optimum pH in the range from 3 to 5.2, the optimum pH of TH2-CP is close to that of the carboxypeptidase from *Actinomucor elegans* (Fu et al. 2011), which has an optimum pH of approximately 7. The optimum temperature for TH2-CP activity was 51 °C (Fig. 3c). Fifty percent of TH2-CP activity was conserved after incubation at 38 °C for 30 min (Fig. 3d).

Effects of inhibitors and divalent mental ions on TH2-CP activity

The effects of several inhibitors and divalent mental ions on TH2-CP activity are summarized in Tables 1 and 2, respectively. It was shown that, except for the chelator (1,10-phenanthroline), which decreased TH2-CP activity by 73 %, other inhibitor of serine peptidases [phenylmethylsulfonyl fluoride (Fahrney and Gold 1963)] and modifiers of the thiol group of cystine (iodoacetic acid, 2-iodoacetamide, and *N*-ethylmaleimide can be considered as inhibitors of cystine peptidases) had no or very little effect on the activity (Table 1). These results correspond to the nature of metallopeptidase of TH2-CP. Concerning the divalent metal ions, besides Ni^{2+} and Zn^{2+}, which decreased TH2-CP activity by 21 and 33 %, respectively, the other ions tested (Co^{2+}, Mg^{2+}, Mn^{2+}, and Ca^{2+}) had very little effect on the activity (Table 2). The decreased activity of TH2-CP by Zn^{2+} may be due to the coordinated effect of the hydroxide ion ($ZnOH^{+}$) on the catalytic zinc ions (Larsen and Auld 1989). This analogy may explain the result obtained for Ni^{2+}.

Substrate specificity and kinetic analysis of TH2-CP

Using dipeptides, tripeptides, and *N*-carbobenzyloxy (Z) derivatives of dipeptides and tripeptides, we investigated the substrate specificity of TH2-CP. Among the substrates tested, four dipeptides (Leu-Pro, Leu-Asp, Arg-Lys, and His-Arg) and three Z-derivatives (Z-Gly-Glu, Z-Gly-His, and Z-Pro-Leu) could not be hydrolyzed by TH2-CP (Fig. 4). However, two tripeptides (Phe-Phe-Phe, 6.8 ± 0.5 U/mg; Try–Tyr–Tyr, 3.7 ± 0.3 U/mg), nine dipeptides (Phe-Phe, 0.5 ± 0.0 U/mg; Phe-Arg, 0.3 ± 0.0 U/mg; Phe-Ser, 0.2 ± 0.0 U/mg; Phe-Ala, 0.1 ± 0.0 U/mg; Phe-Gly, 0.04 ± 0 U/mg; Leu-His, 0.3 ± 0.0 U/mg; Lys-Phe, 0.1 ± 0.0 U/mg; Lys-Leu, 0.03 ± 0.0 U/mg; Arg-Phe, 0.06 ± 0.0 U/mg) and three Z-derivatives (Z-Gly-Gly-Leu, 2.5 ± 0.2 U/mg; Z-Gly-Leu, 1.5 ± 0.1 U/mg; Z-Gly-Phe, 1.2 ± 0.0 U/mg) were hydrolyzed by TH2-CP (Fig. 4).

