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Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin

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Abstract

Ovine and caprine β-lactoglobulin (β-Lg) were isolated from sweet and acid whey by precipitation with trichloroacetic acid. This method allowed a rapid purification of ovine and caprine β-Lg with high purity (higher than 92%), when starting from acid whey. These β-Lg preparations were digested with trypsin, chymotrypsin, proteinase K and thermolysin and the angiotensin converting enzyme (ACE)-inhibitory activity was determined at different hydrolysis times. Consistently, higher activity was found in the hydrolysates prepared with enzymes of microbial origin. Four novel ACE-inhibitory peptides were purified and identified from caprine β-Lg hydrolysed with thermolysin. The identified peptides were caprine β-Lg f(46–53), f(58–61), f(103–105), and f(122–125), with ACE-inhibitory activities (IC_{50}) that ranged from 34.7 to 2470 μM. The structure of the identified active peptides in relation to previous structure–activity studies is discussed.

Keywords: Ovine and caprine whey; β-Lg hydrolysates; ACE-inhibition; Bioactive peptides

1. Introduction

Due to the increasing production of whey as by-product of cheesemaking, the options for processing of whey into a variety of products is being extensively studied. During the last few years, the development of processes to isolate or concentrate added-value molecules with biological activity, which can be used as nutraceuticals in functional foods, has acquired a special relevance (Blendford, 1996). In the Mediterranean countries, cheeses are mainly manufactured from ovine and caprine milk or from a mixture of milks from different species. The use and exploitation of other species' whey is less extended and elimination of this by-product still causes many contamination problems.

Whey proteins can be a source of peptides with biological activity. For instance, potent antibacterial peptides have been found within the sequence of bovine lactoferrin (Bellamy et al., 1992; Recio & Visser, 1999) or bovine α-lactalbumin (Pellegrini, Thomas, Bramaz, Hunziker, & von Fellenberg, 1999). Several studies have also found peptide sequences derived from whey proteins with opioid activity (Chiba & Yoshikawa, 1986) or antihypertensive properties (Mullally, Meisel, & FitzGerald, 1996). Most of the food-derived antihypertensive peptides act by inhibiting the angiotensin-I converting enzyme (ACE). ACE is a peptidyldipeptide hydrolase that catalyses both the formation of the potent vasoconstrictor, angiotensin-II, and the inactivation of bradykinin, a vasodilator peptide. Thus, inhibition of ACE results in a lowering of blood pressure (Koike, Ito, Miyamoto, & Nishino, 1980).

Several authors have achieved the preparation of enzymatic hydrolysates of whey proteins with ACE inhibitory properties by using digestive enzymes (Mullally, Meisel, & FitzGerald, 1997a), microbial enzymes (Abubakar, Saito, Aimar, & Itoh, 1996) or a combination of both (Saito, Abubakar, Itoh, Arai, & Aimar, 1997; Pihlanto-Leppälä, Rokka, & Korhonen, 1998). Recently, some studies have also identified the whey-derived peptides responsible for such activity (Mullally, Meisel, & FitzGerald, 1997b; Abubakar,

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Saito, Kitazawa, Kawai, & Itoh, 1998; Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000). While bovine whey or isolated proteins, bovine β-lactoglobulin (β-Lg) or α-lactalbumin, have been employed as precursor proteins of ACE-inhibitory peptides, whey from other species has not been studied in this regard (Bordenave, Sannier, & Piot, 2000). In this study, we describe the isolation of β-Lg from ovine and caprine whey and the preparation of hydrolysates with ACE-inhibitory activity using enzymes of digestive and microbial origin. Furthermore, different ACE-inhibitory peptides were isolated from the thermolysin digest of caprine β-Lg and these active sequences were identified by N-terminal sequencing in combination with mass spectrometry (MS).

