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Journal of Chromatography A, 1054 (2004) 269–277
JOURNAL OF CHROMATOGRAPHY A
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Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography–tandem mass spectrometry

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Available online 17 June 2004

Abstract

A total of 75 peptides included in the fraction with molecular mass below 3000 from an 8-month-old Manchego cheese could be identified using HPLC coupled *on line* to an ion trap mass spectrometer. Some previously described peptides with antihypertensive and/or angiotensin-converting enzyme (ACE)-inhibitory activity were detected. The formation of five active sequences was followed during cheese ripening in four different batches of Manchego cheese. Two experimental batches of Manchego cheese elaborated with selected bacterial strains with the aim of improve the organoleptic characteristics demonstrated also a good performance in the formation of peptides with ACE-inhibitory activity during cheese ripening.

Keywords: Cheese; Food analysis; Peptides; Enzyme inhibitors

1. Introduction

Proteolysis, together with lipolysis, is essential for the development of texture and flavour in cheese. Proteolysis of different types of cheeses has been extensively studied, and these studies were mainly focused on the knowledge of the maturation process [1,2] or the development of taste [3]. However, it is known that peptides derived from milk proteins may also exhibit many different biological activities. These peptides, which are inactive within the sequence of the precursor protein, can be released by enzymatic proteolysis, for instance, during cheese ripening.

Since 1979, several authors have described bioactive peptides from milk proteins [4]. Among the different types of bioactive peptides (immunostimulating, opioid, antimicrobial peptides, etc.), antihypertensive peptides have been extensively studied due to the high incidence of hypertension, which is currently considered to be one of the most serious chronic illnesses. Most food-derived antihypertensive peptides act by inhibiting the angiotensin-converting enzyme (ACE). ACE is important in blood pressure regulation because it catalyses formation of the potent vasopressor angiotensin II from angiotensin I, and the inactivation of the vasodilator nonapeptide bradikinin.

However, identification of biologically active peptides in food matrices is a challenging task in food technology. Milk protein hydrolysates are known for their complexity and can contain up to hundreds of different peptide sequences. Identification of bioactive peptides in fermented dairy products or milk protein hydrolysates generated by the action of unspecific enzymes is a labour-intensive and difficult task. Despite its complexity, the presence of peptides with antihypertensive activity or in vitro ACE-inhibitory activity has been reported in different types of cheeses [5–7]. In a previous work, we identified 22 peptides in several chromatographic fractions that exhibited potent ACE-inhibitory activity obtained from a ripened Manchego cheese [8]. However, bioactive peptide identification from complex hydrolysates or fermented products comprises several purification steps, by combining different chromatographic techniques. Each separation step requires solvent evaporation and evaluation of the biological activity. In most cases, final fractions often still contain multiple compounds that can cause a discrepancy between the activity found in the purified fractions and

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doi:10.1016/j.chroma.2004.05.022

the activity of the individual chemically synthesised peptides [8–10].

The sequence of numerous milk protein derived peptides with ACE-inhibitory activity is already known [11]. Moreover, several studies have established clear structure–activity relationships for ACE inhibitors [12]. All this knowledge may permit the identification of potentially active peptides using new strategies that facilitate their identification. An alternative strategy could involve identification of the major peptide compounds in complex mixtures by a rapid and reliable analytical technique and the selection of several of the identified peptides based on their structures to be assayed for certain biological activities.

LC–MS–MS is normally used in proteomic studies because it allows a large number of peptides to be sequenced in a relatively short analysis time. There are now several configurations of mass spectrometers that provide MS–MS data with sufficient mass accuracy to deduce peptide sequences of enzymatically digested proteins from low-energy collisionally-induced MS–MS spectra (for reviews see references [13–15]). This technique has already been used for peptide identification in cheese extracts with successful results [3,16].

In the present work, HPLC–MS–MS was used to identify the peptide sequences released during Manchego cheese ripening. The formation of several peptides with demonstrated ACE-inhibitory activity was followed during cheese ripening in four different batches of Manchego cheese.

2. Experimental

2.1. Cheese samples

Several batches of Manchego cheese were prepared as previously described [17]. One batch of Manchego cheese was prepared with raw ewes' milk without addition of bacterial starters (hereafter termed raw-milk cheese). Three batches of Manchego cheese were manufactured from pasteurised ovine milk (15 s at 72 °C), of which, one batch was made from pasteurised ovine milk and inoculated with a commercial mixed-strain starter (Ezal, Dangé-Saint-Romain, France) before rennet coagulation (hereafter termed pasteurised-milk cheese); and two experimental batches of pasteurised-milk cheeses that were referred to as type 1 and 2. Type 1 cheeses were prepared by inoculating milk with *Lactococcus lactis* subsp. *lactis* (80%) and *Leuconostoc mesenteroides* subsp. *dextranicum* (20%), and type 2 cheeses were prepared by inoculating milk with *L. lactis* subsp. *lactis* (80%), *L. mesenteroides* subsp. *dextranicum*(10%), and *Lactobacillus plantarum* (10%). Cheeses of about 1 kg were pressed for 5–6 h at 15 °C, brine-salted at the same temperature for 22 h, and ripened at 12 °C and at 81–85% relative humidity. A whole cheese was taken after 15 days, 2, 4, 8, and 12 months ripening.

