J Sci Food Agric 85:1041–1048 (2005)
DOI: 10.1002/jsfa.2063

Identification of antioxidant and ACE-inhibitory peptides in fermented milk

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Abstract: The angiotensin-converting enzyme (ACE)-inhibitory activity and antioxidant properties of a commercial fermented milk from Europe were evaluated. This dairy product showed moderate ACE-inhibitory activity and $ABTS^{\bullet+}$ radical-scavenging capacity. The peptides from most active fractions collected by reverse phase high-performance liquid chromatography (RP-HPLC) were sequenced by RP-HPLC–tandem mass spectrometry. This technique allowed rapid identification of peptides included in the most active fractions, and various potentially active peptides were recognised according to previous studies of structure–activity relationship. Three of the identified sequences had previously been described as potent ACE inhibitors. The structure of some sequences substantiated the presence of peptides with ACE-inhibitory, antioxidant and immunomodulatory activities.

Keywords: ACE-inhibitory peptides; antioxidant peptides; fermented milk; tandem mass spectrometry

INTRODUCTION

It is accepted that food proteins may act as precursors of biologically active peptides with different physiological effects. Among these activities the inhibition of angiotensin-converting enzyme (ACE) is one of the most comprehensively studied. ACE inhibition in the organism results in a lowering of blood pressure, although it may also influence different regulatory systems involved in immunodefence and nervous system activity.[1]

ACE-inhibitory peptides can be released from the inactive precursor protein during food processing. In fact, fermentation has already been proved to be a successful strategy to produce ACE-inhibitory peptides. For instance, two potent ACE-inhibitory peptides, VPP and IPP, derived from caseins during milk fermentation with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* are responsible for the antihypertensive activity shown by Calpis® sour milk (Calpis Co Ltd, Tokyo, Japan).[2] Selected strains of *Lactobacillus delbrueckii* ssp *bulgaricus* and *Lactococcus lactis* ssp *cremoris* have also been used to produce fermented milks containing ACE-inhibitory peptides.[3] However, only fragmentary information is available on the presence of ACE-inhibitory peptides in European commercial fermented products.[4]

The role of free radicals and active oxygen species in various diseases, including aging, cancer, inflammation and the toxicity of numerous compounds, has been well documented.[5] To replenish the aging-induced loss of defence systems against oxidative stress, the body must be provided with a constant supply of antioxidants through proper diet.[6] The search for natural antioxidants as alternatives to synthetic ones is a subject of great interest nowadays. Several food proteins such as milk caseins[7] and maize zein[8] have been found to possess antioxidant properties. Recently, hydrolysis of food proteins has been performed to release peptides with demonstrated antioxidant activity. Several peptides derived from a digest of soybean β-conglycinin and two peptides from a protein hydrolysate of lecithin-free egg yolk showed strong linoleic acid oxidation-inhibitory activity.[9,10] Similarly, an α_{s1}-casein-derived hexapeptide identified in a casein hydrolysate with pepsin showed strong free radical-scavenging activity.[6] Other peptides derived from a tryptic digest of β-casein exhibited potent inhibition of lipoxygenase activity.[11] One of these antioxidant peptides, β-casein f(177–183), had previously been identified as an antihypertensive agent called casokinin.[12] Thus peptides derived from milk proteins after enzymatic hydrolysis may reveal multifunctional activities. More specifically, ACE-inhibitory and antioxidant activities could be related with intracellular antioxidant peptides that also showed ACE-inhibitory activity.[13] Similarly, the octapeptide captopril, a drug that is commonly used in the treatment of hypertension, also exhibits antioxidant properties.[14] Recently, a

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Contract/grant sponsor: Comisión Interministerial de Ciencia y Tecnología (CICYT); contract/grant number: AGL2001-1261
Contract/grant sponsor: Instituto Nacional de Investigaciones Agrarias; contract/grant number: CAL01-046-C2-1
(*Received 28 June 2004; revised version received 6 September 2004; accepted 7 September 2004*)
Published online 26 January 2005

peptide derived from the peptic hydrolysis of egg albumen has been found to exhibit potent ACE-inhibitory and antioxidant properties.[15] However, there are few data on the release of antioxidant peptides derived from food proteins during fermentation processes.[16]

The aim of the present work was to study the ACE-inhibitory and antioxidant activities of a commercial fermented milk from Europe which is consumed widely in Spain. The peptides from most active fractions collected by reverse phase high-performance liquid chromatography (RP-HPLC) were sequenced and their biological (ACE-inhibitory, immunomodulatory and antioxidant) activities were studied in relation to their sequence and structure.

