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Purification and characterization of CEP from *Lactococcus lactis* ssp. *lactis*

Yuxing Guo [a], Daodong Pan [a,*], Xiaoqun Zeng [a], Masaru Tanokura [b]

[a] Food Science & Nutrition Department of Nanjing Normal University, Nanjing 210097, PR China
[b] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Science, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

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ABSTRACT

The cell-envelope proteinase (CEP) of *Lactococcus lactis* ssp. *lactis* LB12 was released from cells by treatment with lysozyme, purified by ammonium sulfate precipitation and chromatographed on DEAE-Sephadex A-25 and Sephacryl S-300 HR. The purified CEP is a monomer structure and has molecular mass of about 53 kDa. Optimal activity occurred at pH 7.5 and 40 °C. It is a metallopeptidase, activated by Mn^{2+}, Mg^{2+}, Ca^{2+}, inhibited by Co^{2+}, Zn^{2+}, Ni^{2+} and EDTA, and a serine proteinase which is inhibited by PMSF. The sequence of the first 13 amino acids of the N-terminal of the CEP was determined to be Asp-Val-Phe-Ala-Pro-His-Met-Ala-Asn-Val-Ala-Ala-Val, and the whey protein hydrolysate produced by the CEP displayed ACE-inhibitory activity.

1. Introduction

Milk proteins are a rich source of biologically active peptides. At present, numerous peptides exhibiting various activities, such as opiate, antithrombotic, and antihypertensive activity have been reported (Pihlanto-Leppala, Rokka, & Korhonen, 1998). Angiotensin-I converting enzyme (ACE) raises blood pressure by converting the inactive decapeptide angiotensin-I to the potent vasoconstrictor octapeptide angiotensin-II, as well as inactivating the vasodilating nonapeptide. Inhibition of ACE may exert an antihypertensive effect as a consequence of the decrease in angiotensin-II and the concomitant increase in bradykinin (Leclerc, Gauthier, Bachelardb, Santure, & Roy, 2002; Mullally, Meisel, & FitzGerald, 1997; Pihlanto-Leppala, 2001; Pihlanto-Leppala et al., 1998; Van der Ven, Gruppen, De Bont, & Voragen, 2002).

ACE-inhibitory peptides can be produced by the fermentation of milk with lactic acid bacteria (LAB). LAB are nutritionally fastidious bacteria and require large concentrations of free amino acids and peptides, and have proteolytic systems to make use of milk protein as nitrogen sources. The proteolytic systems are composed of a cell-envelope proteinase (CEP) and more than ten intracellular peptidases exhibiting different specificities (Courtina, Nardia, Wegmannc, et al., 2002; Exterkate Fred, 1995; Haandrikman et al., 1991; Law & Haandrikman 1997; Thomas & Pritchard, 1987).

The CEP is a key enzyme in the proteolytic system, as it catalyzes the initial steps in the degradation of casein that are essential for the rapid growth of LAB in milk. In recent years CEP from several different strains has been purified and characterized, these include *Streptococcus thermophils* CNRZ 385 (153 kDa) (Fernandez-Espla, Garault, Monnet, & Rul, 2000), *Lactobacillus delbrueckii* ssp. *lactis* ACA-DC178 (Tsakalidou, Anastasiou, Vandenberghe, Van Beeumen, & Kalantzopoulos, 1999) and *Lactobacillus* casei ssp. *casei* IFPL731 (150 kDa) (Fernandez de Palencia, Pelaez, & Martin-Hernandez, 1997). The isolation of CEP from these LAB is achieved by repeated treatment of the cells with a calcium-free buffer, which results in an autoproteolytic release of an active proteinase from the cells, viz. a proteinase truncated at the C-terminal end. It has been demonstrated that Ca^{2+} protects the cell-envelope enzyme from detachment from the cells. Release of the proteinase has been proposed to occur following the removal of bound calcium, as result of which local molecular unfolding exposes a sequence in the middle domain of the proteinase which is highly susceptible to autoproteolytic attack. However, the removal of relatively weakly bound calcium in the cell-bound CEP initiates a local conformational rearrangement in the proteinase domain which leads to a less active enzyme. The rearrangement apparently distorts the substrate recognition site containing the catalytic triad to the extent that substrate binding and the catalytic process itself is affected (Exterkate Fred, 1995).