2.2. Preparation of water-soluble extracts (WSEs)

WSEs from Manchego cheeses were obtained by following the procedure previously described [8]. The WSEs were ultrafiltered on a hydrophilic M_r 3000 cut-off membrane (Centripep, Amicon, Beverly, MA, USA). The permeates were freeze-dried and kept at −20 °C until use.

2.3. Analysis of peptides by on line RP-HPLC–MS–MS

RP-HPLC separations of the permeates with the peptides with molecular masses below 3000 were performed on an Agilent HPLC system connected *on line* to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik, Bremen, Germany). The HPLC system was equipped with a quaternary gradient pumping system, an *in line* degasser, a variable wavelength absorbance detector set at 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 100 μl. Solvent A was a mixture of water-trifluoroacetic acid (1000:0.37, v/v) and solvent B contained acetonitrile–water–trifluoroacetic acid (800:200:0.27, v/v). Peptides were eluted with a linear gradient of solvent B in A going from 0 to 40% in 60 min at a flow rate of 0.8 ml/min. The flow was split post detector by placing a T-piece (Valco, Houston, TX, USA) connected with a 75 μm i.d. polyether ether ketone (PEEK) outlet tube of an adjusted length to give approximately 20 μl/min of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulizing and drying gas and operated with an estimated helium pressure of 500 Pa. 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 MS analyses and about five spectra in the multiple MS (MS^n) analyses. The signal threshold to perform auto MS^n analyses was 5000 and the precursor ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp going from 0.39 to 2.6 V. Using Data Analysis (version 3.0; Bruker Daltoniks), 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.

3. Results and discussion

3.1. Identification of peptides by HPLC–MS–MS

With the aim of identifying possible bioactive peptides, the permeate of the WSE obtained from 8-month-old Manchego cheese type 1 was subjected to RP-HPLC coupled *on line* to a mass spectrometer. In our case, the mass spectrometer was a quadrupole ion trap capable of multiple

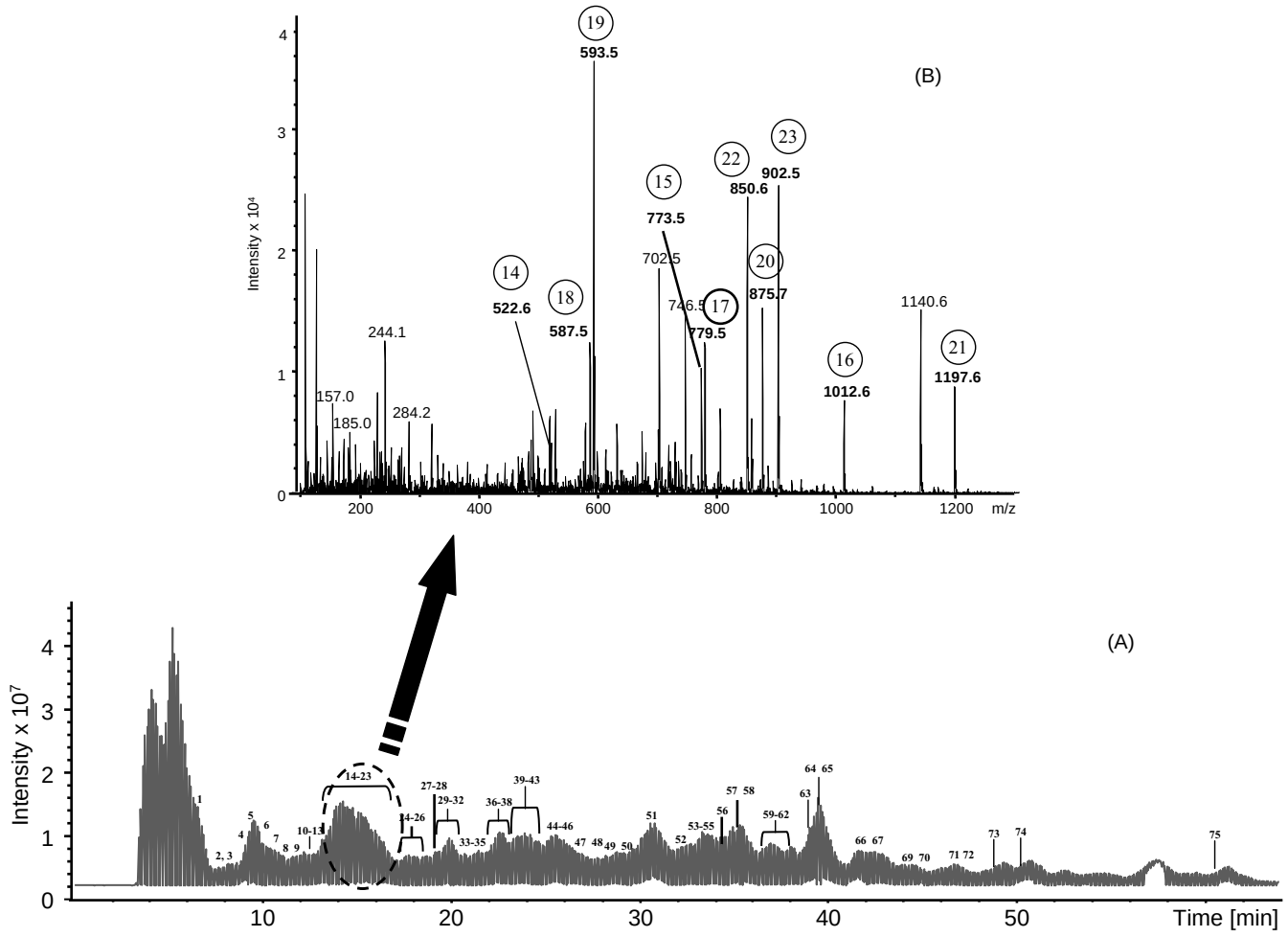

Fig. 1. (A) Total ion current chromatogram corresponding to the M_r 3000 permeate from 8-month-old Manchego cheese type 1. Numbers correspond to the identified peptides following the peptide number of Table 1. (B) Mass spectrum of the selected chromatographic peak in (A). Similarly, numbers in circles correspond to the peptide numbers given in Table 1.

