Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides

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Summary. The aim of this study was to identify whey-derived peptides with angiotensin I-converting enzyme (ACE) inhibitory activity. The bovine whey proteins α-lactalbumin and β-lactoglobulin were hydrolysed with pepsin, trypsin, chymotrypsin, pancreatin, elastase or carboxypeptidase alone and in combination. The total hydrolysates were fractionated in a two step ultrafiltration process, first with a 30 kDa membrane and then with a 1 kDa membrane. Inhibition of ACE was analysed spectrophotometrically. The peptides were isolated by chromatography and identified by mass and sequencing analysis. The most potent inhibitory peptides were synthesized by the 9-fluorenylmethoxycarbonyl solid phase method. Inhibition of ACE was observed after hydrolysis with trypsin alone, and with an enzyme combination containing pepsin, trypsin and chymotrypsin. Whey protein digests gave a 50 % inhibition (IC_{50}) of ACE activity at concentration ranges within 345–1733 μg/ml. The IC_{50} values for the 1–30 kDa fractions ranged from 485 to 1134 μg/ml and for the < 1 kDa fraction from 109 to 837 mg/ml. Several ACE-inhibitory peptides were isolated from the hydrolysates by reversed-phase chromatography, and the potencies of the purified peptide fractions had IC_{50} values of 77–1062 μM. The ACE-inhibitory peptides identified were α-lactalbumin fractions (50–52), (99–108) and (104–108) and β-lactoglobulin fractions (22–25), (32–40), (81–83), (94–100), (106–111) and (142–146).

Oligopeptides derived from food proteins, especially from bovine milk proteins, are known to have biological activity (Korhonen *et al.* 1998), and some of them have antihypertensive properties. One of the principal physiological functions of angiotensin I-converting enzyme (ACE) is to catalyse the removal of the terminal dipeptide from the decapeptide angiotensin I to give the octapeptide angiotensin II, a potent peptide that causes constriction of blood vessels and assists in the regulation of blood pressure. ACE also contributes to the inactivation of bradykinin, a nonapeptide present in blood, by successively removing two dipeptides. Bradykinin is a potent vasodilator and may be involved in the control of blood pressure. In

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consequence, inhibition of ACE may exert an antihypertensive effect. Several endogenous and food-derived peptides that inhibit ACE have been reported (Ariyoshi, 1993), including peptides derived from casein (Maruyama & Suzuki, 1982; Maruyama *et al*. 1985; Meisel & Schlimme, 1994; Nakamura *et al*. 1995*a*; Maeno *et al*. 1996). Recently, ACE-inhibitory peptides derived from whey proteins have also been identified (Mullally *et al*. 1996, 1997*a*; Pihlanto-Leppälä *et al*. 1998). Some of the peptides identified have been shown to have multifunctional activity, such as β-casomorphin and lactorphins, which have opioid and ACE inhibitory activity (Brantl *et al*. 1981; Antila *et al*. 1991; Meisel & Schlimme, 1994; Mullally *et al*. 1996).

The aim of the present study was to prepare protein hydrolysates with different molecular size distributions that might have special biological properties. Particular emphasis was placed on the possible formation of ACE-inhibitors derived from whey proteins.

materials and methods

Substrates and enzymes

Bovine α-la and β-lg were isolated according to Pearce (1983). Successive filtration (0·2 μm ceramic membranes and 10 kDa cut-off membranes) yielded a whey protein concentrate that was used to separate individual whey proteins. α-Lactalbumin was separated from β-lg using pH adjustment, heat treatment and centrifugation (Tupasela *et al*. 1997). Total protein was determined by the Kjeldahl method. The purity of individual protein fractions was analysed by ion-exchange chromatography (Humphrey & Newsome, 1984) with a MonoQ HR 5/5 column (Pharmacia, S-751 82 Uppsala, Sweden) using 20 mM-piperazine buffer, pH 6·5. The protein content of the α-la fraction was 0·73 g/g; 76 % of the total protein was α-la and the rest mainly β-lg. The protein content of the β-lg fraction was 0·85 g/g with 92 % of β-lg and the remainder α-la. Most enzymes used in this study were obtained from Sigma (St Louis, MO 63178, USA): pepsin (EC 3.4.23.1, from porcine stomach, activity 3200–3800 BAFE units/mg protein), trypsin (EC 3.4.21.4, from bovine pancreas, type II, activity 10000–13000 BAFE units/mg protein), α-chymotrypsin (EC 3.4.21.1, from bovine pancreas, type II, activity 40–60 units/mg protein), carboxypeptidase B type I (EC 3.4.17.2) and carboxypeptidase A (EC 3.4.17.1). Elastase (EC 3.4.21.36, from bovine pancreas) was obtained from Boehringer Mannheim (D-68298 Mannheim 31, Germany).