The purpose of this study was to purify and characterize the CEP from *L. lactis* ssp. *lactic* LB12. The cells of the strain were treated with lysozyme which partially digested the cell wall leading to the relaxation of the enzyme–cell wall interaction. The ACE-inhibitory activity of whey protein hydrolysate produced by the purified CEP was also evaluated.

* Corresponding author. Tel.: +86 25 83598286; fax: +86 25 83707623.
E-mail address: daodongpan@163.com (D. Pan).

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2. Materials and methods

2.1. Materials

Lactococcus lactis ssp. *lactis* LB12 isolated from Chinese pickled cabbage was conserved by the Dairy Biotechnology Institute of Nanjing Normal University. DEAE-Sephadex A-25, Sephacryl S-300 high resolution and the high molecular weight (HMW) electrophoresis calibration kit were purchased from Amersham Biosciences (Uppsala, Sweden). MeOsuc-Arg-Pro-Tyr-pNA (MS-Arg) was synthesized by MP Biomedicals (Fountain Pkwy, Solon, USA). Hippuryl-l-histidyl-l-leucine (HHL), angiotensin-I converting enzyme (ACE, obtained from rabbit lung), phenylmethylsulphonyl fluoride (PMSF) and 4-(2-hydroxyerhyl) piperazine-1-erhanesulfonic acid (HEPES) were obtained from Sigma Chemicals Co. Ltd. (St. Louis, MO., USA).

2.2. Preparation of cell-free extract

Lactococcus lactis ssp. *lactis* LB12 was grown in MRS media at 37 °C for 20 h, with shaking at 110 rpm. The cells were harvested by centrifugation at 4500 rpm for 20 min at 4 °C and washed three times with 50 mM Tris–HCl buffer (pH 7.8) containing 30 mM $CaCl_2$. The washed cells were suspended in solution (50 mM Tris–HCl, 100 mM NaCl, 0.5% Triton X-100, 1 mg/ml lysozyme, pH 8.5) and incubated for 3 h at 37 °C. The cell debris was removed by centrifugation at 10,000 rpm for 20 min at 4 °C. The clear supernatant was lyophilized and used as the cell-free extract.

2.3. Measurement of proteinase activity

The mixture, which contained 0.05 ml of 6.4 mM MeOsuc-Arg-Pro-Tyr-pNA (MS-Arg) dissolved in methanol, 2.85 ml of 50 mM Tris–HCl buffer (pH 7.8) and 0.1 ml of enzyme solution, was incubated at 37 °C for 60 min. The reaction was stopped by the addition of 0.5 ml of 30% (v/v) acetic acid. The absorbance of the liberated *p*-nitroaniline was measured at 410 nm (the molar absorption coefficient (ξ_{410} nm) of *p*-nitroaniline was 8800 $M^{-1}cm^{-1}$ during the release of enzyme thus enhancing the conditions of the assay). One unit of enzyme activity (U) was defined as the amount of enzyme enhancing the release of 1 μM of *p*-nitroaniline per min at 37 °C.

2.4. Protein quantification

Protein concentrations were estimated by the Bradford, 1976 using the Coomassie Protein assay reagent with bovine serum albumin as the standard.

2.5. Purification of the enzyme

2.5.1. Ammonium sulfate precipitation

The cell-free extracts were fractionated by salting out with solid ammonium sulfate at 45% (w/v) saturation. The precipitate formed was collected by centrifugation at 10,000 rpm for 20 min at 4 °C, dissolved in 50 mM Tris–HCl buffer (pH 7.8), and then dialyzed overnight against the same buffer at 4°C.