MATERIALS AND METHODS

Materials

Two commercial milk samples, namely pasteurised and ultrahigh-temperature (UHT)-treated milks, and a commercial fermented milk consumed widely in Spain were purchased from a local market.

Water-soluble extracts (WSEs) of the milks were obtained by centrifugation at $10\,000 \times g$ for 30 min at 5 °C and filtration through a Whatman No 40 filter. The pH of the pasteurised and UHT milks was previously adjusted to 4.6. The WSE of the fermented milk was treated with a Sep-pak C-18 cartridge (Waters, Millford, MA, USA) and eluted with acetonitrile/water (70:30 v/v). After removing the acetonitrile, the eluent was freeze-dried and kept at −20 °C until use.

ACE (EC 3.4.15.1, 5.1 U mg^{-1}), hippuryl-histidyl-leucine (Hip-His-Leu) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma (St Louis, MO, USA) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Aldrich Chemie (Steinheim, Germany).

ACE-inhibitory activity

ACE-inhibitory activity was measured by the method of Kim *et al*[17] with some modifications. Briefly, 20 µl of each sample was added to 0.1 ml of 0.1 M potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl and 5 mM Hip-His-Leu. After addition of ACE (5 mU) the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.1 ml of 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.

Inhibitory activity was expressed as the percentage of ACE inhibition at a given peptide nitrogen concentration or as the peptide concentration required to inhibit 50% of the original ACE activity (IC_{50}). The total nitrogen content of the fermented milk WSE was determined by the Kjeldahl method. Aminic nitrogen was measured using the Cd-ninhydrin method. The peptide nitrogen content was calculated by subtracting aminic nitrogen from total nitrogen.

Measurement of antioxidant activity

Antioxidant activity was measured using the method of Re *et al*[18] with some modifications. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration in 10 ml of water) and keeping the mixture in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted in 5 mM phosphate-buffered saline (PBS, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm in a 1 cm cuvette at 30 °C. After addition of 2 ml of diluted $ABTS^{\bullet+}$ solution to 20 µl of samples or Trolox standard (end concentration from 0 to 8 µg ml^{-1}) in PBS the absorbance was recorded every minute for 10 min at 30 °C. Appropriate solvent blanks were run in each assay. Results are the mean values of three triplicates.

The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of antioxidants and of Trolox for the standard reference data. To calculate the Trolox equivalent antioxidant capacity (TEAC), the gradient of the plot of the percentage inhibition of absorbance versus sample concentration was divided by the gradient of the plot for Trolox. This gives the TEAC at the specific time.

Separation of peptides by RP-HPLC

Semipreparative RP-HPLC was performed on a Waters HPLC system equipped with two pumps (module Delta 600), a pump controller (module 600), an autosampler (module 717) and a diode array detector (module 996) in combination with an automatic fraction collector (module II). The data-processing software was Millenium version 32 (Waters).

The WSE of fermented milk was applied to a Prep Nova-Pack HR C_{18} column (60 Å, 4 µm, 7.8 mm × 300 mm, Waters) with a C_{18} cartridge as guard column. Solvent A was a mixture of water and trifluoroacetic acid (TFA) (1000:1 v/v) and solvent B contained acetonitrile and TFA (1000:0.8 v/v). The sample concentration was 40 mg ml^{-1} and the injection volume was 100 µl. The peptides were eluted with a linear gradient of solvent B in A going from 1 to 20% over 55 min and from 20 to 40% over 40 min at a flow rate of 4 ml min^{-1}. The absorbance of the eluent was monitored at 214 and 280 nm. The fractions were collected in time periods of 10 min.