Preparation of hydrolysates from whey proteins

α-Lactalbumin and β-lg were hydrolysed with either a single enzyme or a combination of enzymes. Either native or heat-treated (80 °C, 15 min, pH 7·0) substrate was used. In combined hydrolysis the proteins were first hydrolysed with pepsin at pH 2·0 and then, after raising the pH with 0·1 M-NaOH to 8·0, with other enzymes (see Table 1). Proteins were dissolved at 3 g/l in 0·01 M-HCl, pH 2·0 or water, and the pH adjusted to 8·0 with 0·1 M-NaOH. The enzyme:substrate ratio was 1:200 (w/w) and hydrolysis was at 37 °C.

The molecular size of the hydrolysate fractions was controlled by ultrafiltration (Fig. 1) using a Minitan ultrafiltration system and membranes obtained from Millipore Co. (Bedford, MA 01730, USA). The ultrafiltration runs were performed at room temperature under 1 MPa pressure. The membranes were conditioned by 10 min rinsing, and cleaned after each filtration with 0·1 M-NaOH according to the manufacturer's instructions. The fractions were freeze dried and stored at −20 °C for further analysis.

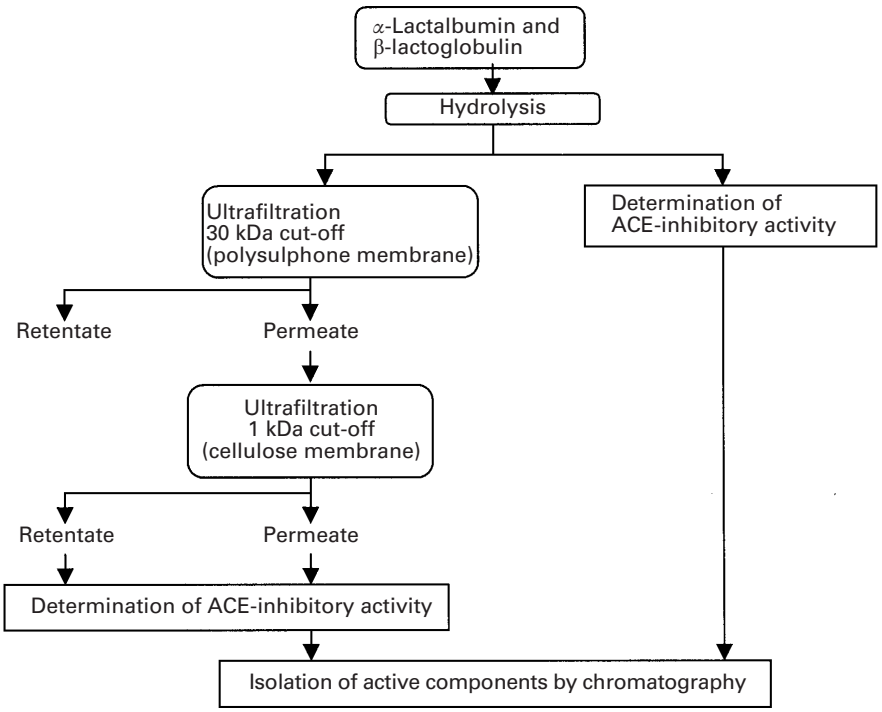

Fig. 1. Schematic outline of the preparation and subsequent fractionation of hydrolysates of α-lactalbumin and β-lactoglobulin.

Chemical characterization of hydrolysates

The molecular mass distribution profiles of the hydrolysates were determined by gel-permeation chromatography. The HPLC equipment consisted of the following: pump (Model 600E; Waters Chromatography division, Milford, MA 01757, USA), automatic sample feeder (Waters Wisp 700), dual wavelength detector (VWM2141; Pharmacia) and Nec data processing equipment (program Maxima; Waters). The column was a Superdex Peptide HR10/30 (Pharmacia), the eluent 0·125 M-potassium phosphate–0·125 M-NaCl, pH 7·0, the flow rate 0·5 ml/min, with detection at 220 nm. Protein standards (Sigma; M_r in parentheses) were β-lg (18400), α-la (14400), aprotinin (6500), bacitracin (1400) and Tyr–Gly (256). Molecular mass distribution was calculated by dividing the total chromatogram area into four ranges of molecular mass (> 5, 5–2, 2–1 and < 1 kDa) and expressed as a percentage of the total area.

