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Yak Milk Casein as a Functional Ingredient: Preparation and Identification of Angiotensin-I-Converting Enzyme Inhibitory Peptides

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Yak milk casein derived from Qula, a traditional Tibetan acid curd cheese, was hydrolyzed by six commercially available proteases (Trypsin, Pepsin, Alcalase, Flavourzyme, Papain and Neutrase). These hydrolysates were assayed for their inhibitory activity of Angiotensin-I-converting enzyme (ACE). The hydrolysates obtained by Neutrase from *Bacillus amyloliquefaciens* showed the highest ACE inhibitory activity. The IC_{50} value of Neutrase-hydrolysate was 0·38 mg/ml. The hydrolysate obtained by Neutrase was further separated by consecutive ultra-filtration with 10 kDa and then with 6 kDa molecular weight cut-offs into different permeated parts and fractionated by gel filtration chromatography with a Sephadex G-25 column. The active fraction was subjected to RP-HPLC, in which five peaks were purified and identified. Amino acid sequence analysis confirmed that the peptides and origins were as follows: YQKFPQY (α_{s2}-CN; f89–95), LPQNIPPL (β-CN; f70–77), SKVLPVPQK (β-CN; f168–176), LPYPYY (κ-CN; f56–61) and FLPYPYY (κ-CN; f55–61). Their amino acid sequences matched well with those of known bioactive peptides from bovine casein. The results indicated that yak milk casein could be a resource to generate antihypertensive peptides and be used as multifunctional active ingredients for many value-added functional foods as well as a traditional food protein.

Keywords: Angiotensin-I-converting enzyme, ACE inhibitory peptide, Qula, yak milk casein.

Abbreviations: ACE, Angiotensin-I-converting enzyme; FAPGG, 2-Furanacryloyl-1-Phe-Gly-Gly; DH, degree of hydrolysis; IC_{50}, inhibitory concentration that inhibits 50% of ACE activity; MWCO, molecular weight cut-off; RP-HPLC, reversed-phase high performance liquid chromatography; ESI-MSn, Electrospray Ionization Tandem Mass Spectrometry.

In recent years, antihypertensive peptides have been extensively studied due to the high incidence of hypertension, which is currently considered to be the most common form of chronic illnesses. Most of the food-derived peptides function as antihypertensive agents by inhibiting the Angiotensin-I-converting enzyme (ACE). ACE is a dipeptidyl carboxypeptidase that plays a major role in the cardiovascular homeostasis by catalyzing the conversion of decapeptide angiotensin-I to octapeptide angiotensin-II and by degrading bradykinin (Meng & Oparil, 1996). ACE inhibitory peptides have been isolated from protein hydrolysates and fermented products (Yamamoto et al. 1994, 1999, 2003; Mullally et al. 1997; Abubakar et al. 1998; Haileselassie et al. 1999; Sandrine et al. 2003; Vermeirssen et al. 2003). Milk proteins are the most important source of ACE inhibitory peptides. Peptides can be produced from milk protein by enzymatic hydrolysis or by fermentation through starter cultures (Korhonen & Pihlanto, 2003). Using commercially available food grade enzymes to hydrolyze casein will be advantageous because these enzymes are cheap and safe. Some bovine casein derived ACE inhibitory peptides have been produced by enzymatic hydrolysis with various proteases, such as Trypsin (Maruyama et al. 1987), sumizyme FP, sumizyme CP and sumizyme RP (Mizuno et al. 2004), pepsin and trypsin (Pihlanto-Leppälä et al. 1998).

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On the Qinghai-Tibetan plateau of China, there are more than 13 million yaks producing a large quantity of milk annually, with 300 000 000 kg in 2004 alone (Liu, 2005). Yak milk and milk products are the major ingredients of the daily diets of Tibetan herders. However yak herders scatter across a large area of the plateau and there is a lack of transportation and storage facility. Therefore it is difficult to collect fresh yak milk for dairy processing. Most of the yak milk is made into a traditional food, ghee, and the rest is acidified naturally, leached, collected and dried, resulting in Qula, a traditional protein ingredient in many Tibetan foods. Qula is also widely used as a raw material for manufacturing industrial casein of different grades.

The total dry matter of yak milk is around 17–18% during the main lactating period, the protein content varies at around 5·5% (Dong et al. 2003). The amino acid composition of total casein in yak milk was similar to that in cow milk (Ochirkhuyag et al. 1997). There are different genetic variants in milk protein, e.g. α_{s1}-casein have A,B,C,D and E genetic variants, variant B predominate in bovine and variant C in yak, variant C differs from B by the substitution of Gly for 192-Glu (Wang & Zhou, 1995). In addition, the milk protein polymorphism analysis shows the allelic combination α_{s1}-CnC-β-CnA2-κ-CnA is the most frequent among the yaks (Grosclaude et al. 1976), which is closer to their bovine counterparts.