The peptide content of collected fractions was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) using bovine serum albumin as standard protein.

Analysis of peptides by on-line RP-HPLC–tandem mass spectrometry (MS/MS)

RP-HPLC separations of the collected peptidic fractions were performed on an Agilent HPLC system

connected on-line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). The HPLC system was equipped with a quaternary gradient pump, at λ_{max} 220 nm, and an automatic injector (all 1100 Series, Agilent Technologies, Waldbronn, Germany). The column used in these experiments was a 250 mm × 4.6 mm Widepore C_{18} column (Bio-Rad, Richmond, CA, USA). The injection volume was 50 µl. Solvent A was a mixture of water and TFA (1000:0.37 v/v) and solvent B contained acetonitrile and TFA (1000:0.27 v/v). The flow rate was 0.8 ml min^{-1}. Peptides of fraction 1 were eluted with a linear gradient of solvent B in A going from 0 to 10% over 60 min. Peptides of fractions 5–7 were eluted with a linear gradient from 0 to 15% of solvent B in A over 5 min followed by a 60 min gradient of solvent B in A from 15 to 35%. The flow was split post-detector by placing a T-piece (Valco, Houston, TX, USA) connected with a 75 µm id peek outlet tube of an adjusted length to give approximately 20 µl min^{-1} of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulising and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (m/z) range 100–2000. About 25 spectra were averaged in the mass spectrometry (MS) analyses and about five spectra in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 10 000 and the precursor ions were isolated within a range of m/z 4.0 and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using Data Analysis™ (version 3.0, Bruker Daltoniks, Bremen, Germany), the m/z spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1, Bruker Daltoniks) was used to process the MS(n) spectra and to perform peptide sequencing.

RESULTS AND DISCUSSION

ACE-inhibitory activity of fermented milk

The ACE-inhibitory activity of the WSEs obtained from commercial products, ie fermented, pasteurised and UHT milks, was measured. The WSE of the fermented milk showed an inhibition percentage of 74.3%, whereas inhibition percentages of the WSEs obtained from pasteurised and UHT milk samples were below 2%. These results indicated that fermentation plays an important role in the release of ACE-inhibitory peptides from milk proteins. Prior to peptide identification a purification step was carried out to eliminate carbohydrates. The retentate containing the peptidic fraction of the fermented milk showed a similar inhibition percentage as before the solid phase extraction, indicating that the ACE-inhibitory activity observed in the WSE is caused by the peptides present in this fraction. The ACE-inhibitory activity of this fraction was also calculated as the protein concentration needed to inhibit 50% of the original ACE activity (IC_{50}) and corresponded to 145.5 µg protein ml^{-1}.

ACE-inhibitory and antioxidant activity of peptidic fractions

Figure 1 shows the UV chromatogram corresponding to separation. Figure 2A shows the measured ACE-inhibitory indices of the collected HPLC fractions divided by their peptide content. The ACE-inhibitory activity of collected fractions ranged from 12 to 80% and their peptide contents were 35–160 mg per 100 g. Fractions 1 and 5–7 showed the highest ACE-inhibitory/peptide content ratios.

The antioxidant activity of these collected HPLC fractions was also determined by $ABTS^{\bullet+}$ radical cation assay. This method is a type of inhibition assay in which the extent of scavenging of a preformed free radical relative to that of a standard antioxidant compound corresponds to the value of antioxidant activity. For our samples the TEAC values were obtained from the capacity of each sample to scavenge $ABTS^{\bullet+}$ at 10 min relative to Trolox. Figure 2B shows the calculated TEAC values of 10 peptidic fractions. The antioxidant activity in relation to Trolox was low in all fractions, with a TEAC value that ranged between 0.1 and 0.26 µg. In relation to other HPLC fractions, fraction 7 showed a notably higher TEAC