The average degree of hydrolysis was evaluated by measuring α-amino groups by the *o*-phthaldialdehyde spectrophotometric method (Frister *et al.* 1988).

Separation of peptides in hydrolysates

The ACE-inhibitory peptides were separated by reversed-phase HPLC. Each sample (~ 40 mg) was dissolved in 1 ml deionized water containing 0·5 ml trifluoroacetic acid (TFA)/l and filtered through a 0·45 μm filter. Portions (100–200 μl) were applied to a reversed-phase column (Super-Pak Pep-S 4·0 × 250 mm, 5 μm, pre-column Pep-S 4·0 × 10 mm, 5 μm; Pharmacia). Solvent A was TFA (0·5 ml/l) and solvent B acetonitrile (900 ml/l containing 0·5 ml TFA/l). A linear gradient was applied from 50 to 600 ml B/l over 45 min at a flow rate of

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1 ml/min. The eluate was monitored at 214 and 280 nm and the fractions were collected on a peak basis and dried in a vacuum. This step was repeated five to ten times, and the fractions from the different chromatographic runs were combined and dried under vacuum. When necessary, the fractions were dissolved in TFA (0·5 ml/l) and applied to a Nucleosil 300–5-C_{18} column (4·0 × 250 mm, 5 μm; Macherey Nagel, D-52348 Düren, Germany) and eluted as in the first step.

The peptides were chemically synthesized with a NovaSyn Gem peptide synthesizer using 9-fluorenylmethoxycarbonyl chemistry (Calbiochem-Novabiochem, Nottingham N67 2QJ, UK).

Characterization of peptides

The amino acid compositions of the peptides and peptide mixtures were analysed by the Pico-Tag method (Millipore Corporation, 1989). The peptides were subjected to gas phase hydrolysis with 6 M-HCl at 112 °C for 24 h under reduced pressure using a Pico-Tag workstation (Waters), derivatized with phenylisothiocyanate, and chromatographed.

The peptides were sequenced by automated Edman degradation using a protein–peptide sequencer (Abi 499 Precise; Perkin Elmer, Foster City, CA 94404, USA). Mass spectroscopy (MS) or mass analysis (MS/MS) were carried out at the Technical Research Centre of Finland, Division of Chemical Technology (PO Box 1401, FIN-02044 Espoo, Finland), using an electrospray mass spectrometer.

Determination of ACE-inhibitory activity

The ACE-inhibitory activity was measured by the spectrophotometric assay of Cushman & Cheung (1971) as modified by Nakamura *et al.* (1995*a*). The method is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine catalysed by ACE. Each assay mixture (250 μl) contained 3·8 mM-Hip-His–Leu, 100 mM-sodium borate buffer, pH 8·3, 300 mM-NaCl, 2 mU ACE (from rabbit lung; Sigma) and 35 μl inhibitor solution. After 30 min incubation at 37 °C, the hippuric acid was extracted with ethyl acetate and measured spectrophotometrically at 228 nm. All analyses were performed in duplicate and three successive assays were carried out with different concentrations. Captopril, known to inhibit ACE, was included in the assays. The inhibitory activity of the hydrolysates and peptide fractions is expressed as the concentration needed to inhibit 50 % of the original ACE activity (IC_{50}).

RESULTS

Chemical characteristics of the hydrolysates

Table 1 shows the degree of hydrolysis and molecular mass distribution in the hydrolysates and the fractions obtained after the second separation step. When a combination of enzymes was used, a higher degree of hydrolysis was found in the hydrolysates. The chromatograms showed that α-la was completely decomposed during pepsin treatment and the hydrolysate was characterized by a high content of polypeptides > 5 kDa. These polypeptides were further degraded into smaller peptides during trypsin treatment. There was no major difference in the molecular mass distribution when heated and unheated α-la were used. According to the chromatogram, part of the β-lg (≈ 30 %) remained intact during pepsin treatment. Heat-treated β-lg was more readily hydrolysed by pepsin than unheated β-lg, more components < 5 kDa being found. The α-la and β-lg hydrolysates obtained after

Table 1. *Enzyme system and hydrolysis times used to prepare the total hydrolysates of whey proteins. Proteins were hydrolysed with a single enzyme or a combination of enzymes first with pepsin and then with other enzymes or enzyme combinations*