2.5.2. DEAE-Sephadex A-25 chromatograph

Four milliliters of crude enzyme preparation were applied to a column (2.60 × 40 cm) of DEAE-Sephadex A-25 previously equilibrated with 50 mM Tris–HCl buffer (pH 7.8). The enzyme was eluted at a flow rate of 25 ml/h with a linear NaCl gradient (250 ml buffer – 250 ml 1 M NaCl buffer). The fractions containing the enzyme were pooled, dialyzed against 50 mM Tris–HCl buffer (pH 7.8) at 4 °C, and concentrated using polyethylene glycol 20,000. The protein concentration and CEP activity were thus analyzed.

2.5.3. Sephacryl S-300 HR chromatograph

The concentrated enzyme solution from the previous step was applied to a Sephacryl S-300 column (1.0 × 40 cm) equilibrated with 50 mM Tris–HCl (pH 7.8). Proteins were eluted with the same solution at a flow rate of 25 ml/h. The fractions containing the enzyme were pooled and concentrated using polyethylene glycol 20,000. The protein concentration and CEP activity were thus analyzed.

2.6. Effects of pH on the enzyme activity

The effects of pH from 5.0 to 9.5 on the enzyme activity were measured in 50 mM Tris–HCl buffer at 37 °C with MeOsuc-Arg-Pro-Tyr-pNA as the substrate. To assess the effect of pH on enzyme stability, the enzyme was dissolved in 50 mM Tris–HCl buffers within the pH range 5.5–9.5 and incubated for 30 min at 37 °C. The residual activity was subsequently measured at 37 °C with MeOsuc-Arg-Pro-Tyr-pNA as substrate.

2.7. Effects of temperature on the enzyme activity and stability

The effects of temperature, from 24 °C to 52 °C, on the enzyme activity were measured in 50 mM Tris–HCl buffer (pH 7.8) with MeOsuc-Arg-Pro-Tyr-pNA as the substrate. The purified enzyme solutions were incubated for 30 min at temperatures ranging from 30 °C to 90 °C to assess the thermal stability of the enzyme. The residual activity was subsequently measured at 37 °C with MeOsuc-Arg-Pro-Tyr-pNA as the substrate.

2.8. Effects of metal ions and inhibitors on enzyme activity

The enzyme was pre-incubated in the presence of various metal ions (Ca^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Ba^{2+}), PMSF and EDTA for 30 min at 37 °C at a final concentration of 1.0 mM and 10.0 mM in 50 mM Tris–HCl buffer (pH 7.8), respectively. The enzyme activity was measured after incubation for 30 min at 37 °C with MeOsuc-Arg-Pro-Tyr-pNA as the substrate.

2.9. Determination of purity and molecular mass

The homogeneities of the active fraction after each purification step were examined by native PAGE with a 4% acrylamide stacking gel and a 10% acrylamide running gel. Proteins in the gels were stained with Coomassie Blue R 250. The molecular mass of the enzyme was determined under denaturing conditions by SDS–PAGE with a 4% acrylamide stacking gel and a 10% acrylamide running gel.

2.10. N-Terminal amino acid sequencing

The purified CEP was electroblotted from SDS–PAGE gel onto polyvinylidene difluoride (PVDF) membranes. The electroblotted proteinase band was cut from the blot and sequenced by using Procise cLC 492 protein sequencing system. Amino acid sequence homology with other CEPs was obtained by using BLAST procedure.

2.11. ACE-inhibitory activity of whey protein hydrolysate

The mixture containing 1 ml whey protein solution (8.0%, w/w) which was adjusted to pH 8.0, and 0.2 ml purified CEP solution was incubated at 37 °C for 3 h. The resulting mixture was then heated at 90 °C for 5 min and the pH was adjusted to 3.8 with 50% lactic

acid solution. After centrifugation at 7000 rpm for 10 min, the supernatant was collected and the pH readjusted to 7.0. The ACE-inhibitory activity of the supernatant was then measured according to the method of Cushman and Cheung (1971) with some modification.

