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Journal of Chromatography A, 1049 (2004) 107–114
JOURNAL OF CHROMATOGRAPHY A
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Application of high-performance liquid chromatography–tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion☆

Blanca Hernández-Ledesma, Lourdes Amigo, Mercedes Ramos, Isidra Recio*

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

Received 8 March 2004; received in revised form 3 July 2004; accepted 12 July 2004

Abstract

Identification of biologically active peptides in food matrices is a challenging task in food technology. In the present study, we propose a strategy for the rapid identification of peptides in complex food fractions and targeting of potentially bioactive peptides according to previous studies of activity-structure relationship. A large number of peptides included in the M_r 3000 permeate of a fermented product and its hydrolysate (obtained by simulated gastrointestinal digestion) could be easily identified using HPLC coupled *on line* to an ion trap mass spectrometer. Three of the identified sequences have previously been described as angiotensin-converting enzyme (ACE) inhibitors. The sequence of some peptides allowed us to anticipate the presence of ACE-inhibitory activity and several peptides were selected to initiate studies on antihypertensive, antioxidant and cytomodulatory activity.

Keywords: Tandem mass spectrometry; ACE-inhibitory activity; Bioactive peptides; Fermented milk; Simulated gastrointestinal digestion

1. Introduction

Milk proteins are precursors of many different biologically active peptides. These peptides, which are inactive within the sequence of the precursor protein, can be released by enzymatic proteolysis during gastrointestinal digestion or food processing. Since 1979, several authors have described bioactive peptides from milk proteins [1]. 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.

Some fermented products have proven to be a source of antihypertensive peptides and in vitro ACE-inhibitory peptides [2,3]. However, other reports have claimed that in some cases milk fermentation is not sufficient to release active sequences from milk proteins but can produce several oligopeptides which will generate the bioactive form after subsequent gastrointestinal digestion [4].

In any case, difficulties in peptide identification limit the knowledge on bioactive peptide formation and release from the precursor proteins. 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 non-specific enzymes is a labour-intensive and difficult task. It comprises several purification steps, generally by combining different chromatographic techniques. Each separation step requires

☆ Presented at the 3rd meeting of the Spanish Association of Chromatography and Related Techniques and the European Workshop, 3rd Waste Water Cluster, Aguadulce, Almeria, 19–21 November 2003.

* Corresponding author. Tel.: +34 91 5622900; fax: +34 91 5644853.
E-mail address: recio@ifi.csic.es (I. Recio).

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doi:10.1016/j.chroma.2004.07.025

solvent removal and evaluation of the biological activity. In most cases, final fractions still contain multiple components and this can cause a discrepancy between the activity found in the purified fractions and the activity of the individual chemically synthesised peptides [3,5].

The sequences of numerous milk protein derived peptides with ACE-inhibitory activity are already known [6]. Also, several studies have established clear structure-activity relationships for ACE inhibitors [7]. All this knowledge may permit the identification of potentially active peptides using new strategies that facilitate identification. One such strategy could involve identification of the major peptide components in complex mixtures by a rapid and reliable analytical technique and the selection of several of the identified peptides for bioactive assays based on their structural similarities to known bioactive peptides.

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 [8]. This information could be used to select potentially active sequences to be individually tested without the need for tedious purification steps.

In the present work, HPLC–MS–MS was used to identify the peptide sequences released by milk fermentation with a strain of *Lactobacillus rhamnosus*. This fermented milk was subjected to a hydrolytic process which simulates physiological digestion and peptides collected in the M_r 3000 permeate were sequenced by HPLC–MS–MS. Several biological activities are proposed for some of the identified sequences in relation to their structures.

2. Experimental

2.1. Fermentation of UHT milk

The following lactic acid bacteria were used to prepare fermented milks: *Lactobacillus plantarum* Colección Española de Cultivos Tipo (CECT) 4645, *Lactobacillus helveticus* CECT 402, *Lactobacillus rhamnosus* CECT 287^T, *Lactobacillus casei* CECT 475^T, *Lactobacillus delbrueckii* subsp. *bulgaricus* CECT 4005^T, *Lactobacillus johnosonii* CECT 289, and *Streptococcus thermophilus* CECT 801 (Colección Española de Cultivos Tipo, Valencia University, Spain). Lactobacilli and *S. thermophilus* were cultured at 37 °C in Man, Rogosa, Sharpe broth (MRS, Pronadisa, Madrid, Spain) and Brain Heart Infusion broth (Pronadisa), respectively. After 24 h of incubation of lactic acid bacteria at 37 °C, colonies were transferred (1%, v/v) to fresh ultra-high-temperature-treated (UHT) skimmed milk, and fermentation was performed at 37 °C for 72 h. Aliquots of each fermented milk were taken during fermentation at 0, 6, 24, 48, and 72 h, and pH, cell growth and ACE-inhibitory activity were measured. The number of lactic acid bacterial cells in the fermented milks was determined by plating on MRS agar and Brain Heart Infusion agar, for Lactobacilli (48 h at 37 °C) and *S. thermophilus* (48 h at 37 °C), respectively.