2. Materials and methods

2.1. Substrate and enzymes

β-Lg was obtained from acid and sweet whey of ovine and caprine milk by the method of Fox, Holsinger, Posati, and Pallansch (1967) as modified by Ebeler, Phillips, and Kinsella (1990). The purity of β-Lg was evaluated by electrophoretic and chromatographic techniques. Isoelectrofocusing (IEF) was carried out using the PhastSystem® (Pharmacia LKB Technology, Upssala, Sweden) with PhastGel IEF 3–9 according to the manufacturer's instructions (Pharmacia, 1984). Capillary electrophoresis (CE) was performed in a Beckman P/ACE System MDQ (Beckman Instruments Inc., Fullerton, CA, USA) using the method of Recio and Olieman (1996). RP-HPLC was carried out as described in Section 2.2.

Both trypsin (EC 3.4.21.4., Type I, 10,900 U mg^{-1} protein) and chymotrypsin from bovine pancreas (EC 3.4.21.1. Type I–S, 44 U mg^{-1} protein) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Proteinase K (EC 3.4.21.14, 20 U mg^{-1}) from *Tritirachium album* and thermolysin (EC 3.4.24.4, 35 U mg^{-1}) from *Bacillus thermoproteolyticus* were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Hippuryl–histidyl–leucine (Hip–His–Leu) and ACE (EC 3.4.15.1, 5.1 U mg^{-1}) were purchased from Sigma.

2.2. Digestion of β-Lg with enzymes

Hydrolysates were prepared using optimal conditions with respect to buffer, pH, type and enzyme:substrate ratio, and temperature for each enzyme, as shown in Table 1. β-Lg was dissolved in the selected buffer (5 mg mL^{-1}) and digested for 48 h at either 37°C (trypsin, proteinase K and thermolysin) or 25°C (chymotrypsin). Portions were withdrawn from the mixtures at 0 and 30 min, and after 2, 8, 24, 36 and 48 h hydrolysis. The hydrolysis was stopped by heating at 95°C for 15 min. The progress of the hydrolysis was monitored by RP-HPLC. Equipment comprised two pumps (module 116; Beckman), and a diode array detector (module 168; Beckman) in combination with an autosampler 717 plus (Waters corp., Mildford, MA, USA), and data processing software (program Gold System, version 7.11, Beckman). Solvents A and B were water:acetonitrile:TFA at ratios of 950:50:1 and 100:900:1, respectively. The column was a Nova-Pack C_{18}, 60 Å, 4 μm, 3.9 × 150 mm (Waters) with a C_{18} cartridge (Waters) as guard column. Samples were eluted with a linear gradient of solvent B in A going from 0% to 50% over 60 min at a flow rate of 0.8 mL min^{-1}. The absorbance of the eluent was monitored at both 214 and 280 nm. Sample concentrations were approx. 3–5 mg mL^{-1} and the injection volume was 50 μL.

2.3. Measurement of ACE-inhibitory activity

ACE-inhibitory activity was measured using the method of Cushman and Cheung (1971) as modified by Kim, Yoon, Yu, Lönnerdal, and Chung (1999). Briefly, 20 μL of each hydrolysate was added to 0.1 ml of 0.1 M potassium phosphate containing 0.3 M NaCl, and 5 mM Hip–His–Leu, pH 8.3. ACE (5 mU) was added and the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.1 m 1 M HCl. The hippuric acid formed was extracted with ethyl acetate, heat evaporated at 95°C for 10 min, redissolved in distilled water and measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate.

Protein content of the samples was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford,

Table 1
Hydrolysis conditions employed for the preparation of the β-Lg digests: composition of the buffer, pH, enzyme/substrate ratio (*E*/*S*) and temperature (*T*)

Enzyme	Buffer	pH	*E*/*S* (w/w)	*T* (°C)
Trypsin	0.02 M Tris-HCl 0.01 M $CaCl_2$	8.0	5/100	37
Chymotrypsin	0.02 M CH_3–$COONH_4$	8.0	5/100	25
Proteinase K	0.02 M Tris-HCl	7.5	5/100	37
Thermolysin	0.02 M Tris-HCl 0.01 M $CaCl_2$	8.0	5/100	37

IL, USA) using bovine serumalbumin as standard protein.

The inhibitory activity of the hydrolysates or collected fractions was expressed as percentage of ACE inhibition at a given protein concentration or as the concentration needed to inhibit 50% of the original ACE activity (IC_{50}).