Aliquots (200 μl) of the buffered substrate solution (6.7 mM HHL in 50 mM HEPES with 300 mM NaCl, pH 8.3) were mixed with 100 μl enzyme and pre-incubated at 37 °C for 5 min. ACE (30 μl, 0.33 U ml^{-1}) was added to start the reaction. After 20 min of incubation at 37 °C, adding 3 ml of 1 N HCl. Hippuric acid released by the action of ACE was extracted with 2.4 ml of ethyl acetate of which 1 ml was evaporated. The sediment was dissolved in 2.5 ml of deionized water and the absorbance was measured at 228 nm. The inhibition activity was calculated using the following equation:

$$\text{Inhibitory activity } (\%) = 100\% \times [(A - B) - (C - D)]/(A - B)$$

where A is the absorbance of a solution containing ACE, but no sample, B is the absorbance of a solution containing ACE that had previously been inactivated by the addition of HCl and no sample, C is the absorbance in the presence of ACE and the sample, and D is the absorbance of a solution containing ACE that had previously been inactivated by the addition of HCl and the sample. For each fraction, inhibitory activity was measured at five concentrations for which triplicate analyses were carried out. Reciprocals of the inhibition activity data were plotted against the reciprocals of the protein concentration in the sample, and linear regression was used to determine the IC_{50} value defined as the concentration of protein needed to inhibit 50% of ACE activity.

3. Results

3.1. Purification of CEP

The DEAE-Sephadex A-25 chromatograph led to the separation of four fractions and the fourth fraction (peak 4) showed the highest enzyme activity (Fig. 1a). The Sephacryl S-300 HR chromatograph yielded four peaks and the first peak (peak 1) showed the highest enzyme activity (Fig. 1b). The enzyme obtained from the final Sephacryl S-300 HR chromatograph step showed a single protein band by native PAGE (Fig. 2a).

The specific enzyme activity observed with MeOsuc-Arg-Pro-Tyr-pNA as the substrate after each purification step is summarized in Table 1. The enzyme was purified about 74-fold from the cell-free extract by ammonium sulfate fractionation and two steps of column chromatography and the recovered activity was about 14.87%.

3.2. Effects of pH and temperature on enzyme activity

The enzyme showed high activity in the pH range 7.5–8.5, with optimum activity at pH 7.5 (Fig. 3a). Less than 10% of the maximum activity was measured at pH 5.0 or 9.5, but more than 50% of the maximum activity was observed at pH 9.0. The enzyme was stable when incubated for 30 min over the pH range 6.5–8.5 (Fig. 3b).

The enzyme showed high activity at temperatures ranging from 36 °C to 48 °C, with maximum activity at 40 °C (Fig. 4a), and retained 95.3, 35.8 and 11.7% of its activity when it was pre-incubated for 30 min at 50, 60 and 70 °C, respectively (Fig. 4b). These data indicated that the enzyme was more stable when heated with the substrate.

3.3. Effects of divalent metal ions and inhibitors on enzyme activity

The effects of various compounds on the enzyme activity are summarized in Table 2. The enzyme was activated by Mn^{2+} and Mg^{2+} and strongly activated by Ca^{2+} to 186% at 10 mM; it was also activated by Co^{2+} and Zn^{2+} at 1 mM but inhibited at 10 mM. Ni^{2+}, EDTA and PMSF caused significant inhibition of enzyme activity. The enzyme was inhibited by Ba^{2+} at a concentration of 1 mM but activated at 10 mM.