The objectives of this study were to use various commercially available enzymes to hydrolyze yak milk casein Qula, to separate and purify the ACE inhibitory peptides in the hydrolysates, and to determine the ACE inhibitory activity of these peptides. In addition, certain peptides were further sequenced and compared with the known sequences of bioactive peptides derived from bovine casein to explore the potential of yak milk casein as a functional ingredient for value-added foods.

Materials and Methods

Sources of experiment materials

Qula from the Tibetan plateau was collected and provided by Hua-Ling Company (Lanzhou, China). Sources of the six commercially available proteases used in this study were as follow: Trypsin (EC 3.4.21.4, 10 000 U/mg protein) and Pepsin (EC 3.4.23.1, 2500 U/mg protein) were purchased from Amresco Company (Solon, Ohio, USA), Alcalase (EC 3.4.21.62, 136 000 U/mg protein) and Flavourzyme (15 000 U/mg protein) were from Novo Company (Novo Nordisk, Denmark), Papain (EC 3.4.22.2, 28 000 U/g protein) and Neutrase (EC 3.4.24.6, 28 000 U/ml protein) were purchased from UnikBio Company (Biocon Company, Guangzhou, China). The origins of the proteases are shown in Table 1. Rabbit lung acetone powder and 2-Furanacryloyl-1-Phe-Gly-Gly (FAPGG) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). The reagents used in the RP-HPLC analysis were HPLC grade chemicals and other reagents used in this study were reagent grade chemicals.

Table 1. The optimal hydrolysis pH and temperature conditions for different enzymes digesting yak milk casein

Enzyme	Origin	pH	Temperature (°C)
Trypsin	Bovine pancrease	8·0	37
Alcalase	*Bacillus licheniformis*	8·0	60
Pepsin	Porcine pancrease	2·0	37
Neutrase	*Bacillus amyloliquefaciens*	7·0	45
Papain	Papaya	7·0	65
Flavourzyme	*Aspergillus oryzae*	7·0	37

Casein preparation

Casein was precipitated from Qula through acidification. Qula was first dissolved in 0·05 M-NaOH and heated to 85 °C until all the particles of Qula were dissolved completely. The solution was cooled to 25 °C and acidified to pH 4·6 using 1 M-HCl to induce casein precipitation. The acid curd was lyophilized, resulting in yak milk casein. This yak milk casein consisted of 872·5 g protein/kg, 19·8 g minerals/kg, 14·9 g fat/kg and 80·5 g moisture/kg.

Hydrolysate preparation

The optimal hydrolysis pH and temperature of the enzymes are shown in Table 1. Solution (50 g/kg) of the yak milk casein was obtained by dissolving in hot water (85 °C), and by adjusting pH to the hydrolysis condition with the addition of 1 M-NaOH or 1 M-HCl (for peptic reaction). The enzymes were added directly to the solution at 100 : 2 ratio of protein substrate to enzyme (w/w). Yak milk casein was hydrolyzed in a thermostat equipped with a stirrer and a pH detector. The pH was monitored by continuous addition of 1 M-NaOH or 1 M-HCl. Each reaction mixture was incubated for 12 h, the hydrolysate was taken at various intervals and the enzyme activity was inactivated by heating to 95 °C for 10 min. The hydrolysates were cooled and the pH was adjusted to 4·6. After centrifugation (4200 ***g***, 20 min), the suspension was collected and its pH adjusted to 7·0. The hydrolysates were then lyophilized and stored at −20 °C.

Cross-digestions of yak milk casein with a combination of two different enzymes were also conducted. The casein was hydrolyzed by the first enzyme for 2 h and then heated at 95 °C for 15 min to inactivate the first enzyme, and then the substrate was adjusted to the optimal conditions for the second enzyme. The second hydrolysis was incubated for 4 h, the hydrolysate was taken at various intervals and the enzyme activity was inactivated by heating to 95 °C for 10 min. These hydrolysates were treated as above.