2.4. Preparation of ACE-inhibitory peptides by RP-HPLC

For the separation of the hydrolysates, the same HPLC equipment as described in Section 2.2 was used. Each hydrolysate was filtered through a 0.45 μm filter (PVDF Durapore, Millipore corp., Bedford, MA, USA) and applied to a column Prep Nova-Pack HR C_{18}, 60 Å, 4 μm, 7.8 × 300 mm (Waters). Solvent A was water/TFA (1000:1) and solvent B was acetonitrile/TFA (1000:1). Sample concentrations were approximately 3 mg mL^{-1} and the injection volume was 200 μL. The peptides were eluted with a linear gradient of solvent B in A going from 0% to 50% over 50 min at a flow rate of 1.5 mL min^{-1}. The absorbance of the eluent was monitored at 220 and 280 nm. The fractions were collected from 8 to 10 separate RP–HPLC runs, pooled, dried under vacuum and redissolved in distilled water. Each fraction was tested for the ACE-inhibitory activity. The active fractions were again applied onto the same column under the same conditions but using a linear gradient from 0% to 20% B over 45 min.

2.5. Mass spectrometry and N-terminal sequence analysis

The mass of each purified peptide was measured by mass spectrometry (MS) with a single quadrupole instrument (Hewlett Packard, Palo Alto, CA, USA). Samples were dissolved to a concentration of approximately 10 μg mL^{-1} in 50% (v/v) aqueous acetonitrile, 1% (v/v) acetic acid, and analysed by infusion of the sample solution (10 μL) using a flow 0.5 mL min^{-1} through the electrospray interface. Nitrogen was used as nebulising and drying gas. The temperature and flow of the drying gas was 320°C and 10 min^{-1}, respectively. The capillary was held at 4.0 kV and the cone voltage was 40 V. By using HPChem Station software (version A.07.01, Hewlett-Packard), the *m/z* spectral data were acquired, processed and transformed to spectra representing mass values.

The N-terminal sequence of the purified peptides was identified by sequence analysis with a Perkin-Elmer/Applied Biosystems Procise 494 microsequencer (Überlingen, Germany) running in pulsed liquid mode.

2.6. Peptide synthesis

Peptides were prepared by conventional Fmoc solid-phase synthesis method with a 431A peptide synthesiser (Applied Biosystem Inc., Überlingen, Germany). The purity of the synthesised peptide was verified by analytical RP-HPLC and MS, as described above.

3. Results and discussion

3.1. Preparation of caprine and ovine β-Lg and β-Lg hydrolysates

The purity of the β-Lg preparations obtained from ovine acid, ovine sweet whey, and caprine acid whey after precipitation with trichloroacetic acid was estimated by gel IEF, CE, and RP-HPLC. Fig. 1 shows the CE patterns of the β-Lg preparations obtained from ovine acid and sweet whey. The β-Lg preparation obtained from ovine acid whey was resolved into two CE-peaks corresponding to β-Lg genetic variants A and B, respectively (Fig. 1a). Assignment of the β-Lg genetic variants was performed by following the peak identification reported previously (Recio, Pérez-Rodríguez, Ramos, & Amigo, 1997). The β-Lg preparation obtained from ovine sweet whey also contained these two peaks, but the band corresponding to variant B was significantly less intense than the variant A peak (Fig. 1b). This difference in ratios was also observed when the samples were analysed by RP-HPLC or IEF. Moreover, when using either RP-HPLC or CE, the β-Lg preparation obtained from ovine sweet whey showed a significant amount of caseinmacropeptide (CMP), since at the trichloroacetic concentration employed (3.1%) both β-Lg and CMP remain in solution (Jollès, Alais, & Jollès, 1961) (Fig. 1b). The β-Lg preparation obtained from caprine acid whey resolved as a single band by IEF and as a single peak by RP-HPLC and CE. In general, β-Lg has been considered to be monomorphic in caprine species (Ng-Kwai-Hang & Grosclaude, 1992). The purity of the desalted and freeze-dried β-Lg preparations was 99.9% when prepared from ovine acid whey, 72.6% when isolated from ovine sweet whey, and 92.9% for caprine β-Lg, as judged from the peak areas determined by RP-HPLC.