After fermentation, the pH of fermented milk was adjusted to 4.0 (if the pH of fermented milk was up to 4.0) by adding 1 M HCl, and the milk was centrifuged at 6000 × *g* for 10 min. The acid-soluble supernatant was used to determine ACE-inhibitory activity.

2.2. Measurement of ACE-inhibitory activity

ACE-inhibitory activity was measured by the spectrophotometric assay of Cushman and Cheung [9] as modified by Hernández-Ledesma et al. [10]. Briefly, the acid-soluble supernatant of each fermented milk was adjusted to pH 8.3 with 1 M NaOH and centrifuged at 6000 × *g* for 10 min. A volume of 15 μL of each supernatant was added to 110 μL of 0.2 M potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl, and 10 mM hippuryl-histydil-leucine (Sigma, St. Louis, MO, USA). Twenty-five microliters of ACE (4 mU) (EC 3.4.15.1, 5.1 U/mg, Sigma) was added and the reaction mixture was incubated at 37 °C for 80 min. The reaction was terminated by the addition of 110 μL 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. The ACE-inhibitory activity was calculated as the protein concentration required to inhibit 50% of the original ACE activity (IC_{50}).

2.3. Simulation of gastrointestinal digestion

Milk fermented with *L. rhamnosus* for 72 h was selected to simulate gastrointestinal digestion. The hydrolysate was prepared from an aqueous solution of product (0.7%, w protein/v). Hydrolysis was carried out according to Alting et al. [11] with some modifications [12]. Samples of hydrolysates were taken after hydrolysis with pepsin for 90 min, and after hydrolysis with Corolase PP for 30, 120 and 240 min. These aliquots and the remaining reaction mixtures were centrifuged at 10,000 × *g* for 30 min and the supernatants were subjected to ultrafiltration through a hydrophilic M_r 3000 cut-off membrane (Centripep, Amicon, Beverly, MA, USA). The M_r 3000 permeate was also obtained from undigested fermented milk. The M_r 3000 permeates were freeze-dried and kept at −20 °C until use.

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

RP-HPLC separations of the M_r 3000 permeates 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 (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–trifluoroacetic acid (1000:0.37, v/v) and solvent B contained acetonitrile–trifluoroacetic acid (1000:0.27, v/v). Peptides were eluted with a linear gradient of 0–45% solvent B over 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 approx. 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 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 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 0.35 to 1.4 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. ACE-inhibitory activity during milk fermentation and simulated gastrointestinal digestion

Cell density, pH and ACE-inhibitory activity were monitored during milk fermentation with different lactic acid bacteria. In all cases, ACE-inhibitory activity increased during milk fermentation although the activity indices at the end of fermentation (72 h) were low or moderate (between 15 and 47%). The highest ACE-inhibitory activity (47%) was reached after fermentation with *L. rhamnosus* and the evolution of cell density, pH and ACE-inhibitory activity during milk fermentation with this strain is shown in Fig. 1. It can

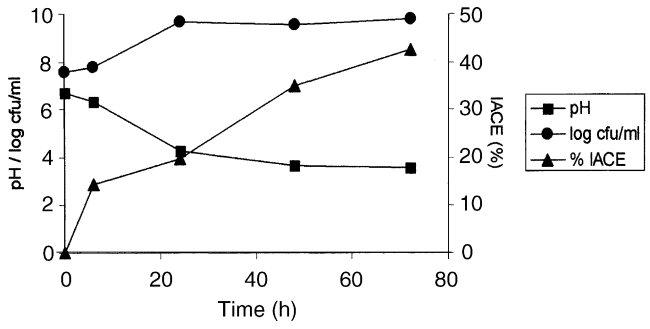

Fig. 1. Evolution of cell density (log cfu/ml), pH and ACE-inhibitory activity (% IACE) during milk fermentation with *L. rhamnosus*.

be concluded that, under these conditions, fermentation is not sufficient to produce potent ACE-inhibitory peptides. Similar results were obtained by other authors [13] although it has also been shown that a strongly proteolytic *L. helveticus* CP790 can produce ACE-inhibitory peptides from caseins [14].