Substrate	Enzyme system	Hydrolysis time, h	Degree of hydrolysis, %	Fraction†	Molecular mass distribution, %‡			
					> 5 kDa	2–5 kDa	1–2 kDa	< 1 kDa
α-Lactalbumin	Pepsin	3	6·8	Hydrolysate	46	33	12	9
				Permeate	—	—	42	58
				Retentate	46	23	24	7
α-Lactalbumin (80 °C, 15 min)	Pepsin	3	7·0	Hydrolysate	47	40	11	3
				Permeate	9	2	26	62
				Retentate	32	31	30	7
α-Lactalbumin	Trypsin	3	6·2	Hydrolysate	56	13	17	14
				Permeate	—	16	31	53
				Retentate	52	15	20	13
α-Lactalbumin	Pepsin, then trypsin	3+7	12·1	Hydrolysate	20	30	39	10
				Permeate	—	10	38	52
				Retentate	10	30	42	18
α-Lactalbumin	Pepsin, then trypsin and chymotrypsin	3+7	13·1	Hydrolysate	18	28	40	14
				Permeate	—	—	14	86
				Retentate	19	11	40	30
β-Lactoglobulin	Pepsin	3	4·2	Hydrolysate	41	40	15	4
				Permeate	ND	ND	ND	ND
				Retentate	35	34	27	7
β-Lactoglobulin (80 °C, 15 min)	Pepsin	3	11·9	Hydrolysate	19	70	8	3
				Permeate	—	14	—	86
				Retentate	19	72	9	—
β-Lactoglobulin	Trypsin	3	6·7	Hydrolysate	29	18	22	31
				Permeate	1	2	27	70
				Retentate	23	27	28	24
β-Lactoglobulin	Pepsin, then trypsin	3+7	11·7	Hydrolysate	23	37	26	14
				Permeate	—	8	35	57
				Retentate	14	33	24	29
β-Lactoglobulin	Pepsin, then trypsin and chymotrypsin	3+7	17·0	Hydrolysate	12	17	37	34
				Permeate	—	2	40	58
				Retentate	9	22	38	31

—, Not detected; ND, not determined.

† A two step ultrafiltration process was used to fractionate the total hydrolysates. The first ultrafiltration was with a 30 kDa cut-off membrane and the permeate fraction was then fractionated with a 1 kDa cut-off membrane.

‡ The molecular mass distribution of hydrolysates and fractions was analysed by gel-permeation chromatography.

combined pepsin, trypsin and chymotrypsin treatment were characterized by a higher content of components < 2 kDa than the hydrolysate obtained after pepsin or trypsin treatment.

ACE-inhibitory activity

Inhibition of ACE was found in several products (Table 2). α-Lactalbumin hydrolysed with pepsin had no inhibitory activity, whereas α-la hydrolysed with trypsin or enzyme combinations did display inhibitory activity. β-Lactoglobulin hydrolysed with pepsin, trypsin and different enzyme combinations also had ACE-inhibitory activity. With both proteins, the most active hydrolysates were obtained after hydrolysis with trypsin, and the permeate fraction obtained after trypsin hydrolysis gave the highest ACE-inhibitory activity (Table 2). In most cases permeate fractions had higher inhibitory potency than the corresponding retentate fractions.

Purification and characterization of ACE-inhibitory peptides from α-lactalbumin

The products from α-la hydrolysed with trypsin and the 1 kDa permeate fraction from α-la hydrolysed with pepsin, trypsin and chymotrypsin were selected for purification of their inhibitory components.

Altogether 20 peptide peaks were collected from the total α-la trypsin hydrolysate in the first chromatography step (Fig. 2*a*). Inhibitory activity was found in two peptide fractions, which we named A2 and A10 (IC_{50} 782 and 576 μg/ml respectively). These were further purified by a second chromatographic stage (Fig. 2, middle trace). From fractions A2 and A10 two active peptide peaks were collected, A2.2 and A10.2, with IC_{50} values of 66 and 357 μg/ml respectively. These peptides were analysed by MS and the results are shown in Table 3. The peptide (Trp–Leu–Ala–His–Lys), which corresponds to α-la residues 104–108, was synthesized and its ACE-inhibitory activity measured. The synthetic peptide had an IC_{50} value of 77 μM.

