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Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides

Xue-Ying Mao [a], Jin-Ren Ni [b,*], Wei-Ling Sun [b], Peng-Peng Hao [b], Li Fan [b]

[a] College of Food Science and Nutritional Engineering, Key Laboratory of Functional Dairy of Chinese Ministry of Education, China Agricultural University, Beijing 100083, China
[b] College of Environmental Engineering, Peking University, Beijing 100087, China

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Abstract

Yak (*Bos grunniens*) milk casein derived from Qula, a kind of acid curd cheese from northwestern China, was hydrolysed with alcalase. The hydrolysates collected at different hydrolysis times (0 min, 60 min, 120 min, 180 min, 240 min, 300 min, 360 min) were assayed for the inhibitory activity of angiotensin-I-converting enzyme (ACE), and the one obtained at 240 min hydrolysis showed the highest ACE inhibitory activity. The active hydrolysate was further consecutively separated by ultrafiltration with 10 kDa and then with 6 kDa molecular weight cut-off membranes into different parts, and the 6 kDa permeate showed the highest ACE-inhibiting activity. This active fraction was further purified to yield two novel ACE-inhibiting peptides, whose amino acid sequences were Pro–Pro–Glu–Ile–Asn (PPEIN)(κ-CN; f156–160) and Pro–Leu–Pro–Leu–Leu (PLPLL) (β-CN; f136–140), respectively. The molecular weight and IC_{50} value of the peptides were 550 Da and 566.4 Da, and 0.29 ± 0.01 mg/ml and 0.25 ± 0.01 mg/ml, respectively.
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Keywords: Qula; Yak (*Bos grunniens*) milk casein; ACE inhibitory peptide; Alcalase

1. Introduction

The currently used synthetic drugs for the treatment of hypertension, e.g., captopril and enalapril have certain side effects, such as coughing, skin discomfort, and, in particular, excessively low blood pressure (Je, Park, Jung, Park, & Kim, 2005). Natural ACE inhibitors such as bioactive peptides, as alternatives to synthetic drugs, have awoken the attention of both food and medical researchers.

Bioactive peptides possess many activities, such as immunoregulatory, opioid, antioxidant, or antihypertensive (Mao, Nan, Li, & Ni, 2005a, 2005b; Martin, Gwenaele, & Said, 1999; Pihlanto, 2001; Sandre et al., 2001). In recent years, many ACE inhibitory peptides have been isolated from various food proteins, such as fish protein (Curtis, Dennis, Waddell, MacGillivray, & Ewart, 2002), cheese whey (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Hernández, Recio, Ramos, & Amigo, 2002; Sandrine, Pascal, Céline, & Adèle, 2003), casein (Maeno, Yamamoto, & Takano, 1996; Mizuno, Nishimura, Matsuura, Gotou, & Yamamoto, 2004; Yamamoto, Maeno, & Takano, 1999), fermented milk products (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000; Yamamoto et al., 1999), corn gluten (Suh & Whang, 1999), bovine plasma (Janitha et al., 2002) and fermented soybean (Gibbs, Zougman, Masse, & Mulligan, 2004). Bioactive peptides can be generated not only during the manufacture of cheese, yogurt and other dairy products, but also during protein hydrolysis by digestive enzymes, such as pepsin, trypsin, or chymotrypsin. They may also be generated by controlled protein hydrolysis. Using commercially available microbially-derived food grade proteases to hydrolyse casein is advantageous because these enzymes are cheap and safe.

Yak milk is a common product in northwestern China, such as Gansu, Xinjiang and Tibet. In these areas, people

* Corresponding author. Tel.: +86 1062755914.
E-mail address: nijinren@iee.pku.edu.cn (J.-R. Ni).

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only use it for the production of butter, and the by-product (Qula, a kind of crude cheese, whose main component is casein) is not fully used. Therefore, improving the value of Qula has received great attention from the Chinese government. Fortunately, the casein content of Qula is greater than 80% on a dry weight basis.

This work was carried out to determine the proper enzymatic conditions to convert Qula into a good source of antihypertensive peptides, and to isolate and characterise ACE inhibitory peptides from yak milk casein hydrolysate. The results would determine the potential of yak milk casein as an ingredient for the production of antihypertensive functional foods.