stages of mass analysis from a single precursor ion. Fig. 1A shows the complex total ion current chromatogram corresponding to the permeate obtained from this extensively ripened Manchego cheese. Despite this complexity, this cheese was selected as starting point for bioactive peptide identification because it had demonstrated to exhibit high in vitro ACE-inhibitory activity [8]. As an example, Fig. 1B shows the mass spectrum obtained from a component of the permeate that eluted between 13 and 17 min. In this region of the chromatogram 10 peptides could be fragmented and identified. A total of 75 peptides were identified in the permeate (marked in the figure).

Identification of peptide components in complex protein hydrolysates in which the sequence of the precursor proteins are known, requires more information than just the masses of the peptides, but does not require complete sequencing of the peptide components. In our case, the identification approach involves the search for the masses and partial sequences (sequences tags) in a database of bovine milk proteins, including sequence modifications due to genetic variants and post-translational modifications (phosphorylation and glycosylation). In most cases, the MS–MS spectrum matched unambiguously one sequence of the group of peptides selected by mass. However, other peptides exhibited fragmentation patterns that significantly deviate from the expected fragmentation pattern and they had to be identified with human intervention. To illustrate this point, Fig. 2 shows, as an example, the MS–MS spectra of three identified peptides. Fig. 2A corresponds to the MS–MS spectrum of the singly charged ion at *m*/*z* 580.5. The fragmentation spectrum contained a major ion at *m*/*z* 451.3, which was identified as a *y*-type fragment ion resulting from the cleavage C-terminal to glutamic acid. This unusual fragmentation pattern had also been previously shown by other authors, who found that peptide ions containing arginine and aspartic or glutamic acid residues result in selective cleavages C-terminal to the acidic residues [18,19]. The assignment of the fragment ions b_2, b_3, y_2 and y_3, together with fragment ions corresponding to losses of water and ammonium from the precursor ion or its fragments allowed unambiguous identification of α_{s1}-CN f(89–92) (ERYL). Similarly, fragmentation of the ion at *m*/*z* 750.5 (Fig. 2B) gave frag-

