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Full Length Research Paper

Purification and characterization of cell-envelopeproteinase from *Lactobacillus casei* DI-1

Guoyu Xing[1], Daodong Pan[1,2]*, Min Tong[1] and Xiaoqun Zeng[2]

[1]Department of Food Science and Nutrition, Nanjing Normal University, Nanjing 210097, China.
[2]Life Science and Biotechnology College, Ningbo University, Ningbo, Zhejiang, China.

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Using a Ca^{2+}-free method, the cell-envelope proteinase (CEP) of *Lactobacillus casei* DI-1 isolated from duck small intestine was released from cells and purified by ammonium sulfate precipitation, and by diethylaminoethyl (DEAE)-Sephadex A-25 and Sephadex G-100 gel chromatography. The purified CEP had a monomer structure with a molecular mass of about 35 kDa. Optimal activity occurred at pH 7.0 and 37°C. The purified CEP was a metallopeptidase, which was activated by Co^{2+}, Ba^{2+}, Mg^{2+} and Fe^{3+}, and inhibited by Ca^{2+}, Zn^{2+}, K^{+}, Ni^{2+}, Mn^{2+}, and ethylenediaminetetraacetic acid (EDTA). It was a serine proteinase which was inhibited by phenylmethylsulfonyl fluoride (PMSF). Its kinetic constant (Km) is 0.29 mM and the first 10 amino acids of the CEP's N-terminal sequences were Asp-Asn-Asp-Phe-Glu-Ile-Phe-Glu-Ser-Ser. The hydrolysates of α-, β- and κ-casein produced by CEP showed different angiotensin-I-converting enzyme (ACE) inhibitory activity; the hydrolysates of β-casein displayed the greatest ACE inhibitory activity.

Key words: Cell-envelope proteinase, purification, characterization.

INTRODUCTION

Lactobacillus casei is a common constituent of mesophilic lactic starters used in the fermentation industry as a probiotic (Hebert et al., 1999; Mirnejad et al., 2010). It is dependent on the small peptides and free amino acids in the culture medium. However, the concentration of free amino acids and peptides present in milk is not sufficient for the growth of all lactic-acid bacteria. These bacteria must degrade milk proteins into the material they need (Tsakalidou et al., 1999). Casein degradation and subsequent utilization of the catabolite products requires a complex proteolytic system consisting of proteinases, peptidases, amino acid and peptide carriers (Fang and Poolman, 1998).

Cell-envelope proteinase (CEP) play an important role in the lactobacillus proteolytic system. CEPs are the critical enzyme in the system (Kunji et al., 1996), since it is the only enzyme that can initiate the breakdown of caseins into oligopeptides. These peptides are then transported into the bacteria and further hydrolyzed by intracellular peptidases (Bockelmann, 1995; Sinsuwan et al., 2008; Delorme et al., 2010). Several functions of CEPs have been found. CEPs' C-terminal is anchored to the cell wall (Fang and Poolman, 1998), which is similar to the surface proteins of Gram-positive bacteria. CEP from several different strains have been purified and characterized, including from *L. casei* subsp. *casei* IFPL731 (Fernandez-de-Palencia et al., 1997), *Streptococcus thermophilus* (Fernandez-espla et al., 2000) and *Lactobacillus helveticus* CRL 1062 (Hebert et al., 1999). CEP of various bacteria strains appears to display different enzymatic properties (Exterkate, 1995).

Since little study has been devoted to the purification and characterization of the CEP of *L. casei*, the purpose

*Corresponding author. E-mail: daodongpan@163.com. Tel: + 86- 574 - 87600737. Fax: + 86 - (0)574 - 87608347.

Abbreviations: CEP, Cell-envelope proteinase; **DEAE,** diethylaminoethyl; **EDTA,** ethylenediaminetetraacetic acid; **PMSF,** phenylmethylsulfonyl fluoride; **ACE,** angiotensin-I-converting enzyme.

of this study was to purify and characterize the CEP of *L. casei* DI-1 isolated from duck small intestine and measure the angiotensin-I-converting enzyme (ACE) inhibitory activities of casein hydrolysate hydrolyzed by purified CEP of *L. casei* DI-1

MATERIALS AND METHODS

We isolated *L. casei* DI-1 from duck small intestine. Diethylaminoethyl (DEAE)-Sephadex A-25, Sephadex G-100 and low molecular weight calibration kits for sodium dodecyl sulphate (SDS) electrophoresis were purchased from Amersham Biosciences Co. Ltd. (Uppsala, Sweden). MeOsuc-Arg-Pro-Tyr-pNA (MS-Arg) was synthesized by MP Biomedicals (Solon, OH, USA). Phenylmethylsulphonyl fluoride (PMSF), hip-puryl-l-histidyl-l-leucine (HHL) and ACE (obtained from rabbit lung) were obtained from Sigma Chemicals Co. Ltd. (St. Louis, MO, USA).