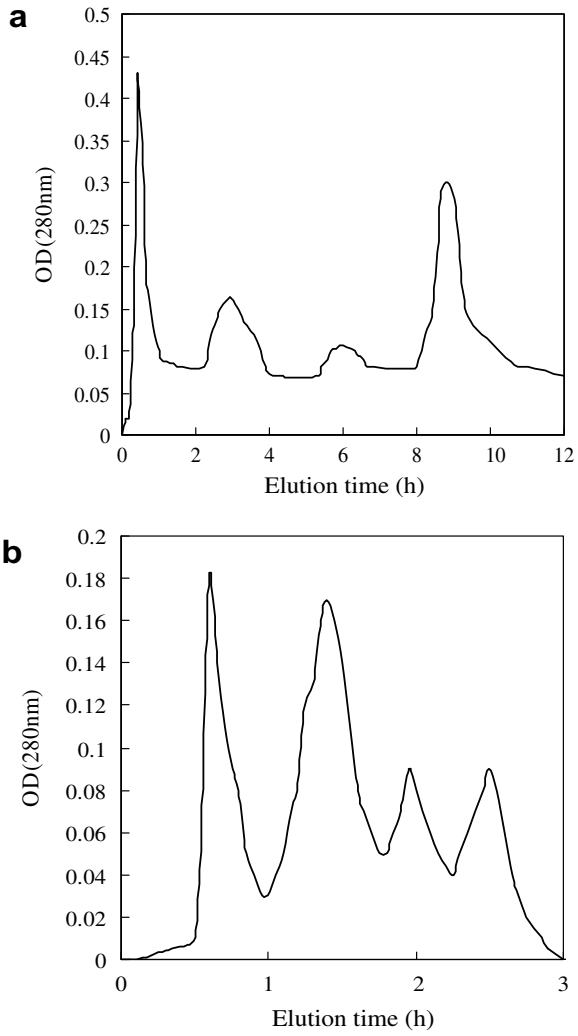

Fig. 1. DEAE-Sephadex A-25 chromatography of the crude enzyme obtained by ammonium sulfate precipitation (a) and Sephacryl S-300 chromatography of the fourth fraction obtained from the DEAE-Sephadex A-25 chromatography (b).

3.4. Molecular mass determination

Electrophoresis using SDS–PAGE showed one band of the enzyme with a molecular mass of ~53 kDa (Fig. 2b). Myosin (22,000), α_2-macroglobulin (1,70,000), β-galactosidase (1,16,000), transferrin (76,000), glutamic dehydrogenase (53,000) were used as molecular size markers.

3.5. N-Terminal amino acid sequencing

The sequence of the 13 amino acids at the N-terminal of the enzyme was determined as Asp-Val-Phe-Ala-Pro-His-Met-Ala-Asn-Val-Ala-Ala-Val.

3.6. ACE-inhibitory activity of the whey protein hydrolysate

Whey protein was hydrolyzed by the purified CEP, and the hydrolysate displayed 45% ACE-inhibitory activity.

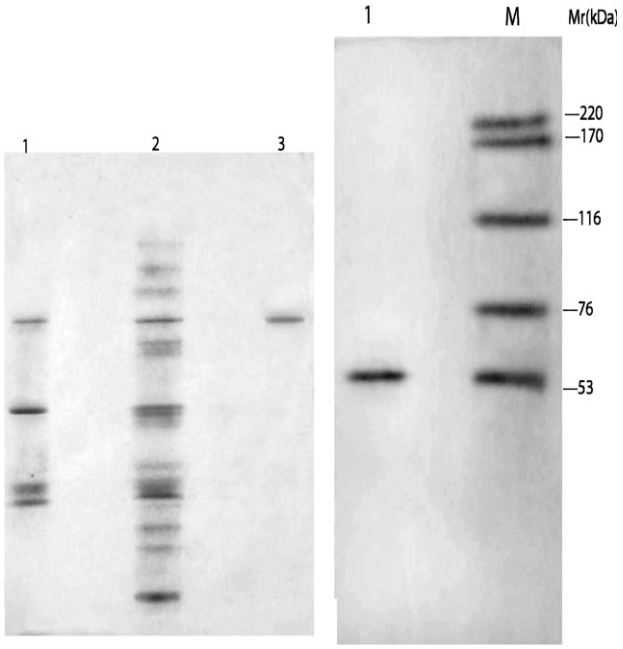

Fig. 2. Native PAGE (a) and SDS–PAGE (b) of fractions during the purification of CEP. (a) Lane 1, after DEAE-Sephadex A-25; Lane 2, concentrated cell-free extract; Lane 3, after Sephacryl S-300 HR. (b) Lane 1, fraction obtained chromatography on Sephacryl S-300 HR; Lane M, molecular weight standards.