Measurement of Degree of Hydrolysis (DH)

The *o*-phthaldialdehyde (OPA) method used to measure DH (Church et al. 1983; Spellman et al. 2003).The OPA reagent was prepared by combining the following reagents

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and diluting to a final volume of 100 ml with water: 75 ml 100 mM-borate buffer (pH 9·5), 5 ml 200 g SDS/l, 80 mg OPA (in 2 ml methanol) and 200 µl β-mercaptoenthanol. The reagent was prepared daily and protected from light. The OPA assay was carried out by the addition of 20 µl hydrolysate (or standard) to 2·4 ml OPA reagent. The absorbance of this solution was measured at 340 nm with an UV spectrophotometer (UNICO WFZUV-2102PC, Shanghai, China), the absorbance value was taken after 5 min. L-Isoleucine (0–2 mg/ml) was used as standard. The DH values were calculated using the following formula: $DH\% = 100(Abs_{hydrolysate} - Abs_{casein})Md/N\varepsilon c$, where M the average molecular mass of casein was calculated to be 23 kDa, ε the molar extinction coefficient at 340 nm (6000/mol/cm) and N the total number of peptide per protein molecule was 200 (Church et al. 1983), d the dilution factor, c the protein concentration.

Measurement of ACE inhibitory activity

ACE inhibitory activity was measured by spectrophotometry using FAPGG as substrate (Vermeirssen et al. 2002). The rabbit lung acetone extract was prepared by dissolving 1·0 g rabbit lung acetone powder in 10 ml 75 mM-Tris-HCl with 300 mM-NaCl buffer at pH 8·3 and by ultra-centrifuging the mixture at 40 000 ***g*** (3K3O Sigma refrigerated centrifuge, Osterode, Germany) under 4 °C for 40 min. The supernatant possessing high ACE activity was stored at 4 °C. The ACE assay was performed at 37 °C in a 75 mM-Tris-HCl with 300 mM-NaCl buffer at pH 8·3, containing 300 µl FAPGG (1·0 mm) and 500 µl diluted rabbit lung acetone extract and 300 µl deionized water (blank) or peptide solution. The ACE inhibitory activity was measured in a heated cuvette holder of a UV spectrophotometer by following the absorption decrease at 340 nm during a period of 7 min and expressed as the absorption decrease per min. The ACE inhibitory activity in the presence of 1 mg peptide/ml was then determined in triplicate.

The yak casein hydrolysates digested by different enzymes for 3 h were used to calculate the IC_{50} value. A plot of inhibitory activity versus log10 of sample concentration was generated using five different concentrations (ranging from 0·10 to 2·0 mg/ml). Each sample was tested in triplicate and the mean value was plotted in the curves. IC_{50} value defined as the concentration of inhibition which gives 50% inhibition of ACE activity was calculated using the linear regression equations of the curves.

Purification of ACE inhibitory peptides

The hydrolysate was subjected to ultra-filtration, using a stirred cell ultra-filtration system fitted with 40-mm diameter polyether-sulfone membranes, with a 10 kDa molecular weight cut-off (MWCO) and then with 6 kDa at 10 °C, 0·25 MPa. The hydrolysate concentration was 40 g/kg. The permeate was lyophilized and stored at −20 °C.

The 6 kDa permeate was fractionated using a SephadexG-25 column (26 × 800 mm, Pharmacia Fine Chemicals AB, Uppsala, Sweden). Gel filtration chromatography was carried out at 10 °C, eluted by distilled water at a flow rate of 0·6 ml/min, and elution was measured with an on-line UV detector at 215 nm. Six fractions were obtained by the separation and each fraction was collected, lyophilized and stored under −20 °C.

The fraction showing the highest ACE inhibitory activity was submitted for RP-HPLC for further separation and purification. RP-HPLC was performed on a system equipped with an intelligent pump (Shimadzu LC-10AT, Kyoto, Japan), a variable wavelength absorbance detector set at 215 nm (Waters 2487 Dual λ Absorbance Detector, Milford, Massachusetts, USA) and Shim-pack PREP-ODS C18 column (20 × 250 mm, Shimadzu). The sample concentration was 1 mg/ml and the injection volume was 200 µl. Peptides were eluted with a linear gradient of 10 to 60% solvent B (acetonitrile : trifluoroacetic acid, 1000 : 0·5, v/v) in solvent A (water : trifluoroacetic acid, 1000 : 0·5, v/v) at a flow rate of 6 ml/min for 35 min. The flow was split by placing a T-piece connected with a tube of an adjusted length to give approximately 1 ml/min of flow directed into the detector. The separation was repeated 8 times and different corresponding eluted fractions were combined and dried under vacuum. Each fraction was assayed for the ACE inhibitory activity. The active fractions were again applied onto a C18 column (4·6 × 250 mm, Kromasil, Bohus, Sweden). The injection volume was 20 µl and the absorbance of the elution was 215 nm, using a linear gradient from 20 to 50% B at a flow rate of 0·5 ml/min for 20 min.