2. Materials and methods

2.1. Materials and chemicals

Qula (casein) was provided by Tongjian Co. (Gansu province, China); Hippuryl-L-histidyl-L-leucine (HHL), angiotensin-I-converting enzyme (ACE), hippuric acid (HA), gastric porcine pepsin (EC.3.4.23.1, 1:10000) and trypsin (EC.3.4.21.4, 1:250, Gibco-BRL, activity 2–4 U/mg) were purchased from Sigma–Aldrich Company (St. Louis, MO). Alcalase was purchased from Huaqiang Biochem. Inc. (activity, 5000 U/g, Beijing, China).

2.2. Preparation of yak milk casein

Casein was precipitated from Qula through pI (isoelectric point) precipitation. Qula was heated at 85 °C in 0.05 M NaOH until dissolved completely. The solution was cooled to 25 °C and acidified to pH 4.6 with 0.5 M HCl, to induce casein precipitation. The acid curd was lyophilised to get yak milk casein.

2.3. Hydrolysis of yak milk casein

Casein was dissolved in demineralised preheated water and brought to the appropriate pH using 1 N NaOH. Casein was hydrolysed by alcalase from *Bacillus licheniformis*. The hydrolysis conditions were as follows: substrate concentration was 6% (w/w), enzyme dose was 2.5% (w/w, defined as enzyme mass/substrate mass × 100%), pH value was 8.0, temperature was 55 °C. At different hydrolysis times (0 min, 60 min, 120 min, 180 min, 240 min, 300 min, 360 min) during hydrolysis, samples were collected and immediately heated in a boiling-water bath for 10 min to inactivate the enzyme. The samples were defined as A0, A1, A2, A3, A4, A5 and A6, respectively. The unhydrolysed casein was removed by pI precipitation. The hydrolysates were collected and lyophilised for further analysis.

2.4. Degree of hydrolysis

The degree of hydrolysis (DH%), derived from the α-amino nitrogen and the total nitrogen, was calculated as follows (Mahmoud, Malone, & Cordle, 1992):

$$\mathrm{DH}\,\% = \frac{\mathrm{AN_h} - \mathrm{AN_c}}{\mathrm{TN} \times P_f} \times 100$$

$\mathrm{AN_h}$ and $\mathrm{AN_c}$ are the percent amino nitrogen of hydrolysate and intact casein, respectively. No significant difference was found between total nitrogen of the intact casein and that of the hydrolysates. Thus, TN refers to the mean percent total nitrogen of the intact casein solution and all hydrolysate samples, and P_f is a correction factor for side chain nitrogen, which cannot be converted to amino nitrogen by hydrolysis of peptide bonds. The P_f factor was calculated from the amino acid profile of casein.

2.5. Measurement of ACE inhibitory activity

The determination of ACE inhibitory activity was performed by a HPLC method, with a modification of the method of (Cushman & Cheung, 1971). HHL (5.0 mM) was dissolved in 90 mM/L Na-borate buffer (pH 8.3) containing 0.4 M NaCl. ACE was dissolved in the same buffer, at a concentration of 60 mU/ml. A mixture containing 150 μl sample and 75 μl ACE solution was incubated at 37 °C for 5 min, 150 μl HHL solution was then added and incubated for 30 min. The reaction was stopped with 50 μl of 0.1% trifluoroacetic acid (TFA). Hippuric acid liberated by ACE was determined by RP-HPLC on a Zorbax Eclipse (2.5 × 150 mm, Agilent, Palo Alto, CA) column. The mobile phase was acetonitrile and Milli-Q water, and the flow rate was 0.8 ml/min. The effluent was monitored with an ultraviolet detector (Agilent) at 228 nm. A series of standard hippuric acid solutions were prepared to construct a calibration curve of peak area versus hippuric acid concentration. The ACE inhibiting activity of the tested substances was calculated as follows:

$$\text{ACE inhibiting activity}\ (\%) = \frac{[\text{hippuric acid}]_{\text{control}} - [\text{hippuric acid}]_{\text{sample}}}{[\text{hippuric acid}]_{\text{control}}} \times 100$$

2.6. Purification of ACE inhibitory peptides

2.6.1. Ultrafiltration

For purification of ACE inhibitory peptides, the active casein hydrolysate was passed through a polyether–sulfone ultrafiltration membrane with 10 kDa and then with 6 kDa molecular weight cut-off membranes (Millipore Co., Billerica, MA) successively. It was fractionated into three fractions (>10 kDa, 6–10 kDa, and below 6 kDa).

The antihypertensive activities of these fractions were tested by HPLC method.