The activity of TH2-CP toward the substrates was in order of Phe-Phe>Phe-Arg>Phe-Ser>Phe-Ala>Phe-Gly, Leu-His>Leu-Pro = Leu-Asp = 0 U/mg, Lys-Phe>Lys-Leu, Arg-Phe>Arg-Lys and Z-Gly-Leu>Z-Gly-Phe>Z-Gly-Glu = Z-Gly-His = 0 U/mg (Fig. 4). The K_m and V_{max} values shown in Table 3 indicate that TH2-CP has a better affinity and reactivity for Z-Gly-Leu than for Z-Gly-Phe. These results indicate that TH2-CP has a preference for C-terminal residues. The comparisons of Phe-Phe with Lys-Phe, Lys-Phe with Arg-Phe, and Z-Pro-Leu with Z-Gly-Leu (Fig. 4) suggest that the specificity of TH2-CP toward the C-terminal residue is affected by the penultimate residue. The increase in catalytic efficiency of TH2-CP by lengthening the peptide substrates was observed in the comparisons of Phe-Phe-Phe with Phe-Phe and Z-Gly-Leu with Z-Gly-Gly-Leu (Fig. 4). This indicates that the specificity of TH2-CP toward the C-terminal residue is also affected by length of the peptide substrate. Although the K_m values showed that TH2-CP had a better affinity for Z-Gly-Leu than for Z-Gly-Gly-Leu, the V_{max} values showed that Z-Gly-Gly-Leu was hydrolyzed faster than Z-Gly-Leu. The result of catalytic efficiency of TH2-CP toward Z-Gly-Leu and Z-Gly-Gly-Leu may be due to the combined effect of affinity and reactivity toward the substrates. The activity of carboxypeptidase from *Actinomucor elegans* toward Z-Gly-Gly-Leu and Z-Gly-Phe was about 0.5 µmol/h/ml at pH 7 and 37 °C (Fu et al. 2011). In contrast, the