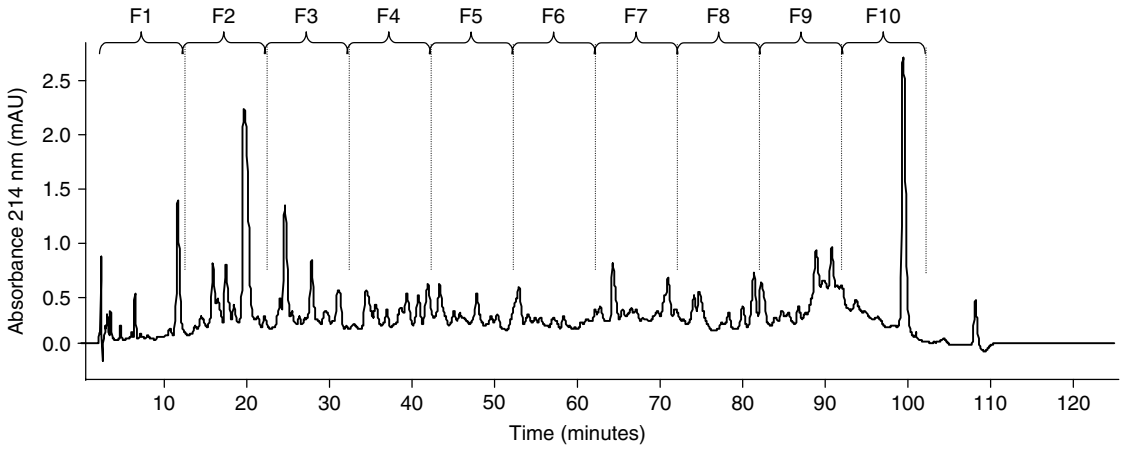

Figure 1. UV chromatogram of semipreparative RP-HPLC of water-soluble extract from fermented milk. Collected fractions were F1–F10.

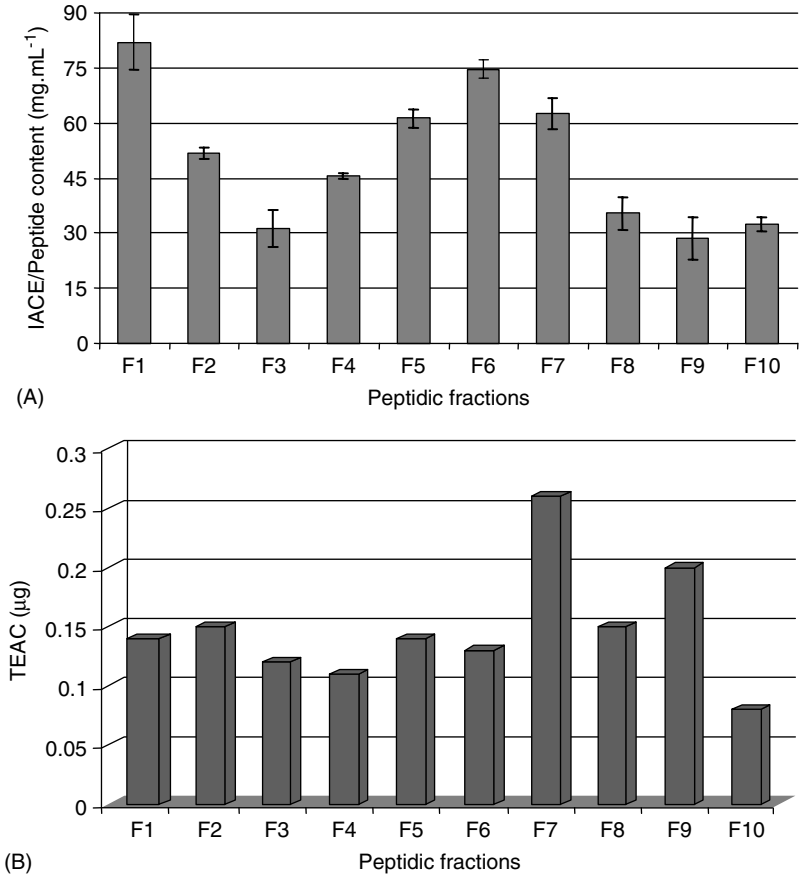

Figure 2. (A) Ratio between ACE-inhibitory indices and peptide content of water-soluble extract of fermented milk fractions collected by semipreparative RP-HPLC. (B) Antioxidant activity (TEAC), expressed as µg of Trolox, of water-soluble extract of fermented milk fractions collected by semipreparative RP-HPLC.