Preparation of cell-free extract

L. casei DI-1 was grown in de Man, Rogosa and Sharpe (MRS media) at 37°C for 20 h, and shaken at 120 rpm. The cells were harvested by centrifugation at 2,400×g for 15 min at 4°C and washed three times with 50 mM Tris-HCl buffer (pH 7.0) containing 30 mM $CaCl_2$. The cells were suspended in specially designated solution (50 mM Tris-HCl, ethylenediaminetetraacetic acid (EDTA)-Na_2, pH 7.0) and incubated in a water bath for 60 min at 40°C. Cell debris was removed by centrifugation at 2,400×g for 15 min at 4°C. The resulting clear supernatant was lyophilized and used as the cell-free extract.

Measurement of proteinase activity

The mixture, which contained 0.05 ml of 6.4 mM MS-Arg dissolved in methanol, 2.85 ml of 50 mM Tris-HCl buffer (pH 7.0) 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. One unit of enzyme activity (U) was defined as the amount of enzyme needed to release 1 µM of *p*-nitroaniline per min at 37°C (Fernandez-de-Palencia et al., 1997).

Protein quantification

Protein concentrations were determined throughout the purification process using the Coomassie blue method. A 1 ml sample was added to 5 ml protein reagent (100 mg of Coomassie blue G-250 dissolved in 50 ml 95% ethanol and 100 ml of 85% phosphoric acid, and distilled water added to 1,000 ml), and measured at 595 nm with 1 ml distilled water as control (Bradford, 1976).

Ammonium sulfate precipitation

The cell-free extracts were fractionated by salting out with solid ammonium sulfate at 40 to 60% (w/v) saturation. The precipitation was collected by centrifugation at 7,600×g at 4°C for 30 min and dissolved in 50 mM Tris-HCl buffer (pH 7.0), then demineralized using a nanofiltration system from Shanghai Laungy Membrane Filtration Technology Co., Ltd. (Shanghai, China). $BaCl_2$ was used to detect the ammonium sulfate, until non-precipitation demineralization was finished.

DEAE-Sephadex A-25 chromatography

Five milliliters of crude enzyme (0.1g/ml) dissolved in 50 mM Tris-HCl buffer (pH 7.0) were applied to a DEAE-Sephadex A-25 column (2.60 × 40 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The enzyme was eluted at a flow rate of 25 ml/h with a linear NaCl gradient (250 ml buffer - 250 ml 0.5 M NaCl buffer). The fractions containing the enzyme were pooled and desalted by ultrafiltration, and then concentrated using vacuum freeze-drying equipment. The protein concentration and CEP activity were then analyzed.

Sephadex G-100 chromatography

The concentrated enzyme solution from the previous step was applied to a Sephadex G-100 column (1.6 × 50 cm) equilibrated with 50 mM Tris-HCl (pH 7.0). Proteins were eluted with the same solution at a flow rate of 25 ml/h. The fractions containing the enzyme were pooled and desalted using a nanofiltration system, and then lyophilized. The protein concentration and CEP activity were then analyzed.

Effects of pH on enzymatic activity

The effects of pH from 5.5 to 8.5 on enzymatic 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 5.0 to 9.0 pH range and incubated at 37°C for 8 h. The residual activity was measured using proteinase activity measurement.

Effects of temperature on enzymatic activity and stability

The effects of temperatures from 27 to 52°C on enzymatic activity were measured in 50 mM Tris-HCl buffer (pH 7.0) with MeOsuc-Arg-Pro-Tyr-pNA as the substrate. The purified enzyme solutions were incubated for 30 min at temperatures ranging from 20 to 90°C to assess the thermal stability of the enzyme. The residual activity was measured using the proteinase activity measurement method.