Peptide analysis

In order to analyse the molecular masses of peptides and the sequences of their active fractions obtained by chromatography, an Electrospray Ionization Tandem Mass Spectrometry (ESI-MS_2) of the fractions was carried out in the National Center of Biomedical Analysis of China, in Beijing.

Determination of protein concentration

The protein content of yak milk casein was determined using the Kjeldahl procedure (AOAC, 2000). The peptide concentration of the hydrolysate of casein and fractions from the various separation steps were measured by *o*-phthaldialdehyde method (Church et al. 1983).

Statistical analysis

Results are reported as mean ± standard deviation. Data were analysed by one-way ANOVA. Differences between the groups were assessed by the Bonferroni test using the SAS program (version 8.2, Cary, NC, USA). We considered the differences between the means to be significant if $P < 0{\cdot}05$.

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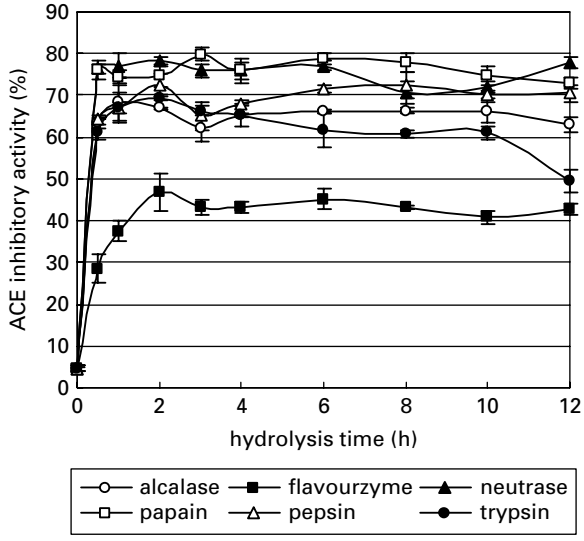

Fig. 1. ACE inhibitory activity (%, means ± SD) changes of yak milk casein hydrolysates released by different enzymes: (○), Alcalase; (●), Trypsin; (△), Pepsin; (▲), Neutrase; (□), Papain; (■), Flavourzyme.

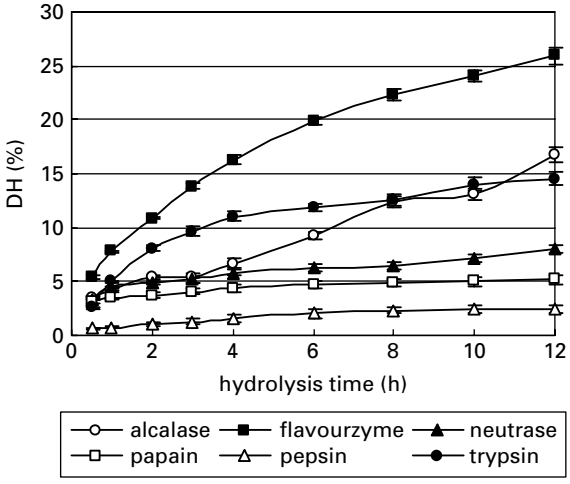

Fig. 2. DH (%, means ± SD) changes of yak milk casein hydrolysates released by different enzymes: (○), Alcalase; (●), Trypsin; (△), Pepsin; (▲), Neutrase; (□), Papain; (■), Flavourzyme.

Results and Discussion

Yak milk casein hydrolysates and ACE inhibition activity assay

ACE inhibitory activities of the hydrolysates obtained with different enzymes versus the hydrolysis times are summarized in Fig. 1. The un-hydrolyzed yak milk casein had a very low ACE inhibitory activity (<5% inhibition), which indicated that the parent protein had a low bioactivity. Robert et al. (2004) identified the ACE inhibitory activity in milk proteins fermented with *Lactobacillus helveticus* NCC 2765, caseinate hydrolyzed by *Lb. helveticus* for 1 and 2 h strongly inhibited ACE activity. In our study, the hydrolysates showed ACE inhibitory activities from the first 30 min of digestion. Neutrase and Papain had the highest and most stable activity after 30 min hydrolysis among all the enzymes. Flavourzyme-derived hydrolysates showed the lowest ACE inhibitory activity. Except Trypsin, the ACE inhibitory activity of hydrolysates digested by the same enzyme from 2 to 12 h did not show differences ($P>0{\cdot}05$). Those of Trypsin reached the highest value at 3 h and then decreased gradually afterwards.