value. In spite of the low TEAC values found, these results indicate that some peptides released from caseins during milk fermentation were able to scavenge the $ABTS^{\bullet+}$ radical cation and therefore to exhibit a degree of antioxidant activity. To date, only a few antioxidant peptides have been identified in fermented dairy products. For instance, a κ-casein-derived peptide with 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical-scavenging activity was recently found in a milk fermented with *Lb delbrueckii* ssp *bulgaricus*.[16]

Identification of peptides by HPLC–MS/MS

The HPLC fractions (1 and 5–7) that showed highest ACE-inhibitory and antioxidant activity were subjected to tandem mass spectrometry using a quadrupole ion trap capable of multiple stages of mass analysis from a single precursor ion. All ions from the total ion chromatogram with a signal higher than 10 000 units were considered for peptide sequencing and most could unambiguously be assigned to a peptide sequence. Only a few detected masses and the corresponding fragmentation spectra obtained by MS/MS could not be matched with any peptide fragment originated by milk protein fermentation, suggesting that peptides derived from the bacterial metabolism would probably also be present in these fractions.

Figure 3A shows the complex UV chromatogram obtained for fraction 6. The mass spectrum of one selected peak (Fig 3B) and the MS/MS spectrum of the singly charged ion (m/z 876.5) and amino acid sequence of the identified peptide with major fragment ions (Fig 3C) showed that the most intense fragment ions corresponded to the loss of water from the precursor ion. The fragment ions were identified as y_3, b_4 and b_6. Ions b_4 and y_3 resulted from cleavage of the peptide bond N-terminal to proline associated with unusual fragmentation events that result in very abundant *b*- and *y*-type fragment ions.[19]

Following this methodology, a total of 35 peptide fragments were identified in the four analysed fractions (Table 1), of which 13 correspond to β-casein, nine to α_{s1}- and α_{s2}-casein and 11 to κ-casein fragments. The dipeptides SN and RY (peptides 1.1 and 1.3, Table 1) could originate from hydrolysis of several caseins. Of many ACE-inhibitory peptides, structure–activity correlation indicated that C-terminal tripeptide residues play a predominant role in competitive binding to the active site of ACE. Aromatic amino acids were the most favourable C-terminal amino acids besides proline, while ACE only weakly binds peptides having terminal dicarboxylic amino acids.[20] Several peptides identified in this study share the characteristic of having hydrophobic residues, such as leucine, phenylalanine or valine, at