Effects of metal ions and inhibitors on enzymatic 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+}, K^{+}, and Fe^{3+}), PMSF and EDTA at a final concentration of 1.0, 5.0 and 10.0 mM in 50 mM Tris-HCl buffer (pH 7.0), respectively. The enzymatic activity was measured after incubation at 37°C for 1 h with MeOsuc-Arg-Pro-Tyr-pNA as the substrate.

Kinetics of CEP

Concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mM MeOsuc-Arg-Pro-Tyr-pNA were prepared as substrate. Enzymatic activity was measured at 37°C with different concentrations of MeOsuc-Arg-Pro-Tyr-pNA, and then absorbance

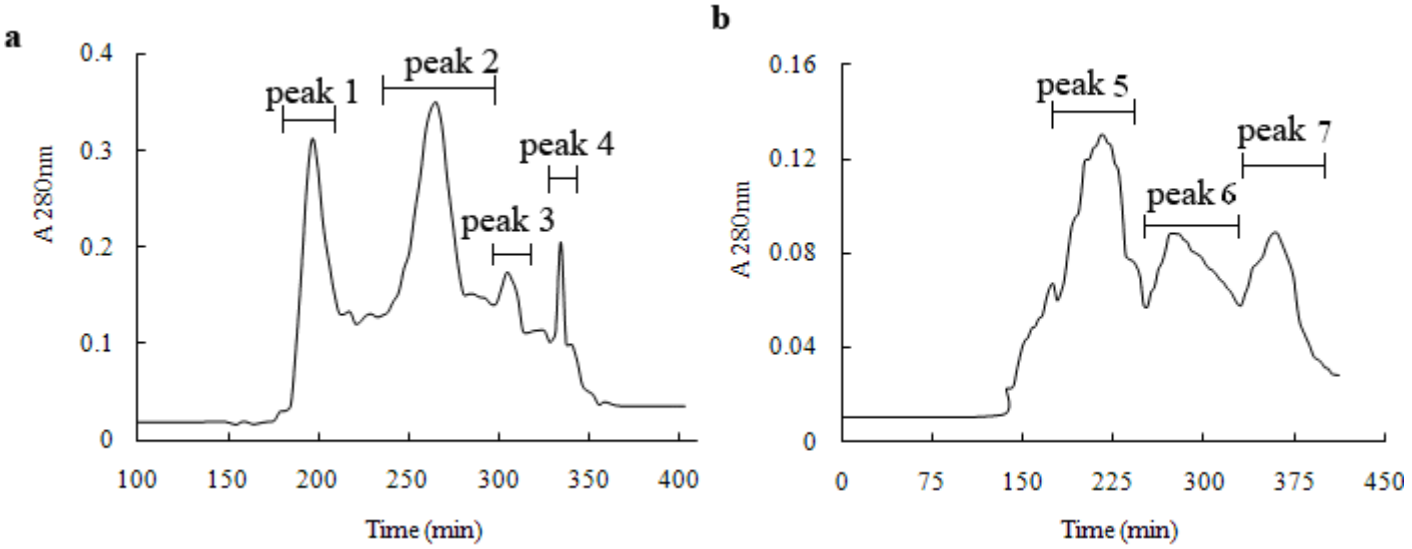

Figure 1. (a) DEAE Sephadex A-25 chromatography of the crude CEP obtained by ammonium sulfate precipitation, (b) Sephadex G-100 chromatography of second fraction (peak 2) obtained using DEAE-Sephadex A-25 chromatography. DEAE, Diethylaminoethyl; CEP, cell-envelope proteinase.

was measured at 410 nm. The reciprocal of the concentration (1/S) was plotted as the abscissa, and the reciprocal of the reaction rate (1/V) was used for the vertical to produce a Lineweaver-Burk diagram and the following equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

The equation can obtain Km and the maximum reaction rate Vmax of *L. casei* DI-1 CEP relative to the substrate MeOsuc-Arg-Pro-Tyr-pNA.

Determination of purity and molecular mass

The active fraction after each purification step was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Laemmli, 1970). The purity and molecular mass of CEP were determined using SDS-PAGE with a 4% (w/v) acrylamide stacking gel and a 12% (w/v) acrylamide running gel. Proteins in the gels were stained using Coomassie blue R-250. For determination of molecular mass (Mr), small molecular mass standard proteins (Sigma) were used.