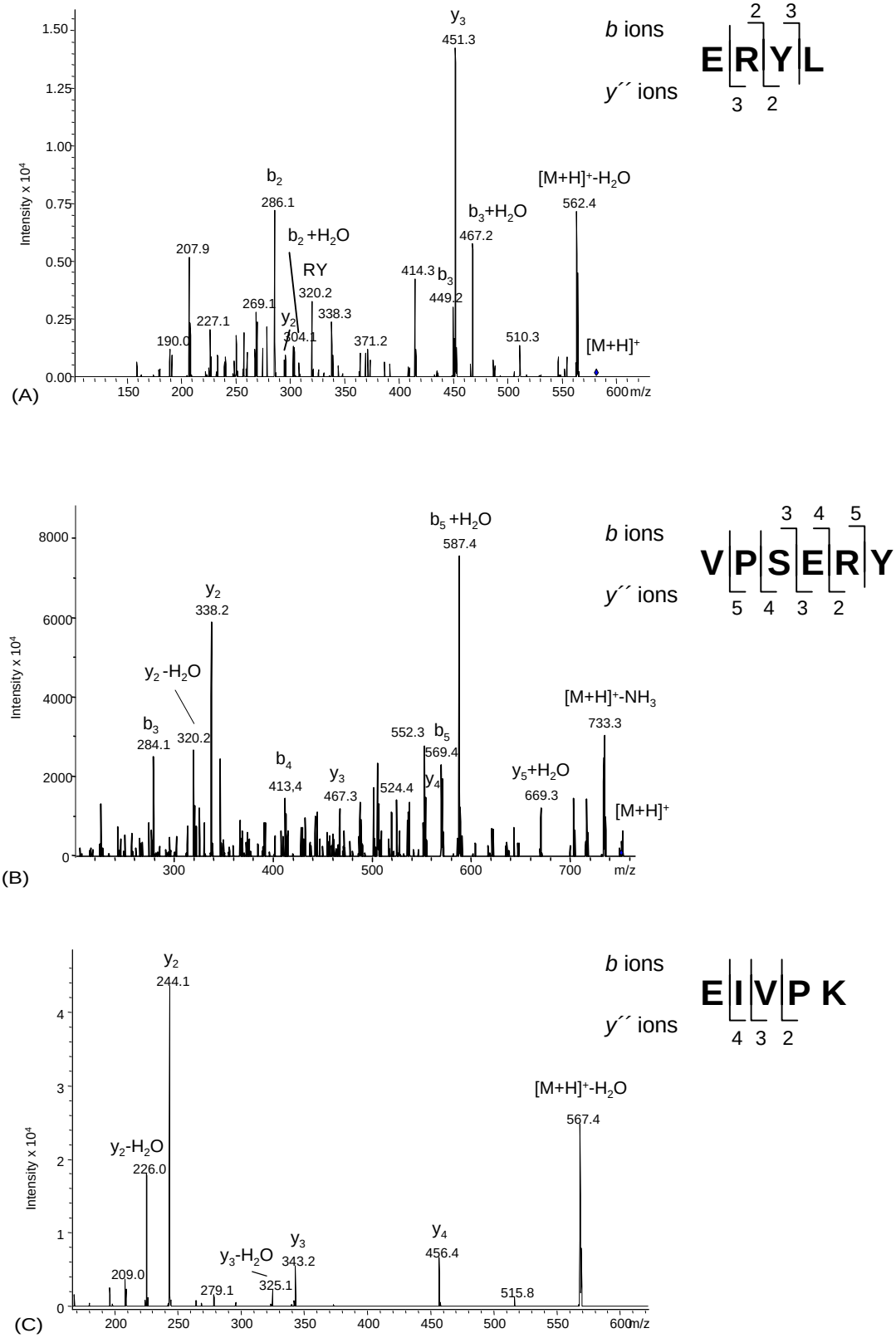

Fig. 2. MS–MS spectrum of ions (A) *m*/*z* 580.5; (B) *m*/*z* 750.5; (C) *m*/*z* 585.4. Following sequence interpretation and database searching, the MS–MS spectrum were matched to (A) α_{s1}-casein f(89–92); (B) α_{s2}-casein f(86–91); (C) α_{s1}-casein f(110–114).

ment ions at *m/z* 338.2 and 587.4, which were remarkably over-represented in the spectrum. These fragment ions could be identified as y_2 and $b_5 + H_2O$ ions of peptide VPSERY, respectively. The fragmentation C-terminal to glutamic acid is favoured due to the presence of arginine given an intense y_2 fragment ion. However, the presence of proline in this peptide did not gave intense N-terminal to proline fragment ions although this amino acid is associated with very abundant *y* and *b* ions that are often easily identifiable because of its intensity [20]. In this spectrum, the ion at *m/z* 669.3 was identified as $y_5 + H_2O$ (i.e., fragment corresponding to a cleavage N-terminal to proline) and the complementary ion, b_1, was not observed because it is often an unstable fragment ion. Fig. 2C shows the fragmentation spectra of the singly charged precursor ion corresponding to peptide EIVPK. In the fragmentation profile of this peptide ion at *m/z* 244.1 that corresponds to the y_2-ion is the most noticeable fragment ion in the spectrum. This fragment was produced by cleavage at the Val-Pro bond and it had previously found that abundant Xxx-Pro relative bond cleavage ratios were observed when Xxx was Val, His, Asp, Ile, and Leu [21]. Moreover, the presence of lysine at the C-terminus of this peptide, favoured the appearance of *y*-type fragment ions in this spectrum.

By using *on line* HPLC–MS–MS, most peptides with intensities higher than 5000 units were identified in the permeate. A total of 75 peptides were identified in the permeate of 8-month-old Manchego cheese (Table 1). Those peptides originated from α_{s1}-casein dominated the degradation products with 43 peptides arising from this protein. Seventeen of the identified peptides corresponded to β-casein fragments, 12 to α_{s2}-casein and only one peptide derived from the κ-CN fraction. The low number of identified peptides from κ-casein can be attributed to its low content in ovine caseins and to the lost of the region corresponding to the caseinomacropeptide [κ-casein f(106–171)] with the whey fraction during cheese clotting. Moreover, the molecule of *para*-κ-casein has been shown to be rather resistant to proteolysis in other types of cheese [22,23]. Dipeptides IR and RL (peptide 4 in Table 1) were undistinguishable by MS and they could be originated from the hydrolysis of several casein fractions. Five phosphopeptides were also identified. The MS–MS spectra of these phosphopeptides were easily identifiable showing prominent ions formed by the loss of 98 Da from the singly charged parent ion, due to β-elimination reaction during the fragmentation process [24]. Phosphopeptide β-CN f(7–18) (peptide 44 in Table 1) had previously been found in human duodenum 20 min after milk ingestion [25]. Therefore, it can be expected that this region of β-CN survives gastric digestion and reaches the intestine where it might promote mineral absorption. Similarly, this phosphopeptide and phosphopeptide β-CN f(13–21) (peptides 44 and 38 in Table 1, respectively) are included in a previously described phosphopeptide [β-CN f(7–21)] found in an anticariogenic preparation of casein hydrolysed with pancreatin [26]. In addition, some of the peptides identified in this study had previously been found in other cheese types. For instance, peptides α_{s1}-CN f(34–38), α_{s1}-CN f(3–9) and α_{s1}-CN f(1–7) (peptides 5, 7 and 20 in Table 1) had been identified in Emmental cheese [2] and the presence of peptides α_{s1}-CN f(109–114) and α_{s1}-CN f(24–30) (peptides 56 and 64 in Table 1) was previously reported in Cheddar cheese [16].