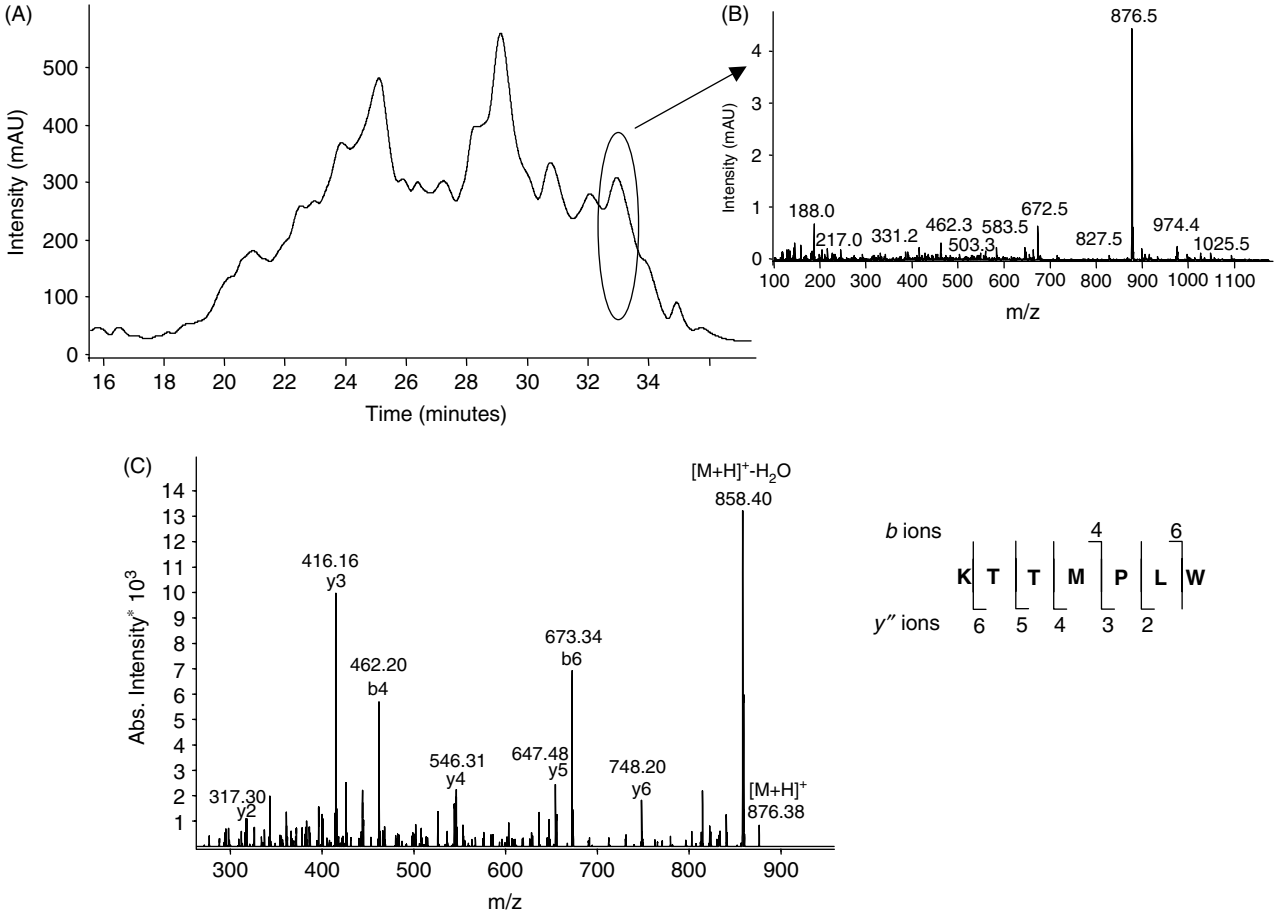

Figure 3. (A) UV chromatogram of semipreparative RP-HPLC fraction 6. (B) Mass spectrum of selected chromatographic peak in (A). (C) MS/MS spectrum of singly charged ion *m/z* 875.6. Following sequence interpretation and database searching, the MS/MS spectrum was matched to α_{s1}-casein f(193–199). The sequence of this peptide is displayed with the fragment ions observed in the spectrum. For clarity, only the *b* and *y″* fragment ions are labelled according to the nomenclature proposed by Roepstorff and Fohlman[30].

C-terminal positions, which favour binding to ACE. Other peptides (7.1 and 7.8, Table 1) with lysine as C-terminal residue and many milk-derived ACE-inhibitory peptides have been reported to contain a positively charged amino acid as a C-terminal residue, which contributes substantially to the inhibitory potency.[21]

Among the identified peptides, three have previously been described as ACE inhibitors. More specifically, the β-CN peptides VPP and NIPPLTQTPV (peptides 1.2 and 6.8, Table 1) demonstrated ACE-inhibitory activity with low IC_{50} values (9 and 173.3 μM respectively).[4,22] In particular, peptide VPP was found to be most potent, having the highest ACE-inhibitory activity (fraction 1, Fig 2) reported so far. This peptide has been proved to exert antihypertensive activity in model animals and humans.[22,23] Peptide RY, also found in fraction 1, showed an amino acid sequence similar to dipeptide obtained from sake and sake lees (IC_{50} 10.5 μM).[24] The presence of these small peptides in fraction justifies the high ACE-inhibitory activity found in this HPLC fraction.