2.6.2. Gel filtration

The most active fraction was loaded onto a DE-52 column (1.6 cm × 30 cm), previously equilibrated with PBS (5 mM, pH 8.0). After washing with PBS (5 mM, pH 8.0) until the UV absorbance returned to baseline, the absorbed peptides were eluted with a linear gradient of NaCl

(0–0.4 mM) in the same buffer at a flow rate of 24 ml/h. The elution profile was monitored at 220 nm, and the active fractions were pooled and lyophilised immediately.

The lyophilised fraction was further purified on a Sephadex G-25 gel filtration column (2.5 cm × 100 cm) equilibrated with distilled water. The column was eluted with distilled water at a flow rate of 16 ml/h and fractions analysed at 220 nm. The fraction exhibiting the highest ACE inhibitory activity was further purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a Zorbax Eclipse XDB-C_{18} (10 mm × 400 mm) column with a linear gradient of acetonitrile (0–60% in 45 min) containing 0.1% trifluoroacetic acid (TFA), at a flow rate of 1.0 ml/min. The eluted peaks were detected at 214 nm.

The peak possessing ACE inhibitory activity was chromatographed again on an analytical Zorbax Eclipse XDB-C18 column (2.1 mm × 150 mm) at 0.4 ml/min and 25 °C using a binary gradient with acetonitrile as an organic modifier. Solvent A contained 0.1% TFA in Milli-Q water and solvent B contained 0.1% (v/v) TFA in acetonitrile. Peaks were detected by UV detector at 214 nm. The active fractions were again collected and lyophilised immediately.

2.6.3. Amino acid sequence determination of the isolated active peptides

The molecular mass of the purified ACE-inhibiting active fractions was determined using LC–MS (HP 1100 series LC–MSD, Agilent) (Robert, Razaname, Mutter, & Juillerat, 2004). The amino acid sequence was determined by liquid chromatography coupled to electrospray ionization mass spectrometry (LC–ESI-MS/MS) using positive ion MS/MS, nebuliser gas (N_2, 40.0 psi), dry gas (N_2, 10.0 l/min, 330 °C). The scan range was set at m/z 50–2000.

2.7. Stability of the isolated ACE-inhibitory peptides for ACE

The ACE inhibitory peptides (10 mg/ml) from yak milk casein hydrolysate were incubated with 20 mU ACE at 37 °C for 3 h, and the reaction was stopped by boiling for 10 min. The ACE-inhibiting activity of the treated peptides was determined by HPLC.

2.8. Statistical analysis

Results of the experiments are given as means ± SD. Differences between treatments were determined by Duncan's multiple-comparison test using SPSS.

3. Results and discussion

3.1. Time profile of ACE inhibitory activity for yak milk casein hydrolysate obtained at different hydrolysis times

Alcalase was used for the production of yak casein hydrolysate. The ACE inhibitory activity of the obtained hydrolysate was measured over a 360 min period, as shown in Fig. 1. It showed that ACE-inhibiting activity increased during the first 240 min of hydrolysis. If the hydrolysis time was too long, the ACE inhibition activity decreased. Yak milk casein hydrolysate obtained by incubation with alcalase for 240 min (A4) had the highest ACE-inhibiting activity. We also observed that the ACE-inhibiting activities of the hydrolysates obtained at 300 min or longer hydrolysis times were significantly lower than that of the 240 min hydrolysate ($p < 0.05$). Fig. 1 also showed that the ACE inhibition activity was correlated with the DH of hydrolysate. When the DH was too high (or when hydrolysis time was longer than 300 min), the ACE-inhibiting factors generated during yak milk casein hydrolysis lost their ability to inhibit ACE activity.

3.2. Separation of active peptide fractions from the hydrolysate by ultrafiltration, gel filtration and HPLC

3.2.1. Ultrafiltration result

In the first isolation step, after separation by 6 kDa and 10 kDa molecular weight cut-off membranes, three components were obtained (>10 kDa, 6–10 kDa, below 6 kDa). The most active component was the one with molecular mass less than 6 kDa (Table 1).

Table 1 showed that the ACE inhibitory activity of the >10 kDa (23.1 ± 0.87%) and 6–10 kDa component (29.2 ± 1.79%) was significantly lower than that of the whole hydrolysate (79.5 ± 0.51%) ($p < 0.01$). The below 6 kDa fraction showed the highest ACE inhibitory activity of 85.4 ± 1.37%, which was significantly higher than that of the whole hydrolysate ($p < 0.01$). It showed that the lower was the molecular weight of the hydrolysate fraction, the higher was its ACE inhibitory activity, and the molecular weight of the most effective fraction was below 6 kDa. This result is similar to that of Yamamoto et al. (1999), Hernández, Amigo, Ramos, and Recio (2004) and Robert et al. (2004).