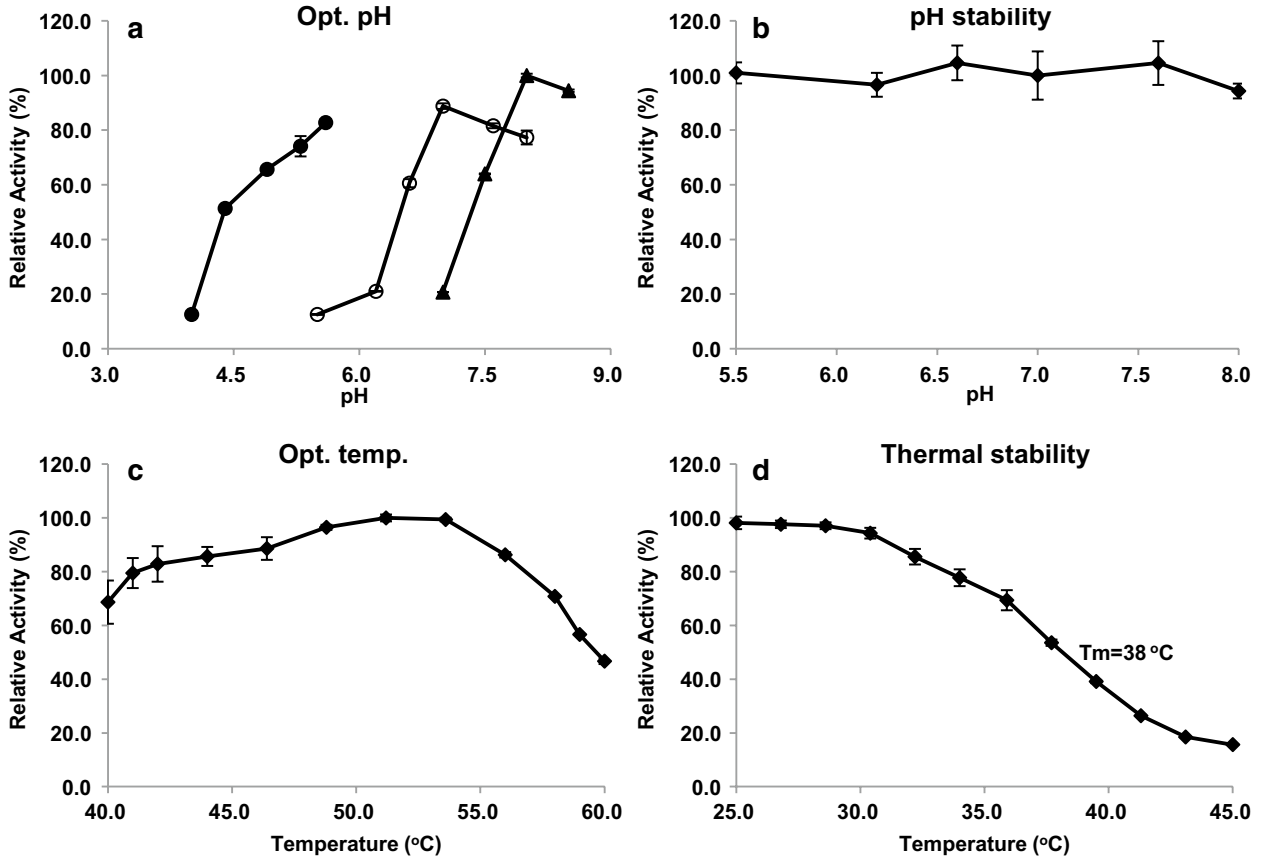

Fig. 3 Optimum pH, pH stability, optimum temperature, and thermostability of TH2-CP. **a** Optimum pH of TH2-CP against Z-Gly-Leu at 40 °C. The activities are presented as relative activity at the pH 8.0 (Tris–HCl buffer). Acetate buffer (*filled circle*), potassium phosphate buffer (*empty circle*), Tris–HCl buffer (*filled triangle*). **b** pH stability of TH2-CP against Z-Gly-Leu at 40 °C. The activities are presented as relative activity at pH 7.0 (potassium phosphate buffer). **c** Optimum temperature of TH2-CP against Z-Gly-Leu and potassium phosphate buffer (pH 7.0). The activities are presented as relative activity at 51 °C. **d** Thermostability of TH2-CP against Z-Gly-Leu and potassium phosphate buffer (pH 7.0). The activities are presented as relative activity at 5 °C. All the data are expressed as the mean ± SD of three independent experiments

Table 1 Effects of various inhibitors on TH2-CP activity

Inhibitor final 1 mM	Residual activity (%)
None	100
Phenylmethylsulfonyl fluoride	100
Iodoacetic acid	98
2-Iodoacetamide	95
N-ethylmaleimide	100
1,10-Phenanthroline	27

The activities presented are as relative to the activity in distilled water (none). Values are the mean of three independent experiments

activities of TH2-CP toward them were 60.3 ± 4.8 and 30.0 ± 1.1 μmol/h/ml at pH 7 and 40 °C, respectively (data not shown). This result indicates that TH2-CP has a much higher activity than carboxypeptidase from *Actinomucor elegans*.

Profiles of casein hydrolysate hydrolyzed by TH2-CP

To evaluate the TH2-CP activity against natural substrates, we analyzed the activity of TH2-CP in combination with Bioprase SP against casein. Figure 5 shows a comparison of free amino acid contents between treatment of casein with and without TH2-CP in combination with Bioprase SP. Except for Cys and Arg, which cannot be detected using the EZ:faast™ kit, the contents of the other 18 standard amino acids increased significantly in the presence of TH2-CP (Fig. 5a).

The comparison of the substrate specificities against the synthetic peptides tested showed that TH2-CP could hydrolyze the C-terminal residues (Pro, Asp, Glu, and Lys) of peptides from casein hydrolysate hydrolyzed by Bioprase SP. This result is most likely due to the different lengths and penultimate residues between the synthetic peptides and the casein hydrolysate hydrolyzed by Bioprase SP. Figure 5b shown that the rates of hydrolysis

Table 2 Effects of divalent metal ions on TH2-CP activity

Divalent metal ion final 1 mM	Residual activity (%)
None	100
Co^{2+}	97
Ni^{2+}	80
Zn^{2+}	68
Mg^{2+}	99
Mn^{2+}	99
Ca^{2+}	99

The activities presented are as relative to activity in distilled water (none). Values are the mean of three independent experiments

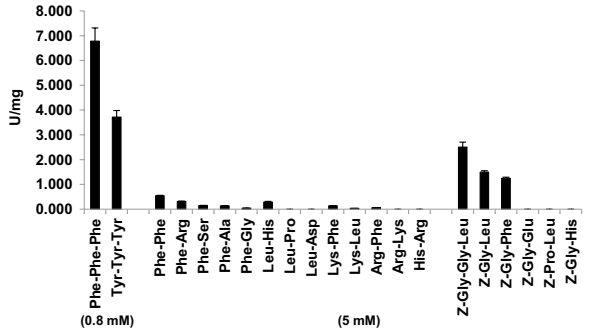

Fig. 4 Substrate specificities of TH2-CP toward synthetic peptides. The activities are measured using sodium phosphate buffer (pH 7.0) at 40 °C. All the data are expressed as the mean ± SD of three independent experiments