N-Terminal amino acid sequencing

The purified CEP was electro-transferred from SDS-PAGE gel onto polyvinylidene difluoride (PVDF) membranes. Proteins were stained using Coomassie blue R-250. The electroblotted proteinase was cut and sequenced using a protein-sequencing system. Amino-acid sequence homology with other CEPs was obtained by using the basic local alignment search tool (BLAST) procedure.

Assay for ACE inhibitory activity

The ACE inhibition activities of hydrolysate from α-, β- and κ-casein produced by CEP were assayed as follows (Cushman and Cheung, 1971): aliquots (200 µL) of the buffered substrate solution (5.0 mM HHL in 100 mM borate buffer with 300 mM NaCl, pH 8.3) were mixed with 80 µL casein hydrolysate and pre-incubated at 37°C for 3 min. Twenty microliters (0.1 U/ml) ACE were added to start the reaction. After 30 min of incubation at 37°C, the enzymatic reaction was stopped by adding 250 µL of 1.0 M HCl and 1.7 ml of ethyl acetate, and the mixture was left to stand for 5 min after shaking for 15 s. Subsequently, 1 ml of the ethyl acetate layer was drawn and kept in an oven at 120°C until the ethyl acetate vaporized, and then 1 ml of distilled water was added and mixed before the absorbance was measured at 228 nm. The level of inhibitory activity was calculated using the following equation: ACE inhibition activity = [(B-A)/B] × 100%, where A is the absorbance of a solution containing ACE, but without the sample; B is the absorbance of a solution with ACE and sample.

Statistical analysis

Experimental data were presented as mean ± standard deviation of the mean for all groups. Data analysis was carried out using SPSS 10.0 (SPSS Inc., Chicago, USA). Student's t-test was used to perform multiple comparisons between means. All data presented are mean values of three determinations and three replicates, unless otherwise stated.

RESULTS

Purification of CEP

As shown in Figure 1a, the crude enzyme after ammonium sulfate precipitation was separated into four fractions in a DEAE-Sephadex A-25 column. The second fraction (peak 2) showed CEP activity (Table 1). The second fraction (peak 2) from DEAE-Sephadex A-25

Table 1. Purification of CEP.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (100%)	Purification (fold)
Cell-free extract	756.3	192.75	0.25	100	1
Ammonium sulfate precipitation	86.89	132.6	1.53	68.84	6.12
DEAE-Sephadex A-25 (Peak 2)	7.57	115.52	15.26	59.93	61.04
Sephadex G-100 (Peak 5)	2.82	47.9	19.01	24.85	76.04

CEP, Cell-envelope proteinase.