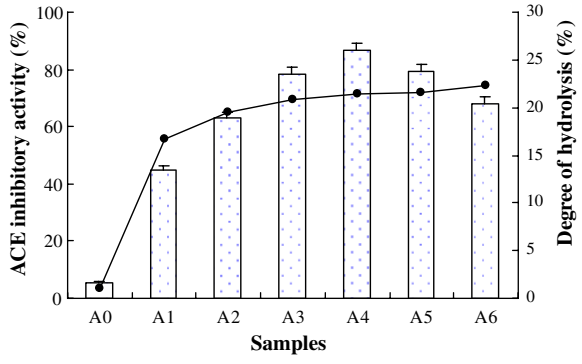

Fig. 1. Time profiles of ACE inhibitory activity (%, means ± SD) of yak milk casein hydrolysate. (Column chart is for ACE inhibition activity; graph chart is for hydrolysis degree of casein hydrolysate). A0, A1, A2, A3, A4, A5 and A6 represent the hydrolysate samples obtained at different hydrolysis times, i.e., 0 min, 60 min, 120 min, 180 min, 240 min, 300 min and 360 min, respectively.

Table 1
The ACE-inhibitory activity of the separated yak milk casein alcalase–hydrolysate fractions obtained by ultrafiltration through 10 kDa and 6 kDa molecular weight cut-off membranes

Hydrolysates	ACE inhibitory activity (%)
Yak casein hydrolysate	$79.5^{b} \pm 0.51$
>10 kDa	$23.1^{a} \pm 0.87$
6–10 kDa	$29.2^{a} \pm 1.79$
Below 6 kDa	$85.4^{c} \pm 1.37$

Values within a column with different letters are significantly different ($p < 0.01$).

3.2.2. Ion exchange and sephadex G-25 gel filtration of active fraction

The 6 kDa permeate was further subjected to DE-52 ion exchange, and fractionated into five portions (see Fig. 2). All fractions were collected, freeze-dried, and the ACE inhibitory activity was measured; the third fraction possessed the strongest ACE-inhibiting activity of 87.5% which was selected for subsequent peptide identification.

The active fraction after ion exchange of casein hydrolysate was subjected to size exclusion chromatography on Sephadex G-25 and was fractionated into four portions, the third fraction (A4-3) had the highest ACE-inhibiting activity of 92.12% (see Fig. 3).

3.2.3. RP-HPLC separation of active fraction

This active fraction A4-3 was further fractionated by RP-HPLC on a C_{18} reversed-phase column, yielding two strong ACE-inhibiting fractions A4-3-II and A4-3-VI (Fig. 4). The purity of these two fractions was determined by HPLC, and detected as two single compounds with retention time of 11.52 min and 24.88 min, respectively.

3.2.4. The molecular masses and amino acid sequences of A4-3-II and A4-3-VI

The molecular masses of the purified ACE inhibitors (A4-3-II and A4-3-VI) from yak casein hydrolysate were estimated to be 550 Da and 566.4 Da as determined by LC–ESI-MS. Their amino acid sequences were Pro–Pro–Glu–Ile–Asn (PPEIN) and Pro–Leu–Pro–Leu–Leu (PLPLL), respectively, as detected by MS–MS. Their

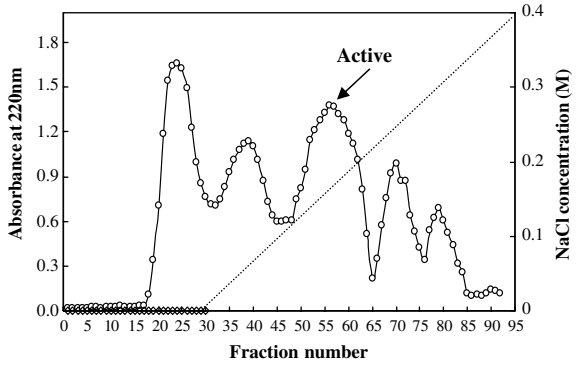

Fig. 2. The DE-52 ion exchange chromatogram of the most active component below 6 kDa eluted with a linear gradient of 0.4 M NaCl.