The DH value increased during hydrolysis (Fig. 2). Casein hydrolyzed by Flavourzyme gave the highest DH value from 5·42 ± 0·21% (0·5 h) to 25·91 ± 0·85% (12 h). Alcalase and Trypsin also showed high DH values. At the same hydrolysis time, the DH value of the casein hydrolysates obtained with different enzymes was significantly different ($P<0{\cdot}05$). Comparing ACE inhibitory activity with DH value showed they appeared not to be correlated, in agreement with reports of Mullally et al. (1997) and Vermeirssen et al. (2003).

In the cross-digestions of yak milk casein with a combination of two enzymes, inhibitory abilities were largely controlled by one enzyme, as shown in Table 2. The Pepsin-Trypsin combination yielded a higher activity than a single enzyme. There were no improvements in the ACE inhibitory activity observed in the other combination digestions and the ACE inhibitory activity decreased significantly ($P<0{\cdot}05$) when casein was hydrolyzed by the second enzyme for 4 h. It appeared that these hydrolysates were further digested and cleaved part of the active peptides, especially in the presence of Trypsin or Pepsin in cross-digestions. Sandrine et al. (2003) suggested that digestive enzyme diminished ACE inhibition by the peptides present in the Emmental cheese water-soluble extract.

The ACE inhibitory activity of the 3 h hydrolysates obtained with six enzymes was compared by determining the IC_{50} values (Table 3). The IC_{50} value of Neutrase-hydrolysate was the lowest and that of the Flavourzyme-hydrolysate was the highest (5·5 times that of Neutrase). The IC_{50} values of ovine and caprine β-lg hydrolysates obtained with microbial proteases were consistently lower than those obtained with digestive enzymes (Hernández-Ledesma et al. 2002). However, the same result was not observed in this study. It might be due to the different substrate protein and enzyme combination. Proteinase K (Abubakar et al. 1998), a protease from *Aspergillus oryzae*, sumizyme FP (Mizuno et al. 2004) and thermolysin (Hernández- Ledesma et al. 2002) were selected as the most potent protease producing ACE inhibitory activity peptides in the respective studies. It has been shown that the choice of enzyme will affect the ACE inhibitory activity of the protein hydrolysate and the form of the active peptide because of the enzyme specificities. In our study,

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Table 2. ACE inhibitory activity of casein hydrolysates prepared by combination of two enzymes following different hydrolysis time

Values are means ± SD for $n=3$

	ACE inhibition (%)			
	1st enzyme hydrolysis time	2nd enzyme hydrolysis time		
	2 h‡	0·5 h	2 h	4 h
Neutrase†-Papain	77·68^{a} ± 1·11	70·20^{b} ± 1·89	72·60^{ab} ± 2·39	70·40^{b} ± 1·22
Neutrase-Trypsin	76·90^{a} ± 1·41	70·80^{ab} ± 1·11	67·60^{b} ± 2·16	66·20^{b} ± 1·39
Neutrase-Pepsin	77·80^{a} ± 1·28	73·56^{ab} ± 1·95	72·05^{b} ± 2·44	71·30^{b} ± 1·37
Neutrase-Alcalase	77·68^{a} ± 1·39	64·47^{bc} ± 2·24	69·96^{b} ± 1·03	61·00^{c} ± 0·21
Papain-Trypsin	72·49^{a} ± 1·22	61·52^{b} ± 0·52	54·64^{c} ± 1·64	51·85^{c} ± 0·29
Papain-Pepsin	73·09^{a} ± 1·69	71·37^{a} ± 3·03	67·28^{ab} ± 1·31	61·85^{b} ± 0·26
Pepsin-Trypsin	58·50^{a} ± 1·22	60·72^{ab} ± 0·46	66·64^{b} ± 1·43	63·85^{b} ± 0·88
Alcalase-Trypsin	66·93^{a} ± 0·27	66·28^{a} ± 0·22	60·83^{b} ± 0·44	58·34^{b} ± 2·90
Alcalase-Pepsin	66·63^{a} ± 0·22	58·95^{b} ± 2·48	55·66^{b} ± 1·46	49·24^{c} ± 0·71

† Enzyme addition order
‡ The hydrolysis condition was adjusted to the optimal condition of the second enzyme
^{a,b,c} Values in the same row followed by a common superscript were not significantly difference ($P<0·05$)

Table 3. The IC_{50} value (amount of hydrolysate needed to inhibit 50% of the ACE activity) of 3 h hydrolysates obtained by treatment of yak milk casein with different enzymes

Enzyme	IC_{50} (mg/ml)
Trypsin	0·847
Alcalase	1·143
Pepsin	1·162
Neutrase	0·384
Papain	0·426
Flavourzyme	2·115

the Papain-hydrolyte had a similar IC_{50} value to Neutrase-hydrolysate, but the protein recovery of the hydrolysate was lower than those of Neutrase (data not shown). Therefore we selected the hydrolysate prepared from Neutrase for further peptide identification.