The 1 kDa permeate fraction from α-la hydrolysed with pepsin, trypsin and chymotrypsin was separated into 16 fractions by reversed-phase chromatography (Fig. 2*b*). Two of these, B11 and B12, had IC_{50} values of 473·5 and 534·4 μg/ml respectively. Peptide sequencing and MS analysis identified these two peptide fractions (Table 3). B11 contained one peptide, whereas B12 was a mixture of three. The peptide corresponding to α-la residues 50–52 (Tyr–Gly–Leu) was synthesized and found to have an IC_{50} value of 409 μM.

Purification and characterization of β-lactoglobulin ACE-inhibitory peptides

The total hydrolysate from β-lg hydrolysed with trypsin and the 1 kDa permeate fraction from pepsin, trypsin and chymotrypsin hydrolysis were selected for the purification of inhibitory peptides.

A total of 16 peptide peaks were collected from the trypsin hydrolysate, three of which – C2, C4 and C5 – proved to have inhibitory activity with IC_{50} values of 404·6, 227·5 and 616·6 μg/ml (Fig. 2*c*) The peptides were identified by MS analyses (Table 3). The peptide corresponding to β-lg residues 22–25 (Leu–Ala–Met–Ala) in peak C4 was synthesized and had an IC_{50} value of 1062 μM.

Reversed-phase chromatography of the 1 kDa permeate fraction gave 17 peptide peaks (Fig. 2*d*) and ACE-inhibitory activity was found in fractions D8, D9, D10 and D12, with IC_{50} values 515, 296, 807 and 1054 μg/ml respectively. MS analyses indicated the presence of one peptide in peaks D8, D9 and D10, whereas D12 contained two.

Table 2. *Angiotensin I-converting enzyme inhibitory activity of samples obtained after hydrolysis of α-lactalbumin and β-lactoglobulin and subsequent ultrafiltration through 30 and 1 kDa molecular mass cut-off membranes*

Enzyme system and hydrolysis times	Fraction	Antiotensin I-converting enzyme inhibition activity†	
		α-Lactalbumin	β-Lactoglobulin
Pepsin (3 h)	Hydrolysate	—	1134
Trypsin (3 h)	Hydrolysate	345	457
	1–30 kDa fraction	1134	685
	< 1 kDa fraction	109	237
Pepsin (3 h), then trypsin (7 h)	Hydrolysate	565	702
	1–30 kDa fraction	667	593
	<1 kDa fraction	837	691
Pepsin (3 h), then trypsin and chymotrypsin (7 h)	Hydrolysate	509	580
	1–30 kDa fraction	485	1006
	< 1 kDa fraction	314	720
Pepsin (3 h), then trypsin, chymotrypsin, carboxypeptidaseA and carboxypeptidase B (7 h)	Hydrolysate	1491	1200
Pepsin (3 h), then trypsin, chymotrypsin and elastase (7 h)	Hydrolysate	1229	1733

† Values are the concentrations in μg/ml needed to inhibit 50% of the original antiotensin I-converting enzyme activity, expressed as peptide or protein content.

DISCUSSION

ACE-inhibitory peptides were produced during the hydrolysis of α-la and β-lg and also in the fractions obtained after the second ultrafiltration. The highest inhibitory activity was produced by trypsin hydrolysis, and there was little or none with pepsin hydrolysis. It seems that the hydrolysis time of 3 h with pepsin was insufficient to release ACE-inhibitory peptides, and a longer hydrolysis period would be needed. Mullally *et al.* (1997*b*) reported that 4 h hydrolysis of whey proteins with pepsin produced an ACE-inhibitory activity very similar to hydrolysis with trypsin. The decrease in ACE inhibitory activity in the presence of elastase or carboxypeptidase in the enzyme mixture was probably due to further degradation of the peptides. This is consistent with the finding of Mullally *et al.* (1997*b*) that elastase does not seem to produce very potent ACE inhibitors. Similarly, lactic acid fermentation has been found to decrease ACE-inhibitory activity in samples predigested with enzymes such as pepsin or trypsin (Saito *et al.* 1997).

We have reported previously that the < 1 kDa fractions from α-la hydrolysed with pepsin or β-lg hydrolysed either with pepsin and trypsin or with pepsin, trypsin and chymotrypsin have no opioid properties although they contain the whey protein-derived opioid peptides α-lactorphin and β-lactorphin (Pihlanto-Leppälä *et al.* 1996). In the present study we found that the ACE-inhibitory activity in the < 1 kDa fraction was in many cases higher than in the other fractions tested. These results, together with those of Mullally *et al.* (1997*b*), indicate that it may be possible to exploit ultrafiltration to enrich ACE-inhibitory peptides derived from whey protein.