Among the identified peptides, several of these had previously demonstrated to exhibit ACE-inhibitory activity. The previously reported ACE-inhibitory peptides and their IC_{50} values are included in Table 1. Several of these peptides had been found in this type of Manchego cheese [8,10] but other sequences had been identified from other species' milk. For instance, peptide LPQNILP (peptide 71 in Table 1) is identical to a fragment of human β-casein, which had been previously synthesised and showed an IC_{50} value of 46 μM [27]. But it has to be noted that its presence in cheese is for the first time reported. In like manner, β-casein f(60–68) (peptide 70 in Table 1) was very similar to one peptide previously found in 8-month-old Gouda cheese made from bovine milk [6]. The peptide found in Gouda cheese showed an IC_{50} value of 14.8 μM although did not demonstrate strong antihypertensive activity in spontaneously hypertensive rats in spite of its low IC_{50} value. However, these results cannot be strictly be extrapolated to the peptide found in our study because there is an amino acid change in this region in these two species (proline at position 63 in bovine β-CN changes to threonine in ovine β-casein) (Table 1). Moreover, this peptide (YPFTGPIPN, peptide no. 70 in Table 1) shows a high homology with a previously identified opioid peptide YPFPGPIPNSL [28]. This peptide (β-CN f(60–68), YPFYGPIPN) shares also the last six residues with a previously reported immunomodulatory peptide (PGPIPN) [29]. Peptide KHPIKHQ (peptide 7 in Table 1) shares the last seven amino acids with a previously identified peptide RPKHPIKHQ [6], which showed an IC_{50} value of 13.4 μM.

Other peptides (peptides 9, 21, 73 and 75 in Table 1) presented high homology with different ACE-inhibitory peptides described in the literature (Table 1). However, comparing them with the previously described peptides, they had not exactly the same last three amino acids which have demonstrated to have great influence on binding to ACE [12], and therefore, the ACE-inhibitory activity of these peptides should be carefully studied.

3.2. Formation of peptides during cheese ripening

Formation of the peptides of interest during Manchego cheese ripening could easily be followed by analysis of the permeates by HPLC–MS and extraction of the characteristic ion/s of the peptide of interest. As an example, Fig. 3 shows the total ion current chromatogram corresponding to the permeate from Manchego cheese type 1 with 8 months ripening and the extracted ion chromatograms of the molecular ions corresponding to peptides α_{s1}-CN f(1–7) (peptide 20 in Table 1) and α_{s1}-CN f(109–114) (peptide 56 in Table 1). Following the same procedure, peptides

Table 1
Peptides identified in the permeate of the water-soluble extract from 8-month-old Manchego cheese type 1 (see Section 2.1)

	Observed mass[a]	Calculated mass[b]	Protein fragment[c]	Sequence	ACE-inhibitory peptides[d]	IC_{50}[e] (μM)	Reference
1	665.5	665.3	α_{s2}-CN f(30-34)	HPRKE			
2	542.5	542.3	α_{s2}-CN f(l–4)	KHKM			
3	671.5	671.3	α_{s2}-CNf(l–5)	KHKME			
4	287.2	287.2	Various fragments	IR/RL	**IR/RL**	695.5/2438.9	[30]
5	616.5	616.3	α_{s1}-CN f(34–38)	KENIN			
6	758.5	758.4	α_{s1}-CN f(3–8)	KHPIKH			
7	886.5	886.5	α_{s1}-CN f(3–9)	KHPIKHQ	RP**KHPIKHQ**	13.4	[6]
8	633.6	633.3	α_{s1}-CNf(1–5)	RPKHP			
9	943.7	943.5	α_{s1}-CNf(3–10)	KHPIKHQG	RP**KHPIKHQ**	13.4	[6]
10	850.5	850.4	κ-CNf(96–102)	ARHPHPH			
11	848.5	848.4	α_{s1}-CN f(55–61)	EDAKQMK			
12	243.1	243.1	Various fragments	PK/PQ			
13	541.5	541.3	β-CN f(176–180)	KAVPQ	KVLP**VPQ**	1000	[31]
14	521.6	521.3	α_{s2}-CN f(29–32)	IHPR			
15	772.5	772.4	α_{s1}-CN f(33–38)	RKENIN			
16	1011.8	1011.6	α_{s1}-CN f(1–8)	RPKHPIKH			
17	778.5	778.3	α_{s2}-CN f(29–34)	IHPRKE			
18	586.5	586.3	α_{s1}-CN f(86–90)	VPSER			
19	592.5	592.3	α_{s2}-CN f(28–32)	AIHPR			
20	874.7	874.5	α_{s1}-CNf(1–7)	RPKHPIK			
21	1196.8	1196.7	α_{s1}-CN f(1–10)	RPKHPIKHQG	**RPKHPIKHQ**	13.4	[6]
22	849.5	849.5	α_{s2}-CN f(28–34)	AIHPRKE			
23	901.5	901.4	α_{s1}-CN f(33–39)	RKENINE			
24	542.5	542.3	α_{s1}-CNf(111–115)	IVPKS			
25	569.7	569.3	β-CN f(95–99)	VPKVK			
26	807.8	807.4	α_{s1}-CN f(79–84)	KYIQKE			
27	608.6	608.3	β-CN f(47–51)	DKIHP	**DKIHP**	577.9	[10]
28	1049.2	1049.5	α_{s1}-CNf(127–135)	NPAHQKQPM			
29	958.7	958.4	α_{s1}-CNf(183–190)	KEDVPSER			
30	1106.7	1106.5	α_{s1}-CNf(126–135)	GNPAHQKQPM			
31	746.5	746.4	α_{sl}-CNf(1–6)	RPKHPI	**RPKHPI**	>1000	[32]
32	1363.8	1363.6	α_{s1}-CN f(124–135)	KEGNPAHQKQPM			
33	1235.7	1235.5	α_{s1}-CN f(125–135)	EGNPAHQKQPM			
34	1205.8	1205.6	α_{s2}-CNf(149–158)	TKKTKLTEEE			
35	698.5	698.4	β-CN f(95–100)	VPKVKE	**VPKVKE**	n.d.	[8]
36	584.4	584.3	α_{s1}-CNf(110–114)	EIVPK	L**EIVPK**	1275.4	[10]
37	875.7	875.4	α_{s1}-CN f(102–108)	KKYNVPQ	**KKYNVPQ**	716.9	[10]
38	1045.7	1045.3	β-CN f(13–21) 1P	VESLSSSEE			
39	802.5	802.3	β-CN f(1–6)	REQEEL	**REQEEL**	n.d.	[8]
40	749.5	749.3	α_{sl}-CN f(86–91)	VPSERY	**VPSERY**	706.1	[10]
41	636.5	636.3	α_{sl}-CN f(89–93)	ERYLG	DV**PSERYLG**		[8]
42	1302.7	1302.5	α_{sl}-CN f(56–67) var D, 1P	DAKQMKAGSNSS			
43	864.5	864.4	α_{sl}-CN f (85–91)	DVPSERY	**DVPSERY**	n.d.	[8]
44	1379.6	1379.3	β-CN f(7–18)2P	NVVGETVESLSS			
45	917.6	917.2	α_{s2}-CN f(55–60) 1P	IRSSSE			
46	1309.9	1309.7	α_{sl}-CN f(1–11)	RPKHPIKHQGL			
47	984.8	984.5	α_{s2}-CN f(192–199)	KPWTQPKT			
48	1121.5	1121.5	α_{sl}-CN f(83–91)	KEDVPSERY	**DVPSERY**	n.d.	[8]
49	579.5	579.3	α_{sl}-CN f(89–92)	ERYL	V**RYL**	24.1	[10]
50	1391.6	1391.5	α_{s2}-CN f(9–19) 2P	SSEEPINISQE			
51	559.3	559.2	α_{sl}-CN f(26–30)	APFPE	**PFPE**	>1000	[33]
					FPE	n.d.	[8]
52	549.5	549.3	α_{s2}-CN f(205–208)	VRYL	**VRYL**	24.1	[10]
53	455.4	455.2	α_{s1}-CN f(106–109)	VPQL	KKYN**VPQL**	77.1	[10]
54	1034.7	1034.5	α_{sl}-CN f(85–93)	DVPSERYLG	**DVPSERYLG**	n.d.	[8]
55	755.5	755.4	β-CN f(47–52)	DKIHPF	**DKIHPF**	256.8	[9]
					RAD**HPF**		[34]
56	697.6	697.4	α_{sl}-CN f(109–114)	LEIVPK	**LEIVPK**	1275.8	[10]
57	799.6	799.3	α_{sl}-CN f(89–95)	ERYLGY			
58	658.5	658.3	α_{sl}-CN f(25–30)	VAPFPE	**PFPE**	>1000	[33]
					FPE		[8]
59	671.6	671.3	β-CN f(199–204)	VRGPFP	**VRGPFP**	592	[10]