Table 1
Purification of CEP

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell-free extract	247.60	61.15	0.25	100.00	1.00
Ammonium sulfate precipitation	8.04	25.89	3.22	42.34	13.04
DEAE-Sephadex A-25	1.67	17.34	10.34	28.36	41.86
Sephacryl S-300	0.49	9.09	18.29	14.87	74.05

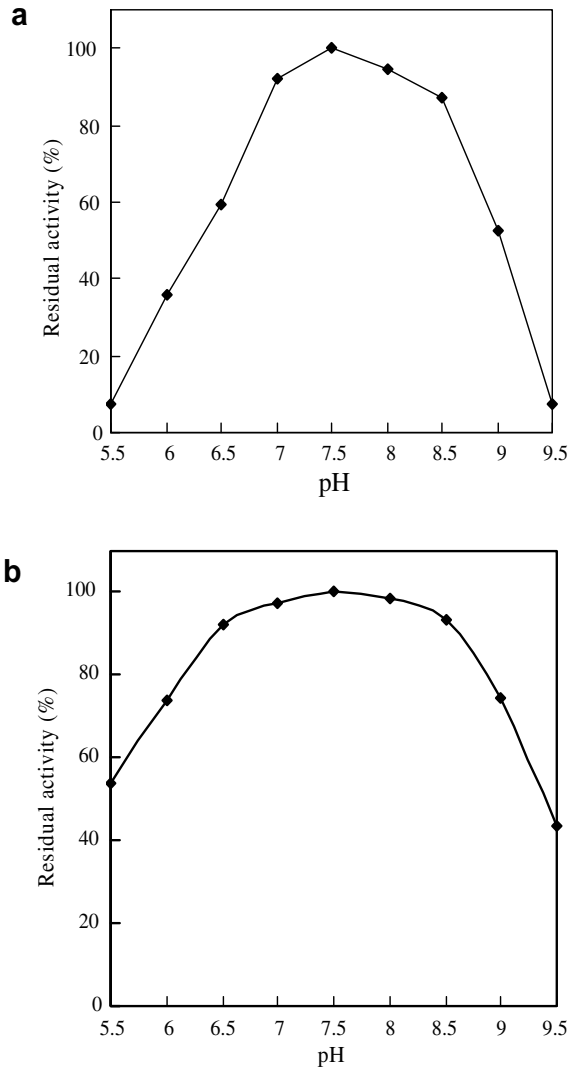

Fig. 3. Effect of pH on activity of the CEP (a) and residual activity of CEP after 30 min incubation at pH ranging from 5.5 to 9.5 (b).

4. Discussion

In this study, CEP from the cell-free extract was purified to homogeneity about 74-fold compared with the cell-free extract and a recovered activity of 14.87% was obtained, and the enzyme could be released from the cell wall by digestion with lysozyme. The fact that high levels of enzyme activity were released by lysozyme treatment suggested that the release of the enzyme from the cell wall may be a result of changes in the interaction between the enzyme and those cell wall components in close association with it.

The purified enzyme was identified as a single band by both SDS–PAGE and native PAGE, which indicated that the enzyme probably consists of a single subunit. The apparent molecular mass was ~53 kDa by SDS–PAGE, and was apparently different from the CEP obtained from other LAB. Laan and Konings (1991) used monoclonal antibodies to investigate the proteolytic degradation of the proteinase from *Lactococcus. lactis* ssp. *cremoris* Wg2, and reported the presence of a 165 kDa enzyme, which had been released into the calcium-free buffer. Coolbear, Reid, and Pritchard (1992) treated cells with lysozyme in the presence of calcium ions to release proteinase from *L. lactis* ssp. *cremoris* H2, and found that a 180 kDa enzyme was released. Nissen-Meyer and Sletten (1991) described the dimeric nature of a free form of the proteinase released from the surface of *L. lactis* ssp. *cremoris* NCDO1201 which had a subunit size of 150 kDa. This enzyme also differed from the CEPs which were extracted from *Lactobacillus helveticus* CP790 (45 kDa) (Yamamoto, Akino, & Takano, 1993), *L. helveticus* L89 (180 kDa) (Martin-Hernandez, Alting, & Exterkate, 1994), *S. thermophils* CNRZ 385 (153 kDa) (Fernandez-Espla et al., 2000) and *Lactobacillus* casei ssp. *casei* IFPL731 (150 kDa) (Fernandez de Palencia et al., 1997) and released into calcium-free buffer.