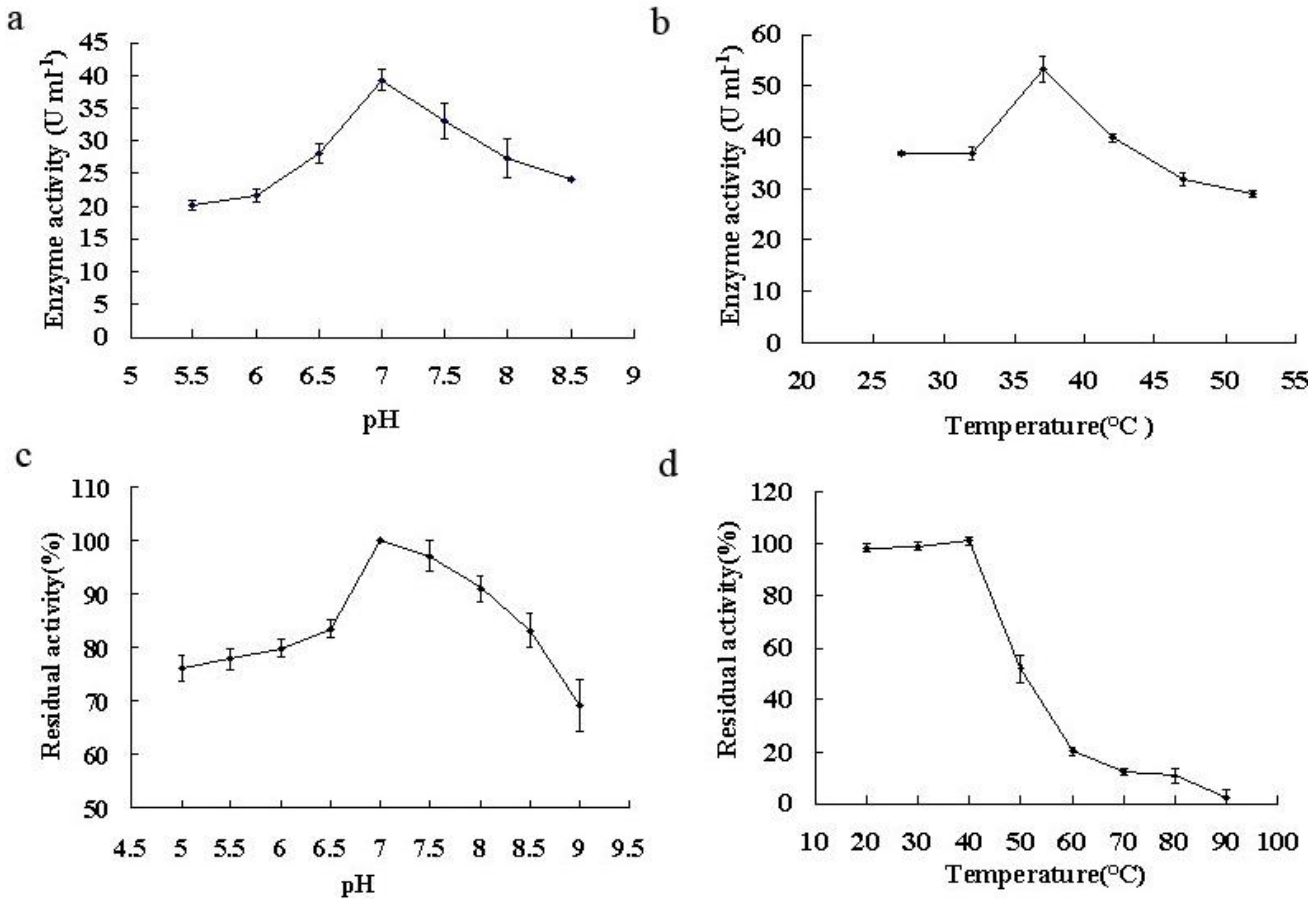

Figure 2. (a) Effect of pH on CEP activity; (b) Effect of temperature on CEP activity; (c) residual activity of CEP after incubation at pH ranging from 5.0 to 9.0; (d) residual activity of CEP after incubation at temperatures ranging from 20 to 90°C. CEP, Cell-envelope proteinase.

column was further separated into three fractions in a Sephadex G-100 column (Figure 1b). The first fraction (peak 5) exhibited CEP activity (Table 1). The CEP activities of these fractions obtained on Cell-free extract, ammonium sulfate precipitation, DEAE-Sephadex A-25 column (peak 2), Sephadex G-100 column (peak 5) are summarized in Table 1. The CEP was purified about 76-fold from the cell-free extract after ammonium sulfate fractionation and two steps of column chromatography. The recovered activity was about 24.85%.

Effects of pH and temperature on enzymatic activity

The enzyme showed high activity at pH levels between 7.0 and 7.5 (Figure 2a), with the optimum activity at pH 7.0. About 55% of the maximum activity was at pH 5.5 and about 65% of the maximum activity was at pH 8.5.

Table 2. Effect of metal ions and inhibitors on CEP enzymatic activity.

Compound	Relative activity (%)		
	1 mM	5 mM	10 mM
None	100	100	100
K^+	92.79	97.30	55.86
Ca^{2+}	80.18	82.88	78.38
Mn^{2+}	114.41	83.78	95.50
Zn^{2+}	93.69	72.97	76.58
Mg^{2+}	81.08	83.78	112.61
Fe^{3+}	90.99	109.01	198.20
Co^{2+}	118.02	111.17	105.41
Ni^{2+}	83.78	45.05	36.94
Ba^{2+}	100.00	146.85	238.74
EDTA	92.81	84.50	71.83
PMSF	99.85	71.83	70.14

EDTA, Ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; CEP, Cell-envelope proteinase.

The enzyme was stable for 8 h over the pH range from 5.0 to 9.0 (Figure 2c). Less than 75% of the residual activity was measured at pH 5.0 or 9.0, but more than 95% of the residual activity was observed at pH 7.5 to 8.0. These findings indicate that the CEP had pH stability.