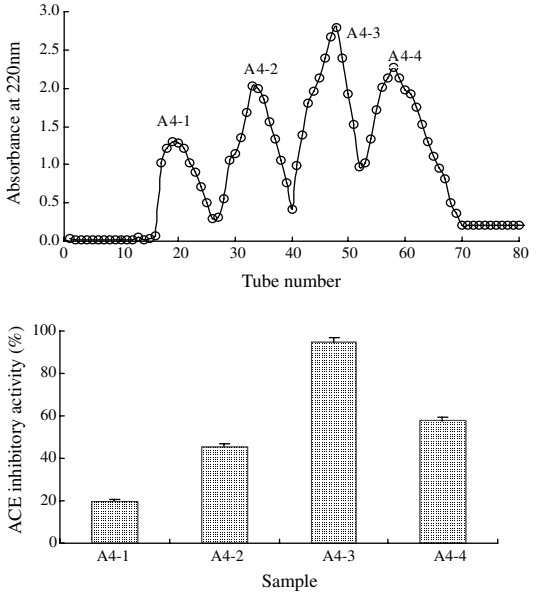

Fig. 3. Elution pattern of the active fraction after DE-52 on a Sephadex G-25 gel filtration column and the ACE inhibitory activity of the separated fractions.

IC_{50} values were 0.29 ± 0.01 mg/ml and 0.25 ± 0.01 mg/ml, respectively.

3.3. Stability of ACE inhibitor from yak casein hydrolysate to ACE

After co-incubation of ACE inhibitor with ACE after 3 h, the ACE-inhibiting activities of them were determined (Table 2). The results showed that the ACE-inhibiting

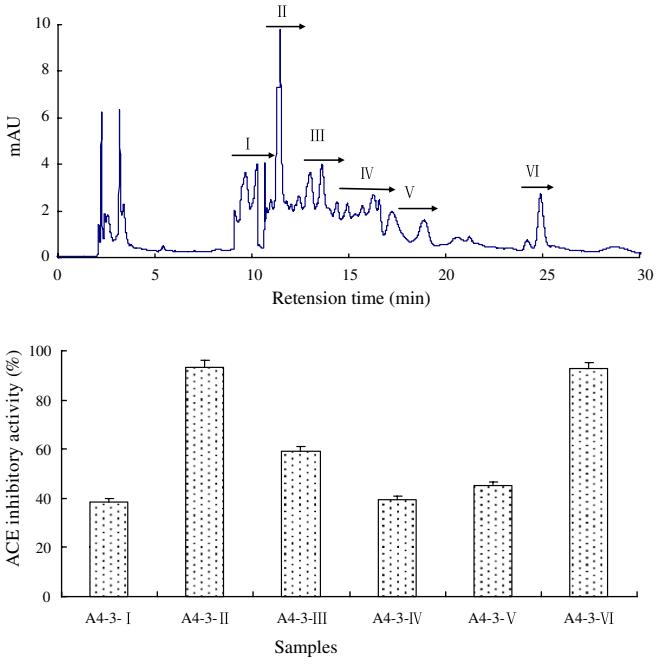

Fig. 4. RP-HPLC chromatograms of A4-3 and the ACE inhibitory activity of the separated fractions.

Table 2
Comparison of ACE inhibitory activity and IC_{50} value of yak milk casein derived peptide before and after co-incubation with ACE

Sample	ACE-inhibitory activity (%[a])		IC_{50} value (mg/ml)[a]	
	Before co-incubation with ACE	After co-incubation with ACE	Before co-incubation with ACE	After co-incubation with ACE
PLPLL	93.20 ± 0.98	92.87 ± 0.81	0.25 ± 0.01	0.25 ± 0.01
PPEIN	92.55 ± 0.87	92.34 ± 0.91	0.29 ± 0.01	0.29 ± 0.01

[a] Not significantly different ($p > 0.05$).

activity and IC_{50} value of PPEIN and PLPLL did not change before and after co-incubation ($p > 0.05$) with ACE, which showed that PPEIN and PLPLL were true ACE inhibitors, and could not be hydrolysed by ACE.

4. Discussion

The amino acid composition of yak milk casein is very similar to that of cow milk (Ochirkhuyag, Chobert, Dalgalarrondo, Choiset, & Haertle, 1997). Casein is divided according to the homology of its primary structures (i.e., amino acid sequences) into four families, i.e., α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN. Moreover, there are different genetic variants in caseins, e.g., α_{s1}-casein has A, B, C, D and E genetic variants, and variant B predominates in bovine milk and variant C in yak (*Bos grunniens*) milk (Eigel et al., 1984). Variant B differed from C by the substitution of 192-Glu for Gly. For α_{s2}-casein, variant C was observed in yak and variant A in bovine milk (Grosclaude, Mahé, Mercier, Bonnemaire, & Teissier, 1976, 1982). Variant C differs from variant A at positions 33, 47 and 130 (Mahé & Grosclaude, 1982). For β-CN and κ-CN, variant A is predominant in yak (*Bos grunniens*) (Grosclaude et al., 1976) and bovine milk.