The ovine and caprine β-Lg preparations were hydrolysed with trypsin, chymotrypsin, proteinase K and thermolysin. Samples were taken at different time intervals. The progress of the hydrolysis reaction was monitored by RP-HPLC and the percentage of ACE inhibition was determined spectrophotometrically. With all four proteases, the RP-HPLC area of the β-Lg peak decreased after 30 min hydrolysis and practically disappeared after 2 h hydrolysis. At longer hydrolysis times, the chromatographic pattern showed an increase in the peak areas of hydrophilic peptides while the peak areas of the more hydrophobic peptides tended to decrease.

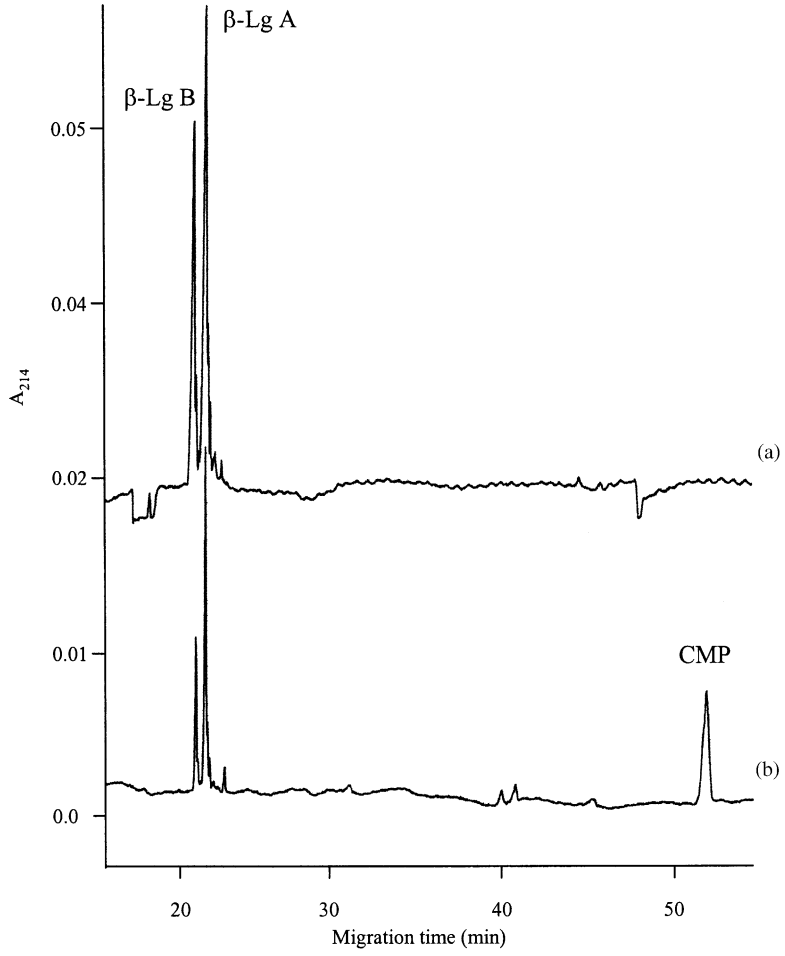

Fig. 1. CE patterns of the β-lactoglobulin preparations obtained from (a) acid ovine whey and (b) sweet ovine whey by precipitation with trichloroacetic acid according to the method of Ebeler et al. (1990). β-Lg A, ovine β-Lg variant A; β-Lg B, ovine β-Lg variant B; CMP, caseinmacropeptide.