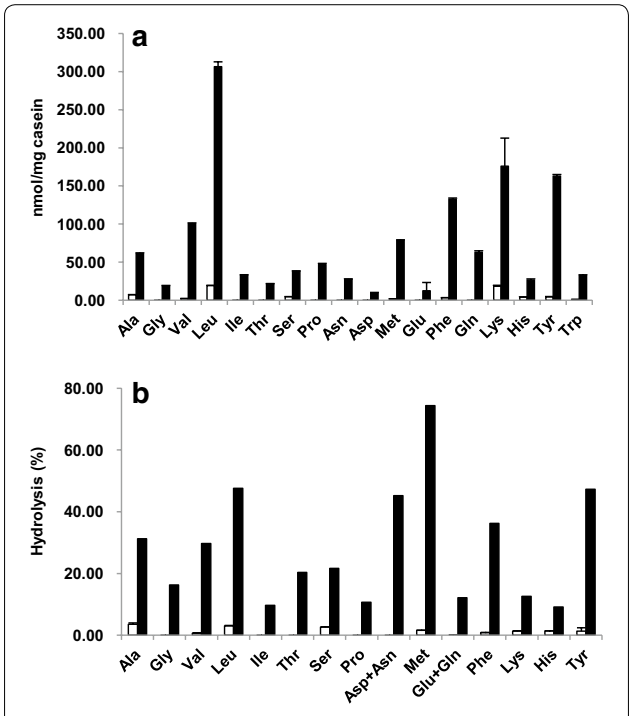

Fig. 5 Profiles of casein hydrolysate by TH2-CP. **a** Productions of amino acid by treating casein with (*black columns*) and without (*white columns*) TH2-CP2 after incubation with Bioprase SP. **b** The degree of hydrolysis by TH2-CP is presented as the relative productions of amino acid by treating casein with 6 M HCl at 110 °C for 15 h. All the data are expressed as the mean ± SD (**a**) or mean (**b**) of three independent experiments

Table 3 Kinetic parameters for hydrolysis of *Z* derivatives by TH2-CP

	K_m mM	V_{max} mmol min^{-1} mg^{-1}	V_{max}/K_m
Z-Gly-Phe	1.49 ± 0.13	1.19 ± 0.06	0.8
Z-Gly-Leu	1.10 ± 0.14	1.40 ± 0.06	1.28
Z-Gly-Gly-Leu	4.97 ± 0.34	4.82 ± 0.39	0.97

K_m and V_{max} values were calculated from the Hanes–Woolf plots obtained using the Michaelis–Menten equation. Values are the mean ± SD of three independent experiments

of Met, Leu, Tyr, Phe, Val, and Ile were 74, 48, 47, 36, 30, and 10 %, respectively. These six amino acids are all hydrophobic amino acids. Protein hydrolysates obtained using proteolytic enzymes are frequently accompanied by bitter compounds formed during hydrolysis (Bumberger and Belitz 1993; Clegg et al. 1974; Matoba et al. 1970). Bitter compounds in dairy products have been well studied in cheese manufacturing. Many bitter peptides in cheese are mainly produced by the proteolytic digestion of casein. The bitterness is generally due to peptides with high contents of hydrophobic amino acids (Matoba and Hata 1972). Additionally, the higher the contents of hydrophobic amino acids at the C-terminus, the higher the level of bitterness (Ishibashi et al. 1987a, b; Kanehisa et al. 1984; Otagiri et al. 1984; Shinoda et al. 1986). The tripeptides Phe-Phe-Phe and Tyr-Tyr-Tyr are bitter, and both of them are fivefold bitterer than caffeine (Ishibashi et al. 1987b). In contrast, their corresponding dipeptides Phe-Phe and Tyr-Tyr have 83 and 43 % the bitterness of caffeine, respectively (Ishibashi et al. 1987b). Among the synthetic peptides tested, Phe-Phe-Phe and Tyr-Tyr-Tyr were the optimum two substrates of TH2-CP (Fig. 4). This finding indicates that TH2-CP can decrease the bitterness of peptides. It was confirmed that carboxypeptidases from wheat, pancreatin, and *Actinomucor elegans* (Fu et al. 2011; Ge and Zhang 1996; Komai et al. 2007; Umetsu et al. 1983),which can effectively hydrolyze hydrophobic amino acids at the C-terminus, can decrease the bitterness of peptides, including the peptides produced by proteolytic digestion of casein. Thus, we consider that the metallocarboxypeptidase TH2-CP from *S. cinnamoneus* TH-2 could also be used to decrease the bitterness of peptides in food industry, since it has a good