Biochemical, immunological and genetic studies on the lactococcal proteinase have been carried out in last ten years (Exterkate Fred, 1995). CEP is synthesized as a pre-protein of approximately 200 kDa in size, which is transported across the membrane to reach the cell wall as the inactive pro-enzyme. The pro-enzyme is then activated by the removal of the pro-sequence. This process is mediated by the product of a closely linked gene, the so-called maturation protein PrtM, which is present as a membrane-bound lipoprotein. The mature, active proteinase (over 1700 residues) contains an N-terminal sequence of about 500 residues. In particular, the segments which constitute the substrate binding site and harbor the catalytic triad showed the highest sequence similarity. The C-terminal sequence consists of a hydrophobic, membrane-spanning sequence with a charged tail (3 kDa) at the extreme C-terminus which may anchor the molecule in the membrane. Adjacent to this membrane anchor a segment of about 140

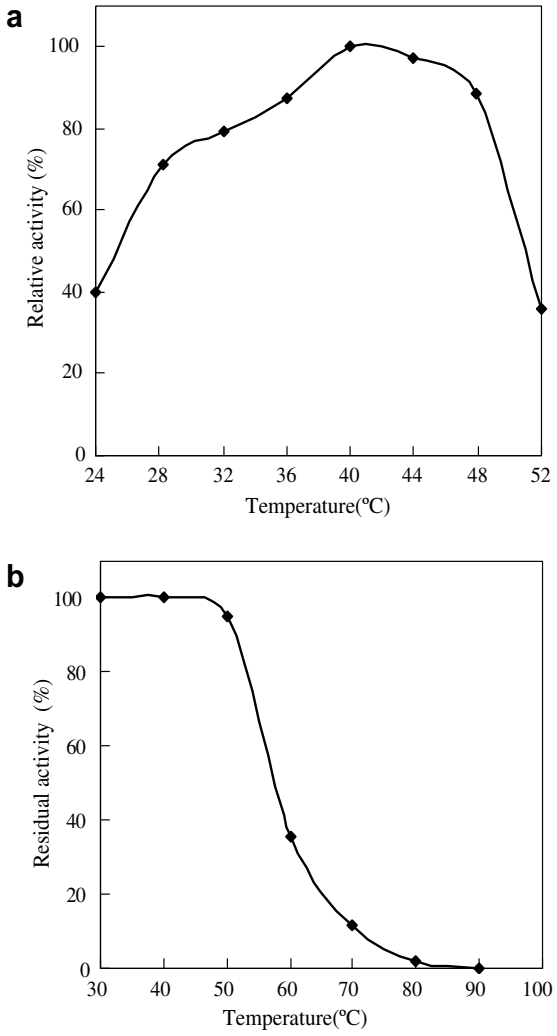

Fig. 4. Effect of temperature on activity of the CEP (a) and residual activity of CEP after 30 min incubation at temperatures ranging from 30 to 90 °C (b).

Table 2
Effect of metal ions and inhibitors on enzyme activity

Compound	Relative activity (%)	
	Compound concentration (mM)	
	1.0	10.0
None	100.0	100.0
Ba^{2+}	72.8	146.2
Zn^{2+}	103.4	53.0
Mn^{2+}	116.8	121.4
Co^{2+}	111.0	82.0
Mg^{2+}	117.2	104.4
Ni^{2+}	76.2	49.8
Ca^{2+}	108.8	186.0
EDTA	96.8	47.4
PMSF	63.4	24.2

residues (approx. 15 kDa) had been predicted to have a flexible extended secondary structure which was believed to traverse and interact with the cell wall anchor. The remaining 165 kDa, viz. the N-terminal proteinase domain and the adjacent middle domain, was probably exposed on the outer surface. It seemed more likely that the ~53 kDa form of the enzyme is released as a consequence of autoproteolysis at a site very close to the catalytic domain sequence.