In order to study whether the proteins and peptides present in this fermented milk can act as precursors of peptides with ACE-inhibitory activity, the milk fermented with *L. rhamnosus* was subjected to a two-stage hydrolysis process that simulates gastrointestinal digestion. The M_r 3000 permeate of each aliquot withdrawn during simulated digestion was obtained. The ACE-inhibitory activity of the M_r 3000 permeates and the total hydrolysates was calculated as IC_{50}. While the activity of the undigested sample and its permeate was very low, i.e., high IC_{50} values (756.7 and 1030.1 μg/mL, respectively), the ACE-inhibitory activity notably increased after hydrolysis with pepsin for 90 min (IC_{50} values of water soluble extract and its permeate of 110.7 and 87.8 μg/mL, respectively). However, the activity of both the total hydrolysate and the M_r 3000 permeate decreased slightly with progression of the hydrolysis catalysed with the pancreatic extract. Despite this decrease observed during the last part of the simulated digestion, the activity found at the end of the simulated digestion (IC_{50} values of water soluble extract and permeate of 174.2 and 198.6 μg/mL, respectively) was higher than that measured in the undigested sample. Similar behaviour was observed in our laboratory during simulated gastrointestinal digestion of commercial fermented milks [12]. With the exception of the sample obtained after 90 min hydrolysis with pepsin, activities of the total hydrolysates were slightly higher than the activities found in the M_r 3000 permeates. This shows that the activity was in part caused by higher molecular mass peptides that remained in the retentate fraction after the ultrafiltration process.

3.2. Identification of peptides by HPLC–MS–MS

With the aim of identifying putative active peptides, the M_r 3000 permeates of the samples obtained during simulated digestion were subjected to RP-HPLC coupled *on line* to ion trap mass spectrometry. Fig. 2A shows the RP-HPLC–UV chromatograms of the permeates obtained from the fermented milk before and after simulated digestion. The permeate corresponding to the undigested fermented milk was injected at a concentration 10 times higher than the digested sample. Even at this concentration, the peptide content of the permeate corresponding to the undigested sample was very low which is in accord with the poor ACE-inhibitory activity found in this sample.

Identification of peptide components in 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 involved the search for the masses and partial sequences