The ACE-inhibitory activity of the ovine and caprine β-Lg hydrolysates obtained with these four proteases versus the hydrolysis time is shown in Table 2. ACE-inhibitory activity was not given by undigested ovine or caprine β-Lg preparations or the proteases when tested at the same concentration as that used to produce the hydrolysates. Generally, the ACE-inhibitory activity of trypsin and chymotrypsin hydrolysates increased with hydrolysis time during the first 24 h. Hydrolysates obtained with thermolysin showed higher ACE-inhibitory percentages at 30 min and 2 h, while those obtained with proteinase K exhibited more variation in the activity with the hydrolysis time. At 36 and 48 h hydrolysis time, the ACE-inhibitory activity of the hydrolysates either remained as high as at 24 h or decreased due to further hydrolysis of the active fragments.

The activity of the 24 h hydrolysates obtained with the four proteases was compared by determining the concentration needed to inhibit 50% of the original ACE activity (IC_{50}) (Table 3). The IC_{50} values obtained with microbial proteases were consistently lower than those obtained with digestive enzymes. This result may indicate the presence of peptide sequences with higher ACE-inhibitory activity or higher concentration of active peptides in the hydrolysates obtained with microbial enzymes. It has to be noted that with all proteases except chymotrypsin, lower IC_{50} values were obtained for the hydrolysates prepared from sweet-whey β-Lg than those achieved for the digests of β-Lg from acid whey. This higher activity may be attributed to the presence of ACE inhibitory peptides derived from the CMP. As expected, the choice of the enzyme employed will determine which peptides will be formed because of differences in enzyme specificities. While trypsin produces peptides with Lys or Arg at the C-terminus, chymotrypsin cleaves the peptide bond after aromatic residues (Trp, Tyr, Phe). Therefore, it might be presumed that more potent ACE-inhibitory peptides could be obtained with chymotrypsin, since it has been found that aromatic residues at the C-terminal position enhance ACE-inhibitory activity (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). However, chymotrypsin hydrolysates generally showed the lowest inhibition

Table 2
Percentage of angiotensin converting enzyme (ACE) inhibitory activity versus hydrolysis time of ovine and caprine β-Lg hydrolysates produced by treatment with trypsin, chymotrypsin, proteinase K and thermolysin

Hydrolysis time (h)	ACE-inhibitory activity (%)											
	Trypsin			Chymotrypsin			Proteinase K			Thermolysin		
	Ovine β-Lg[a]	Ovine β-Lg[b]	Caprine β-Lg	Ovine β-Lg[a]	Ovine β-Lg[b]	Caprine β-Lg	Ovine β-Lg[a]	Ovine β-Lg[b]	Caprine β-Lg	Ovine β-Lg[a]	Ovine β-Lg[b]	Caprine β-Lg
0												
0.5	35.1	44.5	62.7	27.0	38.9	36.0	78.1	80.3	63.5	81.5	84.3	91.2
2	48.5	—	67.3	34.0	56.8	50.5	85.3	91.1	58.0	84.7	82.5	89.6
8	57.5	68.4	64.9	46.9	65.3	55.1	84.0	75.9	73.9	80.0	77.0	84.5
24	77.3	69.8	73.9	51.9	75.6	66.4	79.4	87.8	76.0	72.7	66.2	81.7
36	70.3	74.6	59.7	69.2	62.7	64.7	67.6	88.2	36.0	73.4	75.2	81.1
48	34.2	59.6	41.2	67.9	62.4	66.4	76.8	91.2	37.2	66.4	62.9	83.2

Each value corresponds to the average value ($n = 3$).
— Not determined.
[a] Prepared from ovine acid whey.
[b] Prepared from ovine sweet whey.

Table 3
ACE-inhibitory activity of 24 h hydrolysates obtained by treatment of ovine or caprine β-Lg with trypsin, chymotrypsin, proteinase K and thermolysin

Enzyme	IC_{50} (μg β-Lg hydrolysate mL^{-1})		
	Ovine		Caprine
	Acid whey	Sweet whey	Acid whey
Trypsin	278	196	245
Chymotrypsin	213	296	388
Proteinase K	117	38	118
Thermolysin	152	81	161

The activity is expressed as the concentration in mg mL^{-1} needed to inhibit 50% the original ACE activity (IC_{50}).

percentages at each of the selected hydrolysis times assayed. The exception was at 48 h of hydrolysis, where the inhibition index of the tryptic hydrolysates decreased notably, probably due to further cleavage of active peptides formed. The RP-HPLC analyses of the hydrolysates revealed a lower hydrolysis degree with chymotrypsin than with the other proteases. Hydrolysates obtained with microbial enzymes contained β-Lg fragments of lower molecular masses than those obtained with the digestive enzymes (as judged by RP-HPLC-MS; data not shown). It is known that not only the C-terminal residues are important for ACE-inhibitory activity, but also peptide size seems to be a determinant (Cushman, Cheung, Sabo, & Ondetti, 1977).