The enzyme showed maximum activity at 37°C (Figure 2b). About 52% of the maximum activity was found at 52°C. We found 20.2, 12.3, 10.53 and 2.63% residue of its maximum activity, respectively, when it was pre-incubated for 30 min at 60, 70, 80 and 90°C (Figure 2d).

Effects of divalent metal ions and inhibitors on enzymatic activity

Table 2 shows the effects of various metal ions and inhibitors on enzymatic activity. The enzyme was activated by Mn^{2+} at low concentrations and by Mg^{2+}, Ba^{2+}, Co^{2+} and Fe^{3+} at high concentrations. Ba^{2+} showed the most significant effect. The enzyme was inhibited by Ca^{2+}, Zn^{2+}, EDTA and PMSF, and significantly inhibited by K^+ and Ni^{2+}. The enzyme was activated by Mn^{2+} at low concentrations but inhibited at high concentrations. Mg^{2+} and Fe^{3+} inhibited the enzymatic activity at low concentrations, but promoted activity at high concentrations.

Kinetics of CEP

Based on the Lineweaver-Burk graph (Figure 3), the linear equation for CEP was y=0.4394x+1.5036. Moreover, according to the formula:

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}},$$

the Km of *L. casei* DI-1 CEP was 0.29 mM and the *Vmax* was 0.665.

Molecular mass and N-Terminal amino acid sequencing

The CEP after purified by ammonium sulfate precipitation, DEAE-Sephadex A-25 column and Sephadex G-100 column showed one band using SDS-PAGE and the molecular mass of CEP was ~35 kDa (Figure 4). The sequence of the 10 amino acids at the N-terminal of CEP was Asp-Asn-Asp-Phe-Glu-Ile-Phe-Glu-Ser-Ser.

Assay for ACE inhibitory activity

ACE inhibition of hydrolysate from α-, β- and κ-casein produced by crude and purified enzyme is shown in Figure 5. The ACE inhibitory activities of casein hydrolysates produced by crude CEP were higher than those produced by purified enzyme. However, the ACE inhibitory activities produced by purified CEP displayed more than half of the ACE inhibitory activities produced by crude CEP. Therefore, CEP played a crucial role in the production of ACE inhibitory peptides in the lactic-acid hydrolysis system. *L. casei* DI-1 CEP hydrolyze various caseins, which contribute differently to ACE inhibitory

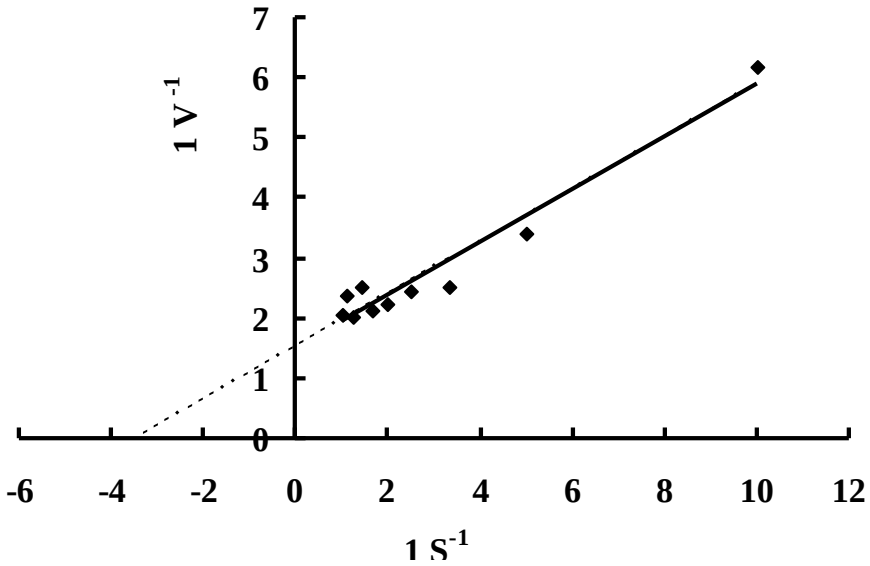

Figure 3. Lineweaver-Burk diagram of CEP from Lactobacillus casei DI-1. CEP, Cell-envelope proteinase.