Purification and characterization of ACE inhibitory peptides

The molecular weight of the identified ACE-inhibitory peptides is mostly smaller than 3 kDa (Abubakar et al. 1998; Pihlanto-Leppälä et al. 1998; Haileselassie et al. 1999; Yamamoto et al. 1999; Hernández-Ledesma et al. 2002, 2004; Robert et al. 2004). Ultra-filtration membranes are reported to be a possible method to enrich ACE inhibitory peptides in protein hydrolysates (Mullally et al. 1997; Hernández-Ledesma et al. 2004). In our study, the 3 h Neutrase-hydrolysate of yak milk casein was further fractioned using ultra-filtration membranes, permeates and retention were used to assay the ACE inhibitory activity (Table 4). The ACE inhibitory activity of the 10 kDa permeates was similar to that of the whole hydrolysate

Table 4. The ACE inhibitory activity of fraction obtained by separated yak milk casein Neutrase-hydrolysate by ultrafiltration through 10 and 6 kDa molecular weight cut-off membranes

Values are means ± SD for $n=3$

	ACE inhibition (%)
casein hydrolysates	77·98^{a} ± 0·56
10 kD retention	25·84^{b} ± 1·21
10 kD permeates	79·96^{a} ± 3·35
6 kD retention	54·15^{c} ± 2·12
6 kD permeates	89·96^{d} ± 1·55

^{a,b,c,d} Values in the same row followed by a common superscript were not significantly difference ($P<0·05$)

while the 6 kDa permeates fraction showed the highest activity ($P<0·05$). It appeared that the lower the molecular weight of a hydrolysate fraction, the higher its ACE inhibition activity.

The ACE inhibitory activities of 6 fractions obtained by gel filtration chromatography (Fig. 3) of the 6 kDa permeates are shown in Table 5. Fraction E5 had the highest ACE inhibitory activity Among these fractions, E3 had the highest concentration (data not shown), but lowest ACE inhibitory activity.

E5 was further purified by RP-HPLC into five different peptide fractions (Fig. 4A). Fraction E5b was then subjected to further purification and 3 major peptide fractions were collected (Fig. 4B). Fractions E5d, E5e and E5b1 to E5b3 were analysed by MS/MS. The result from the MS/MS analysis was matched with the known casein amino acid sequence reported by Fox & McSweeney (1998). The ACE inhibition count at 1 mg peptide/ml of the fractions and the identified peptide sequences are displayed in Table 6.

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Table 5. The ACE inhibitory activity of fraction obtained by separated the 6 kDa ultrafiltration permeates of Neutrase-hydrolysate though the SephadexG-25 column

Values are means ± SD for $n=3$

Fraction†	ACE inhibition (%)
E1	$50{\cdot}90^{a} \pm 1{\cdot}12$
E2	$67{\cdot}19^{b} \pm 1{\cdot}67$
E3	$35{\cdot}18^{c} \pm 3{\cdot}35$
E4	$64{\cdot}03^{bd} \pm 0{\cdot}56$
E5	$77{\cdot}47^{e} \pm 1{\cdot}12$
E6	$60{\cdot}86^{d} \pm 3{\cdot}63$

† Fractions correspond to those given in Fig. 3

[a,b,c,d,e] Values in the same row followed by a common superscript were not significantly difference ($P<0{\cdot}05$)

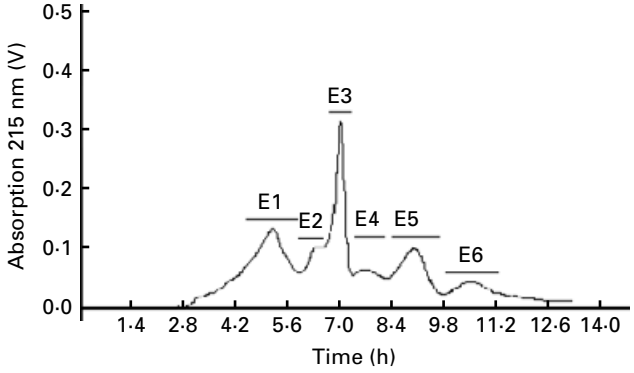

Fig. 3. Gel filtration chromatography of the 6 kDa permeates of yak milk Neutrase-hydrolysate on the SephadexG-25 column. Six fractions were collected and designated as E1–E6.