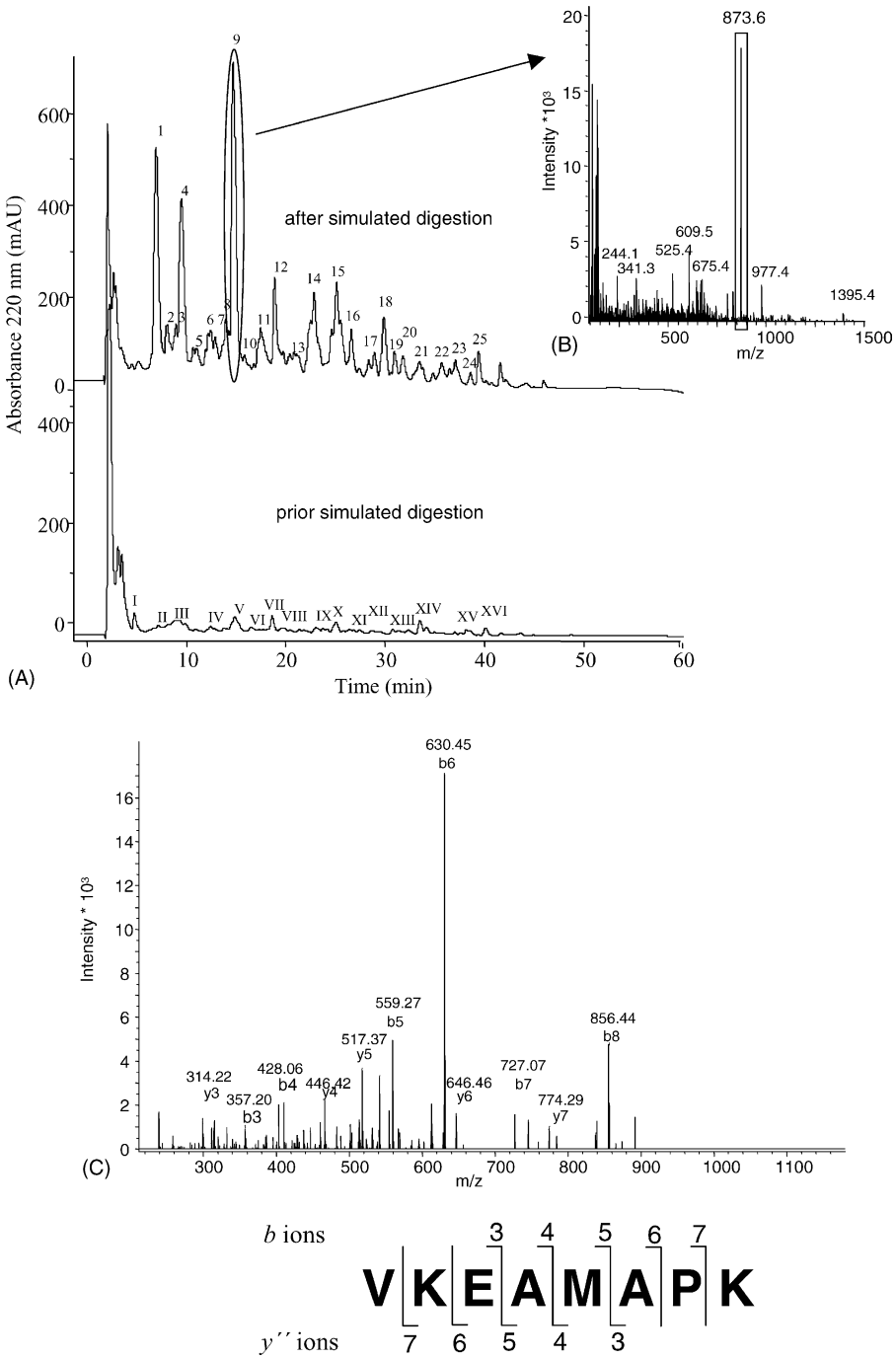

Fig. 2. (A) RP-HPLC–UV chromatograms corresponding to the M_r 3000 permeate from milk fermented with *L. rhamnosus* prior to, and after simulated gastrointestinal digestion. (B) Mass spectrum of the selected chromatographic peak in Fig. 3A. (C) MS–MS spectrum of ion *m/z* 873.6. Following sequence interpretation and database searching, the MS–MS spectrum was matched to β-casein f(98–105). The sequence of this peptide is displayed with the fragment ions observed in the spectrum. For clarity, only the *b* and the *y''* fragment ions are labelled.

(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. Fig. 2B shows the MS spectrum of the most abundant component from the M_r 3000 permeate of the digested sample (peak 9 in Fig. 2A) and the MS–MS spectrum of ion *m/z* 873.6. The fragmentation spectrum contained a major ion at *m/z* 630.45 which was identified as a *b*-type fragment ion resulting from the cleavage N-terminal to Pro (Fig. 2C). This amino acid is associated with very abundant *y* and *b* ions that are often easily identifiable because of their intensity [15]. However, cleavage C-terminal to Pro produces low ion abundance as is the case of b_7 in Fig. 2C, as previously observed in our laboratory [6,12] and by others [16,17]. The

presence of the basic residue Lys near the N-terminus and at the C-terminus favoured the appearance of both *b*- and *y*-type fragment ions [8].

Because milk caseins are rich in Pro residues and this amino acid has been related to several unusual fragmentation events [17], its presence in several of the identified peptides helped in their identification. As an example, Fig. 3 shows the fragmentation spectra of the singly charged precursor ions corresponding to peptides IHPF, VLPVPQ and YQEPVLGPV. In the fragmentation profile of peptide IHPF (Fig. 3A) fragment ions at *m/z* 251.07 and 263.06 that correspond to b_2 and y_2, respectively, are remarkably over-represented in the spectrum. These fragments are produced by cleavage at the His–Pro bond. As well as the Pro effect, it has been shown that the basic His residue is implicated in preferential ion formation at its C-terminal bond [18]. Therefore, it is reasonable that these fragments were the most intense in the spectrum. A similar effect was obtained for VLPVPQ (Fig. 3B), although in this case the most noticeable fragment ions were y_2 and y_4 that corresponded to the cleavage at the Val–Pro and Leu–Pro bonds, respectively. Remarkably, in this spectrum the intensity of fragment ions b_2 and b_4 was not notable although higher intensities could be predicted. As expected, the signal of the fragments formed C-terminal to Pro was low or non-existent. These results are in agreement with those obtained by Breci et al. when studying the N-terminal to proline cleavage in a group of peptide mass spectra acquired in a Finnigan ion trap mass spectrometer [17]. They found that the most abundant Xxx–Pro relative bond cleavage ratios were observed when Xxx was Val,