Other peptides in fractions 6 and 7 showed a high homology with different ACE-inhibitory peptides previously described in the literature (Table 2). For instance, the α_{s1}-casein peptide KTTMPLW (peptide 6.4, Table 1) identified in fraction 6 may exhibit ACE-inhibitory activity like peptide TTMPLW (IC_{50} 51 μM), which was identified after fermentation of casein and subsequent hydrolysis with pepsin and trypsin.[25] Similarly, α_{s1}-casein f(28–34), FPGVFGV, with an ACE-inhibitory activity (IC_{50} 140 μM) reported by Maruyama *et al*,[26] is included in amino acid sequence VAPFPGVFGV identified in fraction 7.

The structure of some of the peptide sequences found in fractions 5–7 may anticipate the presence of other biological activities in these fractions. A summary of the biologically active peptides found and potentially new active sequences is shown in Table 2. Peptide KTTMPLW (peptide 6.4) had a sequence similar to TTMPLW, which exhibited immunomodulatory activity,[27] with the exception of the N-terminal residue. This activity has also been described for β-casein f(193–209), YQEPVLGPVRGPFPIIV, which shares several amino acid residues with peptide GPVRGPFPII (peptide 7.4, Table 2) identified in fraction 7. Also, the β-casein peptide KVLPVPQ (peptide 6.12) may possess antioxidant activity as occurs with peptide VLPVPQ.[11]

The role played by some amino acids in structure–activity relationship has been reported. Histidine

Table 1. Casein-derived peptides identified in fractions collected from semipreparative RP-HPLC of water-soluble extract of fermented milk

Fraction[a]	Peptide no	Observed mass	Calculated mass[b]	Protein fragment	Sequence
F1	1.1	218.9	219.09	Various caseins	SN
	1.2	311.0	311.19	β-CN f(84–86)	VPP
	1.3	337.1	337.18	Various caseins	RY
F5	5.1	584.4	584.32	α_{s1}-CN f(12–16)	PQEVL
	5.2	813.5	813.46	α_{s1}-CN f(15–21)	VLNENLL
	5.3	702.5	702.41	α_{s2}-CN f(172–177)	KFALPQ
	5.4	438.4	438.28	β-CN f(74–77)	IPPL
	5.5	876.4	876.44	β-CN f(124–131)	SLTLTDVE
	5.6	923.5	923.51	κ-CN f(24–30)	KYIPIQY
	5.7	632.4	632.35	κ-CN f(26–30)	IPIQY
	5.8	953.4	953.57	κ-CN f(66–74)	AVRSPAQIL
	5.9	1081.5	1081.62	κ-CN f(66–75)	AVRSPAQILQ
	5.10	873.4	873.43	κ-CN f(75–81)	QWQVLSN
	5.11	1906.8	1906.05	κ-CN f(107–123)	AIPPKKNQDKTEIPTIN
F6	6.1	661.5	661.39	α_{s1}-CN f(19–23)	NLLRF
	6.2	805.4	805.40	α_{s1}-CN f(24–30)	FVAPFPE
	6.3	1000.5	100.51	α_{s1}-CN f(150–156)	FRQFYQL
	6.4	875.5	875.46	α_{s1}-CN f(193–199)	KTTMPLW
	6.5	919.4	919.46	α_{s2}-CN f(133–140)	ENSKKTVD
	6.6	1005.4	1005.39	β-CN f(37–44)	EQQQTEDE
	6.7	890.5	890.52	β-CN f(70–77)	LPQNIPPL
	6.8	1078.5	1078.60	β-CN f(73–82)	NIPPLTQTPV
	6.9	796.5	796.45	β-CN f(84–90)	VPPFLQP
	6.10	925.5	925.49	β-CN f(84–91)	VPPFLQPE
	6.11	786.5	786.43	β-CN f(157–163)	FPPQSVL
	6.12	779.6	779.49	β-CN f(169–175)	KVLPVPQ
	6.13	1100.5	1099.60	κ-CN f(73–81)	ILQWQVLSN
F7	7.1	1089.5	1089.59	α_{s1}-CN f(25–34)	VAPFPGVFGK
	7.2	688.5	688.43	β-CN f(134–139)	HLPLPL
	7.3	340.2	340.18	β-CN f(158–160)	PPQ
	7.4	1051.5	1051.62	β-CN f(199–208)	GPVRGPFPII
	7.5	389.2	389.16	κ-CN f(4–6)	NQE
	7.6	1959.4	1960.05	κ-CN f(23–38)	AKYIPIQYVLSRYPSY
	7.7	844.6	844.51	κ-CN f(26–32)	IPIQYVL
	7.8	340.2	340.21	κ-CN f(109–111)	PPK

[a] Fractions are termed as in Fig 1.
[b] Monoisotopic mass values.