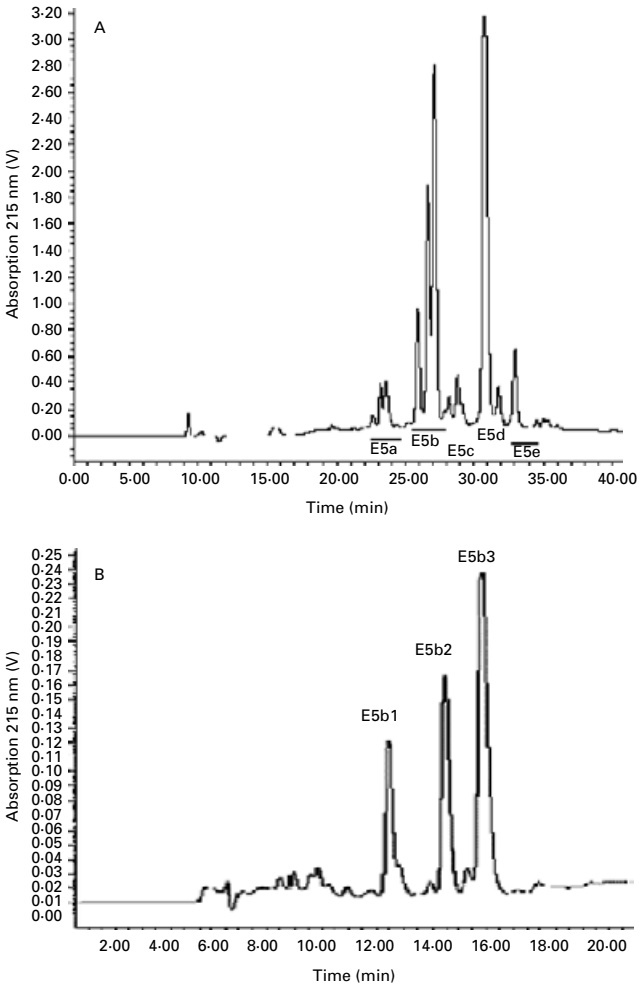

Fig. 4. (A) Separation of peptides from active fraction E5 by RP-HPLC. Five fractions were collected and designated as E5a–E5e. (B) The E5b fraction further Separated by RP-HPLC on a Kromasil C18 column. The peaks were designated as E5b1–E5b3.

The sequence of E5b1, E5b2 and E5b3 were identified as YQKFPQY, LPQNIPPL and SKVLPVPQK following the MS/MS analysis (Table 6). Because of their low contents, the ACE inhibitory activities of these fractions were not detected. Though LPQNIPPL resembled the bioactive peptide sequence structure IPP (β-CN, f74–76) (Nakamura et al. 1995), and SKVLPVPQK shared important structural features with SKVLPVPQ (β-CN, f168–175) (Yamamoto et al. 1994) and VLPVPQ (β-CN, f170–175) (Hernández-Ledesma et al. 2004), which were the previously identified bioactive peptides from bovine casein, it was impossible to determine which peptide maintained the ACE inhibitory activity of fraction E5b. We need to chemical synthesise the peptides found in th study and detect their ACE inhibition in the further study.

Peptides E5d and E5e originated from the same amino acid sequence region of κ-CN (Table 6). The hexapeptides E5d (LPYPYY) might be liberated from heptapeptides E5e (FLPYPYY) during the hydrolysis and had higher ACE inhibitory activities than their precursors. The dipeptide YP (κ-CN, f58–59) originated from bovine κ-casein has been reported by Yamamoto et al. (1999). It had been purified from yogurt-like product fermented by *Lb. helveticus* CPN4 and showed a strong antihypertensive activity in spontaneously hypertensive rats with an IC_{50} value of 720 μM. The κ-casein alignment of yak, cow and other cattle are shown in Fig. 5. It is clear that the peptide sequence FLPYPYY in Yak milk casein exists also in Cow, Zebu and Buffalo milk caseins. It is speculated that hydrolysis of cow milk with Neutrase will also result in the ACE inhibitory peptides.