The inhibitory activity of the hydrolysates in this study derived from several potent inhibitory peptides. The present results and those of our earlier study (Pihlanto-Leppälä *et al.* 1998) suggest that the peptides originating from α-la residues 99–110 may make a considerable contribution to the ACE-inhibitory activity of α-la hydrolysates. An α-la tryptic peptide, f(99–108), which was further

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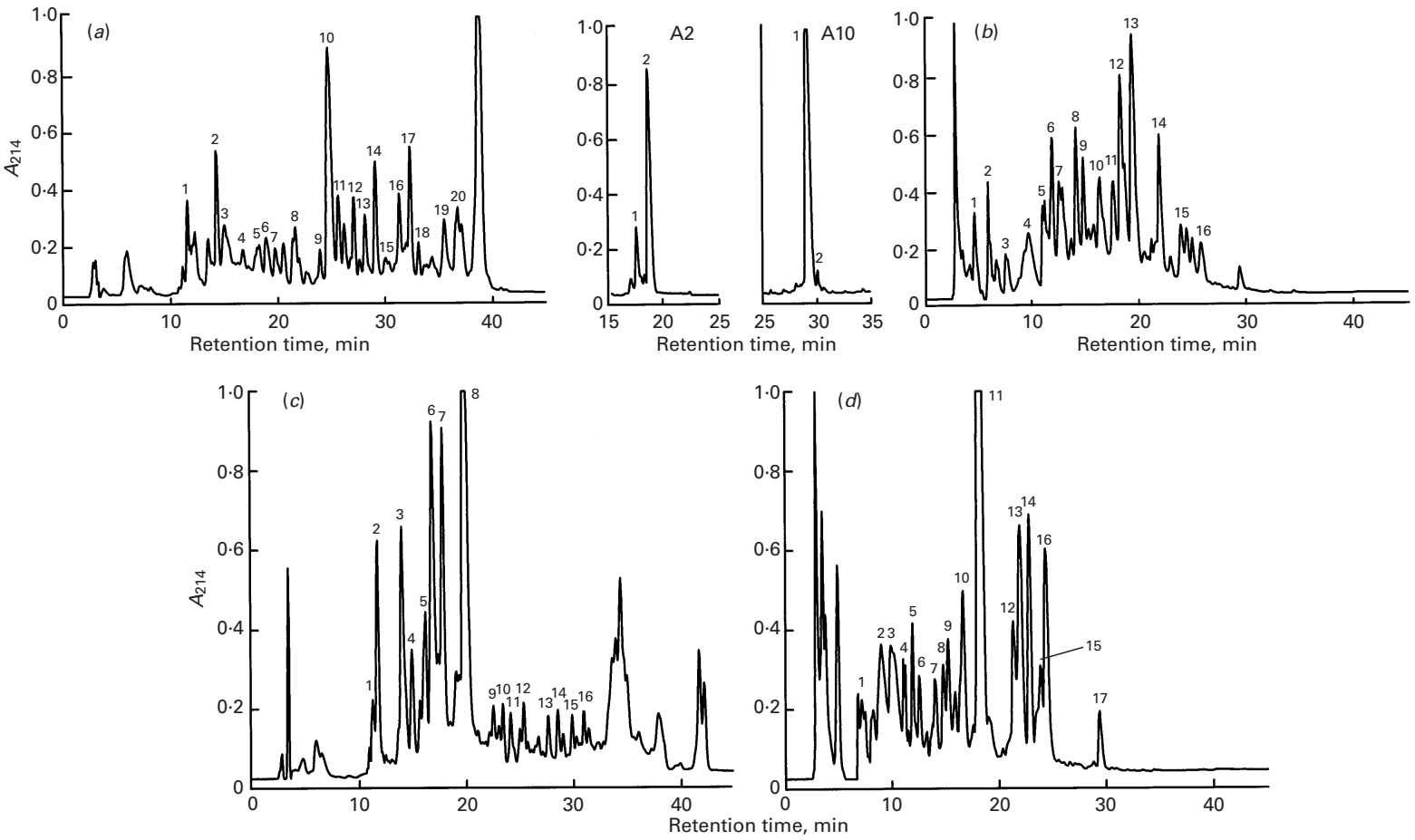

Fig. 2. For legend see opposite.