Table 2. Previously described bioactive peptides that share structure homology with sequences identified in fractions collected from semipreparative RP-HPLC of water-soluble extract of fermented milk. Residues in bold letters indicate sequence homology with peptides found in this study

Peptide no	Sequence	Previously described sequence	Activity[a]	Ref
1.2	**VPP**	**VPP**	Antihypertensive (IC_{50} 9 μM)	22
1.3	RY	**RY**	Antihypertensive (IC_{50} 10.5 μM)	24
5.3	KFA**LPQ**	RPKHPIIKKHQG**LPQ**	ACE-inhibitory (IC_{50} NR)	31
6.4	K**TTMPLW**	**TTMPLW**	ACE-inhibitory (IC_{50} 51 μM)	25
			Immunomodulatory	27
6.8	**NIPPLTQTPV**	**NIPPLTQTPV**	ACE-inhibitory (IC_{50} 173.3 μM)	3
6.9	**VPPFLQP**	TPVV**VPPFLQP**	Antihypertensive (IC_{50} 749 μM)	32
6.12	**KVLPVPQ**	S**KVLPVPQ**	Antihypertensive (IC_{50} 39 μM)	33
		VLPVPQK	Antioxidant	11
7.1	VAP**FPGVFGK**	FPGVFGK	ACE-inhibitory (IC_{50} 140 μM)	26
7.2	**HLPLP**L	**HLPLP**	ACE-inhibitory (IC_{50} 41 μM)	34
7.4	**GPVRGPFPII**	YQQPVL**GPVRGPFPII**V	Immunomodulatory	35
7.9	**PPK**	MAI**PPK**	Antithrombotic	36

[a] The activity is referred to as antihypertensive when demonstrated in laboratory animals or humans. If only demonstrated *in vitro*, the activity is referred to as ACE-inhibitory. NR, not reported.

and proline have been described as the most important residues in the lipoprotein peroxidation-inhibitory activity of peptides isolated from a soybean β-conglycinin hydrolysate.[28] The highest antioxidant activity was found in fraction 7. Seven of the eight peptides identified in it contained at least one proline residue, and six of them had more than two residues of proline. The high content of proline peptides could determine the antioxidant activity found in this fraction. Preliminary results with synthetic peptides demonstrated that tyrosine and tryptophan showed $ABTS^{\bullet+}$ radical-scavenging capacity (unpublished results). These residues are generally accepted to be responsible for antioxidant activities when incorporated into dipeptides.[29] The properties of these amino acids may be explained by the special capability of phenolic and indol groups to serve as hydrogen donors. In κ-CN f(23–38), four of 16 residues were tyrosine, which was also identified in fraction 7 and thus may explain its high antioxidant activity. Some of these peptides are being chemically synthesised to be used as a starting point for further studies on antioxidant peptides derived from milk proteins.

CONCLUSIONS

Milk fermentation has been described as a strategy to release ACE-inhibitory peptides from caseins. The ACE-inhibitory and antioxidant activity was moderate in a commercial fermented milk.

Some of the peptides identified corresponded to previously described ACE inhibitors or showed marked structural similarities with previously identified antihypertensive, immunomodulatory and antioxidant fragments and may be used as multifunctional ingredients of functional foods to control cardiovascular diseases.

ACKNOWLEDGEMENTS

This work was supported by Comisión Interministerial de Ciencia y Tecnología (CICYT) project AGL2001-1261 and Instituto Nacional de Investigaciones Agrarias project CAL01-046-C2-1.

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