Although the IC_{50} value of the total hydrolysates was slightly lower when using proteinase K as the proteolytic enzyme, we selected the hydrolysates prepared from caprine β-Lg with thermolysin for further peptide identification studies. Thermolysin hydrolysis has been used to produce ACE-inhibitory peptides from tuna muscle (Matsumura, Fujii, Takeda, Sugita, & Shimizu, 1993), zein (Miyoshi et al., 1991), chicken muscle and egg ovoalbumin (Fujita, Yokoyama, & Yoshikawa, 2000). Due to the enzyme specificity, these authors obtained mostly tri-peptides with high inhibitory activity containing Pro near or at the C-terminal end. However, the peptides formed from β-Lg with this enzyme have not been studied.

3.2. Purification and characterisation of ACE-inhibitory peptides from caprine β-Lg

The 24 h thermolysin hydrolysate from caprine β-Lg was subjected to RP-HPLC at semipreparative scale by using a Prep Nova-Pack HR C_{18} column. The hydrolysate was separated into 11 different fractions (Fig. 2a). Enough material of each fraction was collected in successive analyses to determine ACE-inhibitory activity and the protein concentration of these fractions was determined by the BCA method. Fractions 1, 4, 5, 6, 10 and 11 showed inhibition percentages higher than 50% with protein concentrations that ranged between 85 and 380 μg mL^{-1}. Identification of the peptides included in these fractions by N-terminal sequencing necessitated further purification of the peptide mixtures. The six active fractions and fraction 9, which had an inhibition index of 45.2% at a protein concentration of 13.6 μg mL^{-1}, were again applied into the same RP-HPLC column and peptides were eluted using a shallower acetonitrile gradient. Profiles corresponding to fractions 1 and 6 revealed the presence of a complex mixture of peptides; fraction 4 was resolved and collected into two different fractions (termed as 4.1 and 4.2 in Fig. 2b); and fractions 5, 9, 10 and 11 were