Haileselassie et al. (1999) treated the enzyme-modified cheese with Neutrase® for 8 h and detected β-casomorphin with a sequence of YPFPGPI (β-CN, f60–66). We did not obtain the same peptide in this study, probably because of different hydrolysis conditions. In addition, we have not identified the sequences of all peptides in the hydrolysates and a part of the peptides could be lost during chromatographic purification. The heating steps during hydrolysis could cause loss of activity as well.

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Table 6. Molecular mass determination and peptide identification of the fractions of the yak milk casein Neutrase-hydrolysates obtained by RP-HPLC with ACE inhibitory activity

¶ Values are means ± SD for $n = 3$

Fraction no.†	Peptide concentration (μg/ml)	ACE inhibition (%)¶	Observed mass	Calculated mass‡	Sequence position§	Amino acid sequence
E5a	220·35	$31·38^{a} \pm 1·14$	—††	—	—	—
E5b	215·5	$73·92^{b} \pm 2·44$	—	—	—	—
E5b1	—	—	973·52	973·47	α_{s2}-CN (f89–95)	YQKFPQY
E5b2	—	—	891·27	891·53	β-CN (f70–77)	LPQNIPPL
E5b3	—	—	995·56	995·62	β-CN(f168–176)	SKVLPVPQK
E5c	225·05	$59·69^{c} \pm 1·64$	—	—	—	—
E5d	118·25	$83·16^{d} \pm 1·37$	815·56	815·39	κ-CN (f56–61	LPYPYY
E5e	112·23	$70·56^{b} \pm 2·49$	961·3	961·4	κ-CN (f55-61)	FLPYPYY

† Fraction no. corresponds to the fraction number given in Fig. 3 & 4
‡ Average mass values
§ Protein sequence according to Fox and Mcsweeney (1998)
[a,b,c,d] Values in the same row followed by a common superscript were not significantly difference ($P < 0·05$)
†† — not determined

```
Cow AAQ87922      MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPIQ  50
Yak AAF63191      ------------------------------RCEKDERFFSDKIAKYIPIQ
Yak AAB86616      --------------------------------------------------
Zebu AAQ73172     --------------------------------------------------
Buffalo AAU95771  MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQ

                  YVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVL
Cow AAQ87922      100
Yak AAF63191      YVLSRYLSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVL
Yak AAB86616            NNQFLPYPYYAKPAAVRSPAQILQWQVL
Zebu AAQ73172     -VLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVL
Buffalo AAU95771  YVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVL

Cow AAQ87922      SNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGE  150
Yak AAF63191      SNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGE
Yak AAB86616      SNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGE
Zebu AAQ73172     SNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGE
Buffalo AAU95771  PNTVPAKSCQAQPTTMTRHPHPHLSFMAIPPKKNQDKTEIPTINTIVSVE

Cow AAQ87922      PTSTPTTEAVESTVATLEDSP----EVIESPPEINTVQVTSTAV        200
Yak AAF63191      RTSTPTTEAVESTVATLEASP----EVIESPPEINTVQVTSTAV
Yak AAB86616      PTSTPTTEAVESTVATLEASPEASPEVIESPPEINTVQVTSTAV
Zebu AAQ73172     PTSTPTIEAVESTVATLEASP----EVIESPPEINTVQVTSTAV
Buffalo AAU95771  PTSTPTTEAIENTVATLEASS----EVIESVPETNTAQVTSTVV
```

Fig. 5. Yak (Bos grunniens), cow (Bos Taurus) and other cattle kappa casein alignment. They shared a common ACE inhibitory peptide fragment FLPYPYY. The amino acid sequence obtained from protein database of NCBI (website: http://www.ncbi.nlm.nih.gov).

The relationship between structure and activity in different ACE inhibitory peptides indicated that binding to ACE was strongly influenced by the C-terminal tri-peptide sequence of the substrate (Vermeirssen et al. 2004). It has been reported that hydrophobic (aromatic or branched-chain aliphatic) amino acid residues at each of the three C-terminal positions of synthesized peptides were effective in enhancing binding to the ACE (Cheung et al. 1980). Researchers suggested that ACE inhibitory peptides from milk protein had a proline residue at the C-terminal end of the peptide (Yamamoto et al. 1999; Mizuno et al. 2004). Most of the peptides from the enzymatic hydrolysates in this study further confirmed this suggestion.

In conclusion, yak milk casein hydrolysates obtained with Neutrase, a protease from *Bacillus amyloliquefaciens*, show high ACE inhibitory activity *in vitro* and some peptides were identified from yak milk casein hydrolysates.

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These findings could expand the application of yak milk casein as multifunctional active ingredients to many value-added functional foods.

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