The enzyme showed an optimal activity at pH 7.5, which was similar to the optimum condition for CEP from *S. thermophils* CNRZ 385 (Fernandez-Espla et al., 2000). However, the optimum pH of some of the CEP purified from LAB, such as *L. helveticus* CP790 (Yamamoto et al., 1993) and *L. delbrueckii* ssp. *lactis* ACA-DC178 (Tsakalidou et al., 1999), was in the range from 5 to 6.5. The optimum temperature of the enzyme was 40 °C, the same as that for the CEP isolated from *S. thermophils* CNRZ 385 (Fernandez-Espla et al., 2000), *L. helveticus* CP790 (Yamamoto et al., 1993), and *L. delbrueckii* ssp. *lactis* ACA-DC178 (Tsakalidou et al., 1999). The CEP in this study retained above 90, 30 and 10% activity during preincubation at 50, 60 and 70 °C for 30 min, respectively. Our enzyme was found to be more stable than that isolated from *Lactobacillus casein* ssp. *casein* IFPL731 (Fernandez de Palencia et al., 1997), which exhibited a 50% loss of activity at 35 °C and a total loss at 50 °C when pre-incubated for 30 min.

The enzyme was activated by Mn^{2+}, Mg^{2+}, Ca^{2+} and inhibited by Co^{2+}, Zn^{2+}, Ni^{2+} and EDTA, which indicates that CEP is a metallopeptidase. Because its activity was inhibited by the serine proteinase inhibitor PMSF, CEP was hypothesized to be a serine proteinase. The enzyme was strongly activated by Ca^{2+}, which suggested that the CEP is a Ca^{2+} dependent enzyme (Exterkate Fred, 1995).

The sequence of the first 13 amino acids at the N-terminal of the CEP was Asp-Val-Phe-Ala-Pro-His-Met-Ala-Asn-Val-Ala-Ala-Val. The amino acids sequence was homology with other CEPs of LAB obtained by using NCBI BLAST procedure. The sequence differed from the CEP of *Lactobacillus johnsonii* NCC 533 (Asp-Thr-Ser-Ala-Asn-Asn-Met-Ala-Asn-Val-Ser-Thr-Val) (Van der Kaaij, Desiere, Mollet, & Germond, 2004), *Lactobacillus casei* ATCC 334 (Asp-Ala-Lys-Ala-Asn-Asn-Met-Ala-Asn-Val-Gln-Ala-Val) (Makarova et al., 2006), *L. lactis* ssp. *cremoris* (Asp-Ala-Lys-Ala-Asn-Asn-Met-Ala-Asn-Val-Gln-Ala-Val) (Christensson, Pillidge, Ward, & O'Toole, 2001) and *L. delbrueckii* ssp. *bulgaricus* ATCC 11842 (Asp-Glu-Ser-Ala-Asp-Gln-Met-Ala-Gln-Val-Gln-Asp-Val) (Van de Guchte et al., 2006).

Among LAB, *L. helveticus* has been shown in past reports to possess strong proteolytic activity in milk-based media and some strains of *L. helveticus* (CPN4, CP790, CP611, CP615, JCM1006, JCM 1004, LBK 16H) are known to produce potent ACE-inhibitory peptides during milk fermentation (Leclerc et al., 2002; Pan, Luo, & Tanokura, 2005; Yamamoto et al., 1993). In the present study, the whey protein hydrolysate produced by the purified CEP displayed ACE-inhibitory activity, which suggested that *L. lactis* ssp. *lactis* LB 12 may produce ACE-inhibitory peptides during milk fermentation.

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