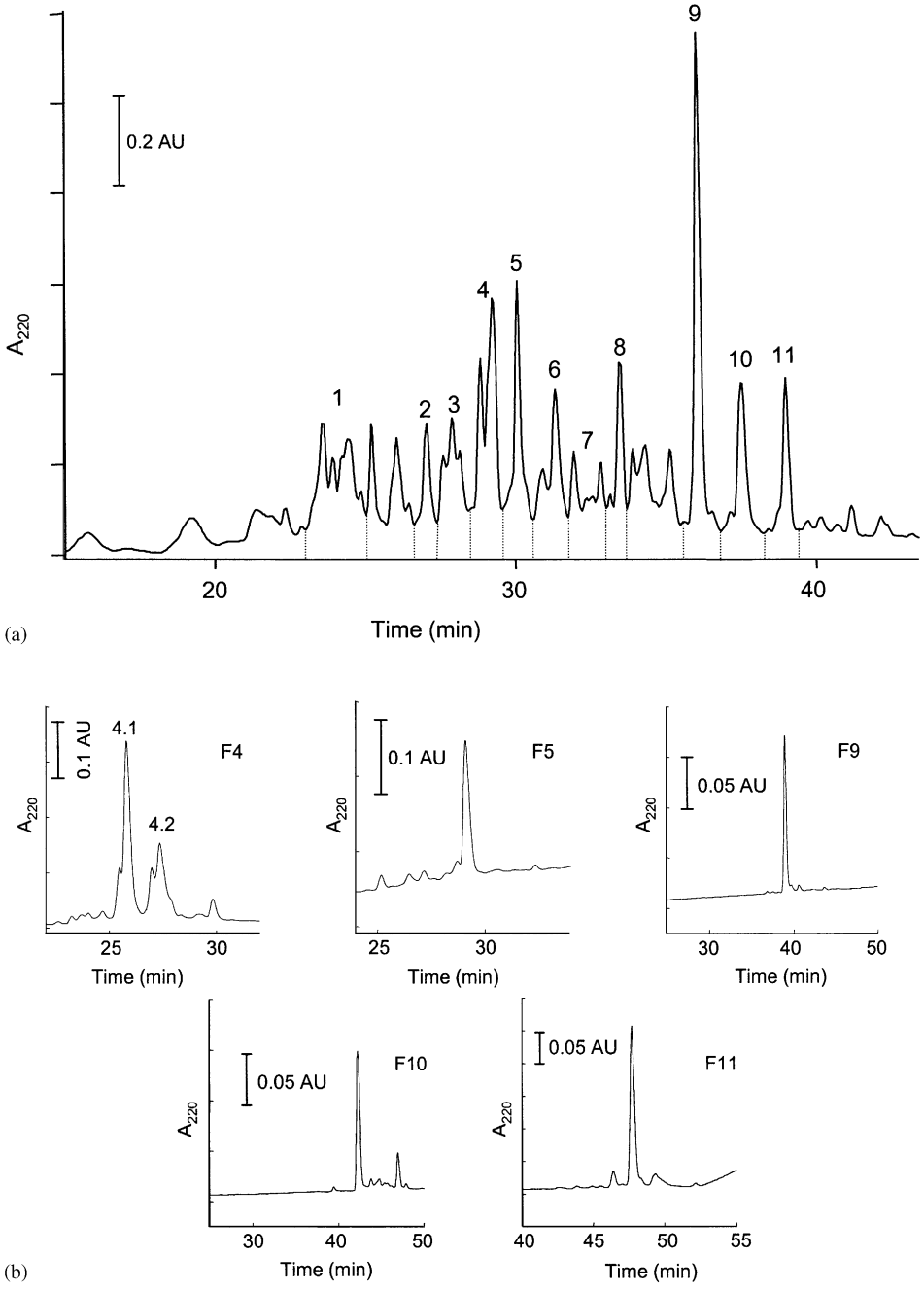

Fig. 2. (a) Separation of the 24 h-thermolysin hydrolysate of caprine β-lactoglobulin by RP-HPLC on a Prep Nova-Pack HR C_{18} column. The numbered fractions were collected and tested for ACE-inhibitory activity. (b) Separation of the most active fractions (F4, F5, F9, F10, and F11) by RP-HPLC on the same column by using a shallower gradient (for experimental conditions, see Section 2.4).

resolved as a major component, which was collected for MS and N-terminal sequence analyses, and one or two minor components, which were discarded (Fig. 2b). The results obtained by combining the N-terminal sequence and molecular mass data were matched with the known amino acid sequence of caprine β-Lg (Preaux, Braunitzer, Schrank, & Stangl, 1979), and these peptide sequences are summarised in Table 4. ACE inhibition indexes were comprised between 45.2% and 75% with protein concentrations of between 13.6 and 372 $\mu g\,mL^{-1}$. The identified major component from fractions 5, 9, 10 and 11 were chemically synthesised and their ACE-inhibitory activities were calculated as IC_{50} (Table 4). From the IC_{50} values it can be concluded that peptides LQKW and LLF were the most potent ACE-inhibitors, while the activity of LVRT was, in comparison, very low. The IC_{50} value of peptide LKPTPEGN could not be determined because this peptide showed a

Table 4
Molecular mass determination and peptide identification of the caprine β-Lg fragments with ACE-inhibitory activity obtained by hydrolysis with thermolysin