Table 1 (*Continued*)

	Observed mass[a]	Calculated mass[b]	Protein fragment[c]	Sequence	ACE-inhibitory peptides[d]	IC_{50}[e] (μM)	Reference
60	977.7	977.4	α_{s1}-CN f(85–92)	DVPSERYL	**VPSERYL**	232.8	[10]
61	862.3	862.5	α_{s1}-CN f(86–92)	VPSERYL	**VPSERYL**	232.8	[10]
62	1234.2	1234.6	α_{s1}-CN f(83–92)	KEDVPSERYL	**VPSERYL**	232.8	[10]
63	1106.2	1106.5	α_{s1}-CN f(84–92)	EDVPSERYL	**VPSERYL**	232.8	[10]
64	757.5	757.4	α_{s1}-CN f(24–30)	VV APFPE	**PFPE**	>1000	[33]
					FPE		[8]
65	988.5	988.5	α_{s1}-CN f(102–109)	KKYNVPQL	**KKYNVPQL**	77.1	[10]
66	650.0	650.3	β-CN f(l 11–115)	PFPKY			
67	1291.9	1291.7	β-CN f(164–175)	SLSQPKVLPVPQ	**KVLPVPQ**	1000	[31]
68	721.6	721.3	α_{s1}-CN f(93–98)	GYLEQL			
69	513.6	513.6	α_{S1}-CN f(98–101)	LLRL			
70	1004.8	1004.5	β-CN f(60–68)	YPFTGPIPN	**YPFPGPIPN**	14.8	[6]
71	793.8	793.4	β-CN f(70–76)	LPQNILP	**LPQNILP**	46	[27]
72	551.5	551.3	β-CN f(135–139)	LPLPL			
73	784.6	784.4	β-CN f(199–205)	VRGPFPI	**VRGPFP**	592	[10]
74	1037.6	1037.6	β-CN f(195–204)	VLGPVRGPFP	PKHKEM**PFP**	n.d.	[35]
					VRGPFP	592	[10]
75	938.5	938.5	β-CN f(197–205)	GPVRGPFPI	**VRGPFP**	592	[10]

n.d., not determined.

[a] Observed mass.

[b] Calculated monoisotopic mass.

[c] Protein sequences were obtained from the Swiss Institute for Bioinformatics (Swissprot database).