degraded to f(104–108) by the residual chymotryptic activity present in the enzyme used, was found to have ACE-inhibitory activity. Our earlier study also showed that α-la f(105–110) from the same region has ACE-inhibitory activity (IC_{50} = 621 μM). The contribution of α-la f(99–103) and f(108–110) to the ACE-inhibitory activity of peak B12 requires further study. Mullally *et al.* (1996) have reported ACE-inhibitory activity in α-la f(50–53) (IC_{50} = 733·3 μM) and the dipeptides Tyr–Gly and Leu–Phe (IC_{50} values 1522·6 and 349·1 μM respectively). In our study we found that the peptic tripeptide Tyr–Gly–Leu (α-la f(50–52)) had ACE-inhibitory potency (IC_{50} = 409 μM) similar to that of Leu–Phe. β-Lactoglobulin hydrolysed with trypsin produced several ACE-inhibitory peptides, β-lg f(81–83), f(22–25) and f(32–40). The release of these peptides was related to both the tryptic and chymotryptic activity of the enzyme used. The β-lg peptide f(142–148) reported by Mullally *et al.* (1997*a*) had a higher ACE-inhibitory activity with an IC_{50} value (42·6 μM) lower than those found in the present study (Table 3). The N-terminal peptide β-lg f(142–146) had lower ACE-inhibitory activity than β-lg f(142–148), indicating that the C-terminal amino acid sequence contributes to the inhibitory activity of this peptide.

There has been much speculation concerning the substrate specificity of ACE. The C-terminal amino acid contributes most to the binding of the peptide substrates with the active site of ACE. Peptides containing hydrophobic amino acids at each of the three C-terminal positions have been found to be potent ACE inhibitors. The N-terminal amino acid also contributes to the ACE-inhibitory activity, the most favourable being the branched-chain aliphatic amino acids valine and isoleucine (Cheung *et al.* 1980). A positive charge, as with C-terminal arginine and lysine, has been shown to contribute to the ACE-inhibitory activity of several casein-derived ACE-inhibitory peptides (Schlimme & Meisel, 1993). The ACE-inhibitory peptides identified in this study are consistent with the known substrate specificity of ACE, since in most cases the C-terminal amino acid was hydrophobic, either lysine or arginine.

ACE is present in a large number of tissues including plasma, kidney, lung and brain. In order to exert an antihypertensive effect *in vivo*, the ACE-inhibitory peptides have to be absorbed from the intestine and delivered to the target organ. ACE-inhibitory peptides with antihypertensive activity have mainly been detected in the sequences of β-casein and α_{s1}-casein. In man, fermented milk containing two ACE-inhibitory tripeptides, Val–Pro–Pro and Ile–Pro–Pro, has been reported to produce a significant reduction in systolic blood pressure compared with a control group (Hata *et al.* 1996). The antihypertensive effect of casein-derived peptides has been demonstrated in studies with spontaneously hypertensive rats (Karaki *et al.*

Fig. 2. Purification of the inhibitors of angiotensin I-converting enzyme from hydrolysates of α-lactalbumin (α-la) and β-lactoglobulin (β-lg). (*a*) α-Lactalbumin hydrolysed with trypsin for 3 h. (*b*) A 1 kDa permeate fraction obtained after hydrolysis of α-la with pepsin for 3 h was then hydrolysed with trypsin and chymotrypsin for 7 h. Portions (200 μl of ~ 40 mg sample/ml) were separated by reversed-phase HPLC on a Super-Pak Pep-S column and eluted with acetonitrile (900 ml/l) containing 0·5 ml trifluoroacetic acid/l in a linear gradient (50–600 ml/l over 45 min) at a flow rate of 1 ml/min. The second purification was by reversed-phase chromatography using a Nucleosil C_{18} column as indicated in the middle chromatograms. The labelled peaks were collected and the peptides with inhibitory properties towards angiotensin I-converting enzyme were α-la f(104–108) (A2.2), f(99–108) (A10.2), f(50–52) (B11) and the peptide mixture containing β-lg f(15–19) and α-la f(99–103) and f(108–110) (B12). (*c*) β-Lactoglobulin was hydrolysed with trypsin for 3 h. (*d*) A 1 kDa permeate fraction obtained after hydrolysis of β-lg with pepsin for 3 h was then hydrolysed with trypsin and chymotrypsin for 7 h. The samples were separated as for α-la. The labelled peaks were collected and the peptides with inhibitory properties towards angiotensin I-converting enzyme were β-lg f(81–83) (C2); f(22–25) (C4); f(32–40) (C5); f(106–111) (D8); f(142–146) (D9); f(94–100) (D10) and the peptide mixture containing β-lg f(15–19) and f(143–148) (D12).