Fraction no.	Observed mass	Calculated mass[a]	Sequence position	Amino acid sequence[b]	Peptide content ($\mu g\,mL^{-1}$)	% ACE inhibition[c]	IC_{50}[d] (μM)
4	—	—	—	—	189.2	65.7	nd
4(4.1)	479	479.2	136–139	FDKA	—	—	—
	560	560.3	128–132	VDKEA	—	—	—
4(4.2)	714	713.4	43–48	VEELKP	—	—	—
	600	600.3	123–127	VRTPE	—	—	—
5	854	854.4	46–53	LKPTPEGN	372.0	51.0	>2700
9	392	391.3	103–105	LLF	13.6	45.2	79.8
10	488	487.3	122–125	LVRT	85.6	53.1	2470
11	573	573.3	58–61	LQKW	13.1	75.0	34.7

[a] Average mass values.
[b] Caprine β-Lg sequence according to Preaux et al. (1979).
[c] Each value corresponds to the mean of three measurements.
[d] Concentration (μM) of synthetic peptide needed to inhibit 50% of the original ACE activity.
nd—not determined.
Fraction no. corresponds to the RP-HPLC fraction numbers given in Fig. 2 and numbers between parenthesis indicate the fraction collected after the second RP-HPLC separation.

maximum inhibition percentage of 45–47% for all the concentrations tested (up to 2700 μM).

Other studies performed using chemically synthesised di-peptides showed that, of the naturally occurring amino acids, Trp was the most effective at the C-terminal end for enhancing binding to the ACE, and Phe is also among the best (Cheung et al., 1980). Cheung et al. (1980) reported IC_{50} values of 16 and 930 μM for the di-peptides RW and IF, respectively. Although the contribution of the C-terminal amino acids of di-peptides with ACE-inhibitory activity may not always be strictly extrapolated to longer peptides, it has to be stressed that, considering the protein concentration, the most potent caprine β-Lg fragment identified in this study corresponded to that found in fraction 11 [LQKW, β-Lg f(58–61)] which ended with RW. The di-peptide KW has been found in an alkaline protease hydrolysate of sardine muscle and this fragment showed a potent ACE-inhibitory activity, with an IC_{50} value of only 1.63 μM (Matsufuji et al., 1994). In the same manner, the active peptide found in fraction 9 [LLF, β-Lg f(103–105)] also possesses favourable C-terminal residues for binding to ACE. The tetra-peptide YLLF, that shares the three C-terminal residues with this peptide identified in fraction 9, was found to have ACE-inhibitory activity (Mullally et al., 1996) and opioid-agonistic activity (Chiba & Yoshikawa, 1986).

Fraction 4, which also showed inhibitory activity, was resolved as two components in the second RP-HPLC separation. However, MS analyses revealed the presence of two peptides in each sub-fraction and therefore the activity could not be attributed to a specific fragment. Based on the structure–activity relationships found for peptides with ACE-inhibitory activity, it can be presumed that β-Lg f(123–127), VRTPE, was not responsible for the activity since it is known that the enzyme binds only weakly peptides with C-terminal dicarboxylic amino acids (e.g. Glu) or Pro at the penultimate positions (Cheung et al., 1980). However, the amino acid sequence of β-Lg f(43–48) suggests that this peptide, which ended with LKP, might contribute to the ACE-inhibitory activity observed in this fraction. Rohrbach, Williams, and Rolstad (1981) showed by using N-blocked synthetic peptides that, in terms of binding specificity to ACE, Pro as C-terminal amino acid and a positively charged amino acid in the penultimate position had a positive influence for peptide-enzyme binding.

In conclusion, higher activities were observed for the caprine and ovine β-Lg hydrolysates obtained with microbial enzymes than those prepared with digestive enzymes, suggesting a more efficient cleavage for the formation of ACE-inhibitory peptides with the enzymes of bacterial origin. Two novel caprine β-Lg-derived peptides with potent ACE-inhibitory activity were identified from the hydrolysate prepared with thermolysin as proteolytic enzyme. Of special interest is peptide LLF, included within the sequence of opioid peptide β-lactorphin (YLLF), which is considered a "strategic zone" of β-Lg, partially protected from digestive breakdown. However, further research is needed to confirm the resistance of the formed peptides to digestive enzymes and to extrapolate this in vitro activity to an in vivo effect.

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