hydrolytic activity against the C-terminal hydrophobic amino acids of peptides.

Conclusions

A metallocarboxypeptidase from *S. cinnamoneus* TH-2 (TH2-CP) has the pH optima of 7.0 and 8.0 in potassium phosphate buffer and Tris–HCl buffer, respectively. However, its stability in potassium phosphate buffer was much higher than that in Tris–HCl buffer. Moreover, the optimum temperature for activity was 51 °C, and 50 % of activity was conserved after incubation at 38 °C for 30 min. Using Z-Gly-Gly-Leu as a substrate, the K_m and V_{max} values were 4.97 ± 0.34 mM and 4.82 ± 0.39 μmol $min^{-1}mg^{-1}$, respectively. TH2-CP can hydrolyze 18 amino acids from the C-terminal residues of casein hydrolysate using the endoprotease from *Bacillus* sp. TH2-CP prefers hydrophobic amino acids at the C-terminal position of casein peptides as substrates. This property indicates that TH2-CP could be used for decreasing the bitterness of peptides in food industries.

Authors' contributions

TH designed the experiments and modified the manuscript. KW carried out all experiment except the determination of the N-terminal amino acid sequence for TH2-CP, which was carried out by JA. KW drafted the manuscript. MU gave advice to KW regarding his experiment and also modified the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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References

Arima J, Iwabuchi M, Hatanaka T (2004) Gene cloning and overproduction of an aminopeptidase from *Streptomyces septatus* TH-2, and comparison with a calcium-activated enzyme from *Streptomyces griseus*. Biochem Biophys Res Commun 317:531–538

Arima J, Uesugi Y, Iwabuchi M, Hatanaka T (2006) Study on peptide hydrolysis by aminopeptidases from *Streptomyces griseus*, *Streptomyces septatus* and *Aeromonas proteolytica*. Appl Microbiol Biotechnol 70:541–547

Arolas JL, Vendrell J, Aviles FX, Fricker LD (2007) Metallocarboxypeptidases: emerging drug targets in biomedicine. Curr Pharm Des 13:349–366

Bumberger E, Belitz HD (1993) Bitter taste of enzymic hydrolysates of casein. Z Lebensm Unters Forsch 197:14–19

Clegg KM, Lim CL, Manson W (1974) The structure of a bitter peptide derived from casein by digestion with papain. J Dairy Res 41:283–287

Fahrney DE, Gold AM (1963) Sulfonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, α-chymotrypsin, and trypsin. J Am Chem Soc 85:997–1000

Fu J, Li L, Yang XQ (2011) Specificity of carboxypeptidases from *Actinomucor elegans* and their debittering effect on soybean protein hydrolysates. Appl Biochem Biotechnol 165:1201–1210

Ge SJ, Zhang LX (1996) The immobilized porcine pancreatic exopeptidases and its application in casein hydrolysates debittering. Appl Biochem Biotechnol 59:159–165

Hatanaka T, Negishi T, Kubota-Akizawa M, Hagishita T (2002) Purification, characterization, cloning and sequencing of phospholipase D from *Streptomyces septatus* TH-2. Enzyme Microb Technol 31:233–241

Hatanaka T, Arima J, Uesugi Y, Iwabuchi M (2005) Purification, characterization, cloning and sequencing of metalloendopeptidase from *Streptomyces septatus* TH-2. Arch Biochem Biophys 434:289–298