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Table 3. *Angiotensin I-converting enzyme inhibitory peptides identified in the active fractions after HPLC separation of hydrolysates of α-lactalbumin (α-la) and β-lactoglobulin (β-lg)*

Origin of the material for peptide separation	Peak	Molecular mass		Peptide fragments	Sequence	IC_{50}†
		Experimental	Theoretical			
α-Lactalbumin with trypsin	A2.2	654·2	653·4	α-la f(104–108)‡	Trp–Leu–Ala–His–Lys	77 μM¶
	A10.2	1200·9	1200·3	α-la f(99–108)‡	Val–Gly–Ile–Asn–Tyr–Trp–Leu–Ala–His–Lys	327 μM
1 kDa permeate from α-la with pepsin, then trypsin and chymotrypsin	B11	352·5	351·4	α-la f(50–52)§	Tyr–Gly–Leu	409 μM
	B12	533·0	532·3	β-lg f(15–19)§	Val–Ala–Gly–Thr–Trp	534 μg/ml
		565·0	564·6	α-la f(99–103)‖	Val–Gly–Ile–Asn–Tyr	
		332·0	330·4	α-la f(108–110)‡	Lys–Ala–Leu	
β-Lactoglobulin with trypsin	C2	393·3	392·5	β-lg f(81–83)‡	Val–Phe–Lys	1029 μM
	C4	405·2	404·5	β-lg f(22–25)§	Leu–Ala–Met–Ala	1062 μM¶
	C5	970·4	970·0	β-lg f(32–40)‡	Leu–Asp–Ala–Gln–Ser–Ala–Pro–Leu–Arg	635 μM
β-Lactoglobulin with pepsin, then trypsin and chymotrypsin	D8	654·0	653·2	β-lg f(106–111)‡	Cys–Met–Glu–Asn–Ser–Ala	788 μM
	D9	568·3	568·4	β-lg f(142–146)§	Ala–Leu–Pro–Met–His	521 μM
	D10	853·2	852·4	β-lg f(94–100)‡	Val–Leu–Asp-Thr–Asp–Tyr–Lys	946 μM
	D12	533·3	532·3	β-lg f(15–19) ‡	Val–Ala–Gly–Thr–Trp	1054 μg/ml
		766·3	765·4	β-lg f(143–148)‡	Leu–Pro–Met–His–Ile–Arg	

† Concentration of an angiotensin I-converting enzyme inhibitor needed to inhibit 50% of its activity.
‡ Identity of peptides based on the amino acid composition and m/z value of peptide peak.
§ Identity of peptides based on sequence analysis.
‖ Identity of peptides based on mass spectrometric analysis.
¶ Values obtained with synthetic peptide.

1990; Yamamoto *et al.* 1994; Nakamura *et al.* 1995*b*; Maeno *et al.* 1996). So far only one whey-derived peptide (β-lg f(78–80): Ile–Pro–Ala) has been found to have an antihypertensive effect in spontaneously hypertensive rats (Abubakar *et al.* 1998). The ACE-inhibitory peptides that have been found to have antihypertensive effect in spontaneously hypertensive rats have IC_{50} values within the range 5–150 μM. The IC_{50} values for the whey peptides reported here are somewhat higher, but it is still possible that these peptides produce an antihypertensive effect (Table 3). The highest ACE-inhibitory activity in this study was with the pentapeptide α-la f(104–108), but it is unlikely that this peptide could have an antihypertensive effect *in vivo*. Tetrapeptides or longer peptides are not thought to be absorbed intact from the intestine. It is more likely that these peptides are decomposed to tripeptides or dipeptides, which can be absorbed. Accordingly, the activity of the degradation products of pentapeptide α-la f(104–108) may be worth further investigation. ACE-inhibitory tripeptides such as α-la f(50–52) and β-lg f(81–83) are more potent *in vivo*, as they can be absorbed intact from the intestine to reach their target organ. Studies on the antihypertensive effect of the peptides identified in this study are already in progress.

Food-derived peptides are considered to be milder and safer and to have fewer side effects than the drugs currently used in the treatment of hypertension. However, there is no actual scientific evidence to support this claim. Both this aspect and the *in vivo* effect of peptides will have to be clarified before whey-derived peptides can be exploited in human nutrition for the prevention and treatment of hypertension. Further development of the techniques for producing and enriching these peptides is also still needed.

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