[d] Previously described ACE-inhibitory peptides that share at least three C-terminal residues with those found in this study. Residues in bold letters indicate sequence homology with the peptide found in this study.

[e] Protein concentration needed to inhibit 50% the original ACE activity.

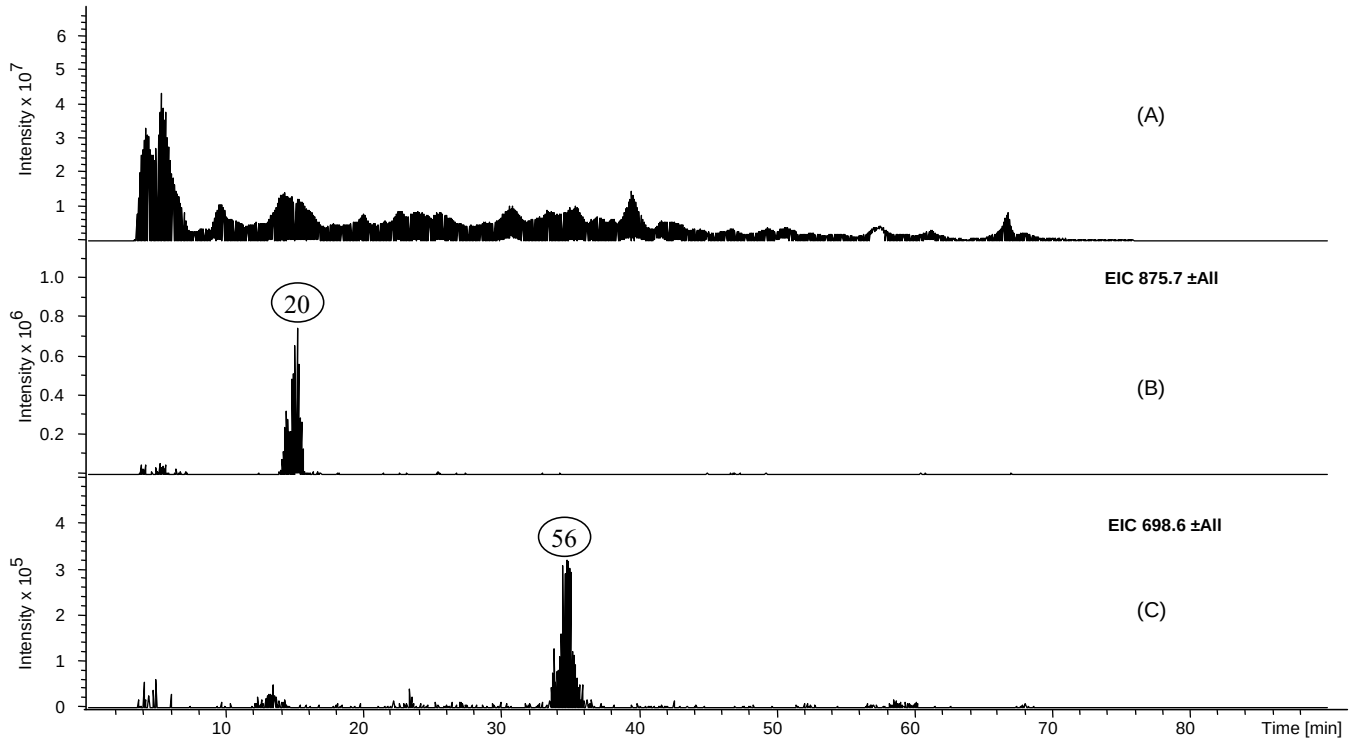

Fig. 3. (A) Total ion current chromatogram corresponding to the M_r 3000 permeate from 8-month-old Manchego cheese type 1. (B) Extracted ion chromatogram of ion *m*/*z* 875.7 and (B) of ion *m*/*z* 698.6 which correspond, respectively, to peptides α_{s1}-CN f(1–7) and α_{s1}-CN f(109–114). Numbers in chromatograms (B) and (C) correspond to the peptide numbers given in Table 1.