In this study, we identified two novel ACE-inhibitory peptides, PPEIN (κ-CN; f156–160) and PLPLL (β-CN; f136–140). PLPLL resembles the sequence structure NLHLPLPLL, which was derived from sodium caseinate hydrolysates treated by *Lactobacillus helveticus* NCC 2765 (Robert et al., 2004). Robert et al. (2004) thought NLHLPLPLL was from the amino acid residue of β-CN (f144–155). However, we think that PLPLL is from β-CN (f136–140). Anyway, PLPLL or the sequence including it possesses high ACE-inhibiting activity. The IC_{50} value of NLHLPLPLL was much higher than that of ENLHLPLPLL and VENLHLPLPLL (Robert et al., 2004), which meant that, in the case of this peptide chain, the shorter was the peptide chain, the higher was its ACE-inhibitory activity. We can conclude from the above results that the active core may be small. The relationship between activity and structure of PLPLL is now in progress in our laboratory.

Most of the reported ACE-inhibitory peptides are from α-CN and β-CN, except IPP (κ-CN f108–110). In this study, we obtained a novel ACE-inhibiting peptide PPEIN (κ-CN; f156–160) from κ-CN. In cheese production, chymosin will cut κ-CN at Phe_{105}–Met_{106}, so κ-CN (f106–169) will be expelled along with whey. In Qula, a kind of acid curd, κ-CN remained in yak casein portions. We luckily obtained PPEIN from an amino acid residue of κ-CN (f156–160). Moreover, it has been reported that hydrophobic amino acid residues at the C-terminal positions of peptides were effective in enhancing binding to the ACE (Cheung, Wang, & Ondetti, 1980). PPEIN does not closely conform to this rule. However, Gobbetti, Ferranti, Smacchi, Goffredi, and Addeo (2000) pointed out that ACE-inhibitory peptides derived from caseins contain a high proportion of hydrophobic peptides (>60%). PPEIN, which can be seen from its amino acid composition of 60% hydrophobic amino acid residues and 40% hydrophilic ones, keeps to this rule. PLPLL exhibited hydrophobic nature, since it is composed of a majority of hydrophobic amino acid. During purification on RP-HPLC column this peptide eluted with a long retention time, confirming its hydrophobic nature (Fig. 4). Also, according to previous papers, ACE-inhibitory peptides have at least one proline residue (Abubakar et al., 1998; Nakamura et al., 1995). Similar results were observed in this study. Two isolated ACE-inhibitory peptides were composed of Pro–Pro or Pro–Leu at their N-terminals.

Some milk casein-derived ACE inhibitory peptides have been identified from its pepsin or trypsin hydrolysate. In this study, we used alcalase to hydrolyse yak milk casein and obtained novel ACE-inhibiting peptides. This study also showed that the ACE-inhibitory activity of yak milk casein hydrolysate varied with the hydrolysis time, and that of the hydrolysate obtained at 240 min hydrolysis was the highest.

The objective of this work was to investigate the potential inhibiting effect of yak milk casein enzymatic hydrolysate on ACE. Our present results demonstrate that yak milk casein–alcalase hydrolysate has high and stable ACE-inhibiting activity, but yak milk casein itself showed very low antihypertensive activity. The molecular weight of the active fraction was lower than 6000 Da. The molecular masses of the purified ACE inhibitors from yak casein hydrolysate were 550 Da and 566.4 Da, and their amino acid sequences were Pro–Pro–Glu–Ilu–Asn (PPEIN) and Pro–Leu–Pro–Leu–Leu (PLPLL), respectively. Moreover, these peptides showed high stability to ACE, which meant that they were true ACE inhibitors. These results provide a good foundation for the value-added utilisation of yak milk casein as a natural antihypertensive ingredient. The ACE-inhibiting activity of yak milk casein enzymatic hydrolysates makes it potentially commercial attractive in the future as a 'health-enhancing ingredient' in the production of functional foods. Further studies on the*in vivo*

antihypertensive activity of active yak casein peptides and their functional mechanisms are currently in progress in our laboratory.

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