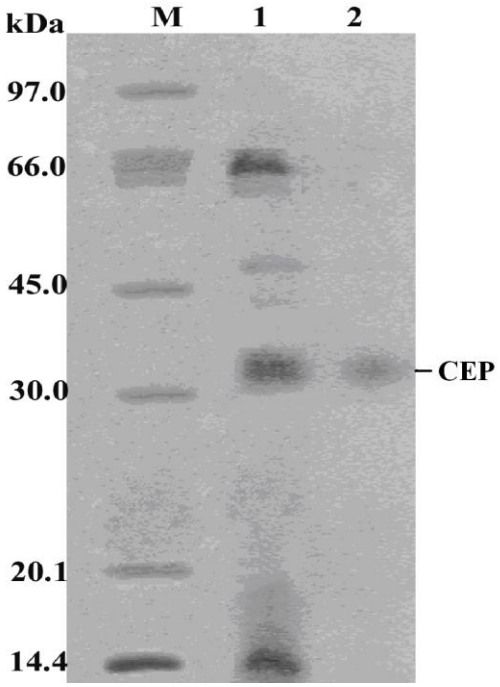

Figure 4. SDS-PAGE of fractions during CEP purification. Lane M, Molecular weight standards. Phophorylase b (Mr 97,000), albumin (Mr 66,000), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 30,000), trypsin inhibitor (Mr 20,100), and α-lactalbumin (Mr14,400) were used as molecular size markers. Lane 1, Peak 2 obtained from chromatography on DEAE-Sephadex A-25 (Figure 1a); Lane 2, peak 5 obtained from chromatography on Sephadex G-100 (Figure 1b). SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; CEP, cell-envelope proteinase.

activity. The hydrolysate of β-casein displayed the greatest ACE inhibitory activity, while the hydrolyzate of κ-casein displayed the least ACE inhibitory activity.

DISCUSSION

The optimum temperature and pH for CEP from *L. casei* IFPL731 are pH 6.0 at 40°C, respectively. CEP enzymatic activity decreases by half when it is kept at 35°C for 30 min and is completely lost when kept at 50°C for 30 min (Fernandez-de-Palencia, 1997). In this study, the optimum temperature and pH of CEP from *L. casei* DI-1 were found to be pH 7.0 to 7.5 at 37°C. Its activity barely decreased when it was kept at 35°C for 30 min, but it lost half its enzymatic activity when it was kept at 50°C for 30 min. Our study found that CEP from the strain of *L. casei* DI-1 displayed more temperature stability than that of CEP from *L. casei* IFPL731. The optimum pH and temperature of CEP for *L. helveticus* CRL 1062 is 6.5 to 7.0 and 42°C (Hebert et al., 1999), for *Lactobacillus delbrueckii* subsp. *lactis* ACA-DC 178 is 6.0 and 40°C (Tsakalidou et al., 1999), for *Streptococcus thermophilus* CNRZ 385 7.5 and 37°C (Fernandez-espla et al., 2000), for *Lactobacillus acidophilus* CH2 and V74 6.5 and 50° C, and for *L. delbrueckii* BGPF1 and BGRA43 are 6.5, 40°C, and 6.5 at 45°C, respectively (Fira et al., 2001). These findings show that there are differences in the optimum pH and temperature of CEP from various lactic-acid bacteria strains.

Our results show that the metal ions Mn^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+} and Fe^{3+} activated the enzyme, while K^{+}, Ca^{2+}, Zn^{2+}, and Ni^{2+}, EDTA and PMSF inhibited enzymatic activity.