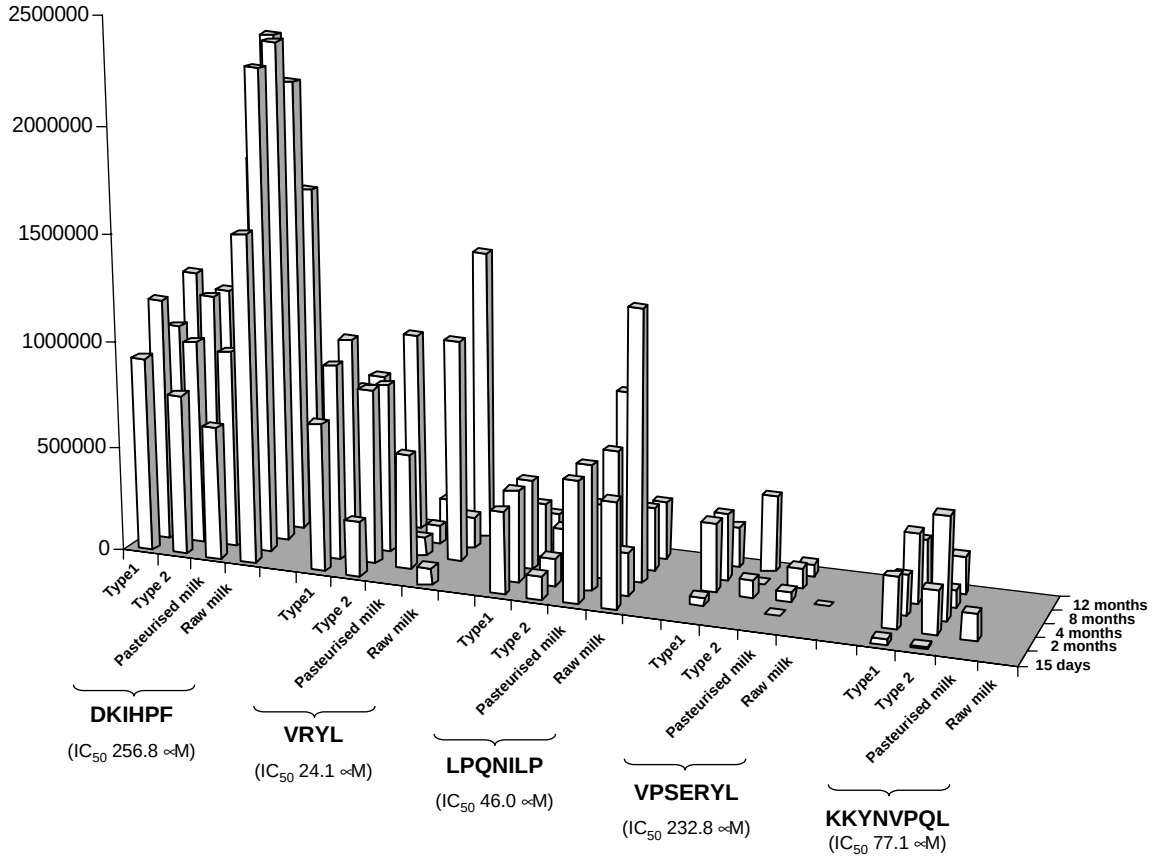

Fig. 4. Estimated amount of peptide (in arbitrary units) after ion extraction of their corresponding characteristic ions (molecular ion and doubly charged ion, when present) in the M_r 3000 permeates from the four batches of Manchego cheese considered during cheese ripening. Type 1, corresponds to experimental Manchego chesse type 1; type 2, corresponds to experimental Manchego cheese type 2; Pasteurised milk, corresponds to Manchego cheese manufactured from pasteurised ovine milk and inoculated with a commercial mixed-strain starter; and Raw milk, corresponds to Manchego cheese prepared with raw ewes' milk without addition of bacterial starters. For details over cheese manufacture see Section 2.1.

found in the 8-month-old Manchego cheese that exhibited IC_{50} values lower than 500 μM (a total of five peptides), were searched for in the all the Manchego cheese types considered in this study: raw-milk cheese, pasteurised-milk cheese and experimental cheeses type 1 and 2. Quantification of the area of the extracted ions gave us an estimation of the amount of the peptide present in the WSE of the corresponding cheese during cheese ripening. Fig. 4 shows the estimated amount of peptides DKIHPF, VRYL, LPQNILP, VPSERYL and KKYNVPQL in the four batches of Manchego cheese during ripening. The most active peptide, i.e., VRYL (IC_{50} 24.1 μM) was detected in all types of Manchego cheese considered in this study along the ripening time although its concentration strongly varied with the type of cheese and the maturation time. The concentration of this peptide was high in the two types of experimental cheeses prepared with selected bacterial strains and its concentration remained practically constant with ripening time. The exception to this behaviour was Manchego cheese type 2 with 8 months ripening time that showed a remarkably lower concentration. The concentration of this peptide was relatively low in the pasteurised-milk Manchego cheese and it varied strongly with the ripening time in the Manchego cheese elaborated with ovine raw milk.

Peptide KKYNVPQL (IC_{50} 77.1 μM) appeared selectively in the two experimental batches although its concentration varied with the ripening time and it was essentially absent from both raw-milk and pasteurised-milk Manchego cheese. Only a small amount of this peptide could be detected in 2-month-old pasteurised-milk Manchego cheese.

Although a general trend was not observed for all active peptides followed in this study, most of them reached maximal concentrations in the raw-milk Manchego cheese followed by Manchego cheese type 1. As a general rule, pasteurised-milk Manchego cheese showed lower concentration in these active peptides than the two experimental batches and raw-milk Manchego cheese except peptide LPQNILP that showed similar values in type 1 and pasteurised-milk Manchego cheese.

4. Conclusions

This report presents an alternative procedure in the search for biologically active peptides derived from food proteins.

The formation of peptides during cheese ripening could easily be followed by the use of a HPLC system coupled *on line* to a tandem mass spectrometer. This technique allowed us to identify peptides with ACE-inhibitory, opioid, and immunomodulatory activity in the permeate of an 8 months ripened Manchego cheese. Different phosphopeptides could also be identified in this fraction. Some active peptides were selected and their presence along ripening in different types of Manchego cheese could be assessed. Our results demonstrate that the two experimental batches of Manchego cheese elaborated with selected bacterial strains, which had previously been designed to prepare good quality Manchego cheeses, contain various ACE-inhibitory peptides in addition to other biologically active sequences.

Acknowledgements

This work has received financial support from the project AGL 2001-1261. J.Á.G.-R. was the recipient of a fellowship from Comunidad Autónoma de Madrid, Spain. The authors thank Dr. L. Cabezas for providing the cheese samples.

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