Hatanaka T, Onaka H, Arima J, Uraji M, Uesugi Y, Usuki H, Nishimoto Y, Iwabuchi M (2008) pTONA5: a hyperexpression vector in Streptomycetes. Protein Expr Purif 62:244–248

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward H, Schrempf CJ (1985) A laboratory manual. The John Ines Foundation, Norwich, pp 70–84

Ichishima E (1972) Purification and characterization of a new type of acid carboxypeptidase from *Aspergillus*. Biochim Biophys Acta 258:274–288

Ishibashi N, Arita Y, Kanehisa H, Kouge K, Okai H, Fukui S (1987a) Bitterness of leucine-containing peptides. Agric Biol Chem 51:2389–2394

Ishibashi N, Sadamori K, Yamamoto O, Kanehisa H, Kouge K, Kikuchi E, Okai H, Fukui S (1987b) Bitterness of phenylalanine- and tyrosine-containing peptides. Agric Biol Chem 51:3309–3313

Kanehisa H, Miyake I, Okai H, Aoyagi H, Izumiya N (1984) Studies of bitter peptides from casein hydrolyzate. X.[1)] synthesis and bitter taste of H-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val-OH Corresponding to C-terminal Portion of *β*-casein[2)]. Bull Chem Soc Jpn 57:819–822

Komai T, Kawabata C, Tojo H, Gocho S, Ichishima E (2007) Purification of serine carboxypeptidase from the hepatopancreas of Japanese common squid *Todarodes pacificus* and its application for elimination of bitterness from bitter peptides. Fisheries Sci 73:404–411

Larsen KS, Auld DS (1989) Carboxypeptidase A: mechanism of zinc inhibition. Biochemistry 28:9620–9625

Lee SH, Taguchi H, Yoshimura E, Minagawa E, Kaminogawa S, Ohta T, Matsuzawa H (1996) The active site of carboxypeptidase Taq possesses the active site motif His-Glu-X-X-His of zinc-dependent endopeptidases and aminopeptidases. Protein Eng 9:467–469

Liu F, Tachibana S, Taira T, Ishihara M, Kato F, Yasuda M (2004a) Purification and characterization of a high molecular mass serine carboxypeptidase from *Monascus pilosus*. J Ind Microbiol Biotechnol 31:572–580

Liu F, Tachibana S, Taira T, Ishihara M, Yasuda M (2004b) Purification and characterization of a new type of serine carboxypeptidase from *Monascus pilosus*. J Ind Microbiol Biotechnol 31:23–28

Matoba T, Hata T (1972) Relationship between bitterness of peptides and their chemical structures. Agric Biol Chem 37:1423–1431

Matoba T, Hayashi R, Hata I (1970) Isolation of bitter peptides from trypsin hydrolysate of casein and their chemical structure. Agric Biol Chem 34:1235–1243

Otagiri K, Shigenaga T, Kanehisa H, Okai H (1984) Studies of bitter peptides from casein hydrolysate. IV.[1)] relationship between bitterness and hydrophobic amino acids moiety in the C-terminal of BPIa (Arg-Gly-Pro-Pro-Phe-Ile-Val)[2)]. Bull Chem Soc Japan 57:90–96

Shinoda I, Nosho Y, Otagiri K, Okai H, Fukui S (1986) Bitterness of diastereomers of a hexapeptide (Arg-Arg-Pro-Pro-Phe-Phe) containing D-phenylalanine in place of L-phenylalanine. Agric Biol Chem 50:1785–1790

Umetsu H, Matsuoka H, Ichishima E (1983) Debittering mechanism of bitter peptides from milk casein by wheat carboxypeptidase. J Agric Food Chem 31:50–53

Vendrell J, Querol E, Avilés FX (2000) Metallocarboxypeptidases and their protein inhibitors structure, function and biomedical properties. Biochim Biophys Acta 1477:284–298

Yokoyama S, Oobayashi A, Tanabe O, Sugawara S, Araki E (1974) Production and some properties of a new type of acid carboxypeptidase of *Penicillium molds*. Appl Microbiol 27:953–960