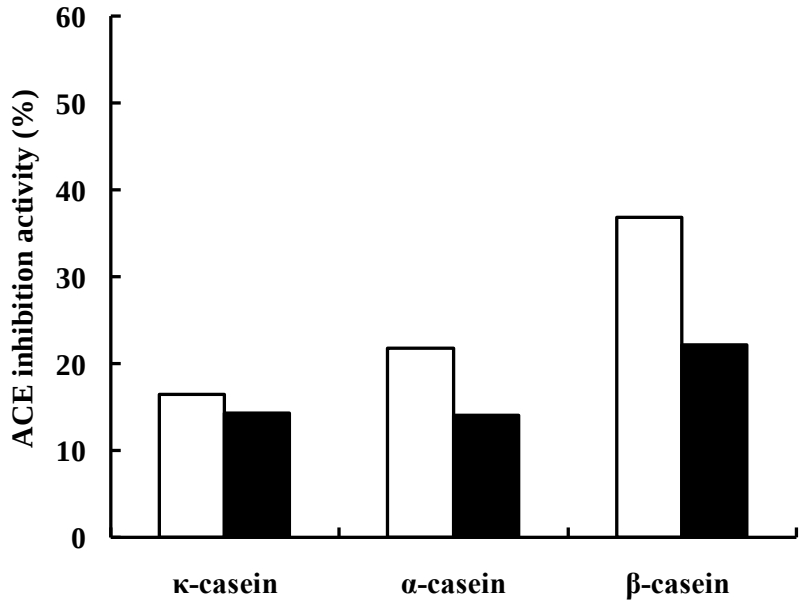

Figure 5. ACE inhibitory activity of casein hydrolyzed by CEP of crude enzyme (□) and purified enzyme (■).CEP, Cell-envelope proteinase; ACE, angiotensin-I-converting enzyme.

These findings are in agreement with some other research findings, including the finding that the CEP of *L. acidophilus* CH2 and V74 were inhibited by K^+ and Zn^{2+}; *L. acidophilus* CH2 and *L. delbrueckii* BGRA43 were slightly inhibited by Ca^{2+} (Fira et al., 2001); and that the CEP of *L. delbrueckii* subsp. *lactis* ACA-DC 178 was strongly inhibited by PMSF and not significantly influenced by EDTA (Tsakalidou et al., 1999), while the CEP of *S. thermophilus* CNRZ 385 was strongly inhibited by serine proteinase inhibitors such as PMSF (Fernandez-espla et al., 2000). However, our findings varies with some other research, such as the findings that the CEP of *S. thermophilus* CNRZ 385 was highly activated by Ca^{2+} ions (Fernandez-espla et al., 2000), and CEP from *Virgibacillus* sp.SK37 was activated at low Ca^{2+} concentrations, but inhibited at high concentrations (Sinsuwan et al., 2008).

It has been shown that CEP is not only a serine protease enzyme but also a kind of metal enzyme. The maintenance of the conformation of some CEP active sites appears to have some connection with metal ions. Serine residues may be the components of the enzyme active site or may help maintain the stability of the enzyme's conformation (Wang and Zou, 1999). The molecular mass of the CEP we characterized was 35 kDa, which was different from other lactic-acid bacteria strains' CEPs. Fernandez-espla et al. (2000) used alumina powder and lysozyme to distill the CEP of *S. thermophilus*, and estimated the molecular mass to be 153 kDa. Genay et al. (2009) concluded that the molecular mass of CEP from *L. helveticus* CNRZ32 is 204 kDa. Kojic et al. (1991) mentioned that the CEP of *L. casei* HN14 has a molecular size of 145 kDa. Therefore, the molecular mass of *L. casei* DI-1 CEP appears to be smaller than other CEP.

The sequence of the first 10 amino acids at the N-terminal of the CEP we characterized was Asp-Asn-Asp-Phe-Glu-Ile-Phe-Glu-Ser-Ser. It displayed a high homology with other CEPs of lactic-acid bacteria strains obtained by using the NCBI BLAST procedure. Among lactic acid bacteria (LAB), *L. helveticus* has been shown in past reports to possess strong proteolytic activity in milk-based media and some strains of *L. helveticus* are known to produce potent ACE-inhibitory peptides during milk fermentation (Leclerc et al., 2002; Yamamoto et al., 1994, 1999). ACE-inhibitory peptides can lead to the drop of blood pressure, and the results have been proven in hypertensive human subjects and Spontaneous Hypertensive Rats (Yamamoto et al., 1994, 1999). Our present results demonstrate that *L. casei* DI-1 also contribute to the ACE inhibitory peptides production and it was a new discovery.

As already demonstrated for lactic-acid bacteria, CEPs may be involved in the development of various healthful properties in dairy products via bioactive peptide production (Hayes et al., 2007; Pan et al., 2005). CEP is a key enzyme in the proteolytic system, as it hydrolyses caseins into oligopeptides. These oligopeptides may have ACE inhibitory activities. To prove this assumption, the CEP of *L. casei* DI-1 was purified and α-, β- and κ-casein were hydrolyzed. We found that CEP of *L. casei* DI-1 can hydrolyze α-, β- and κ-casein into ACE inhibitory activity peptides. The CEPs of LAB are highly strain-specific to

produce ACE inhibitory activity peptides. Further research is however needed to study the relation between structure of CEPs from LAB and function.

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