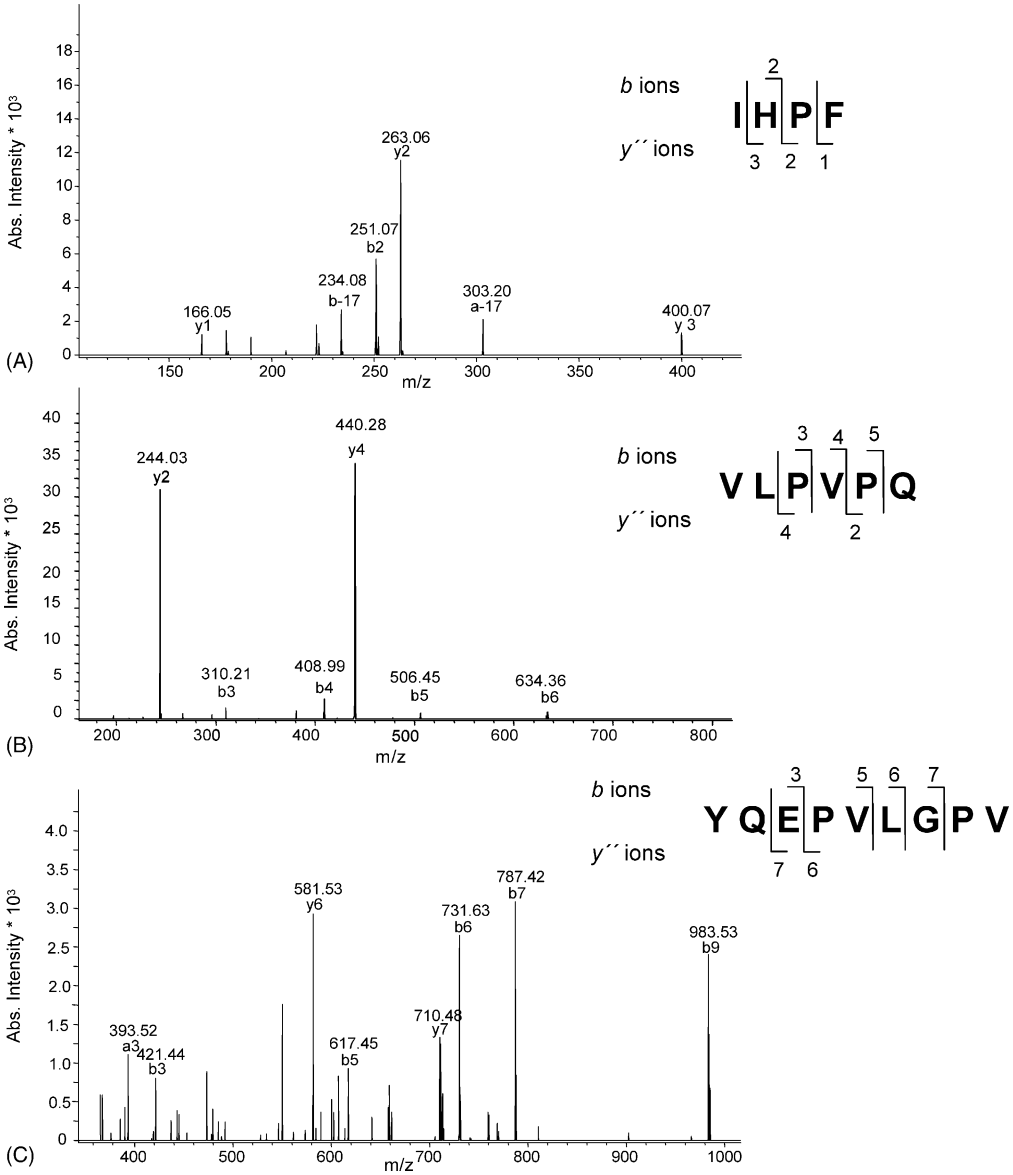

Fig. 3. MS–MS spectrum of ions (A) *m/z* 513.4; (B) *m/z* 689.5; (C) *m/z* 1001.6. Following sequence interpretation and database searching, the MS–MS spectrum were matched to (A) β-casein f(49–52); (B) β-casein f(133–138); (C) β-casein f(193–201).

Table 1
Casein-derived peptides identified in the M_r 3000 permeate obtained from milk fermented with *L. rhamnosus* during 72 h

Peak number[a]	Observed mass	Calculated mass[b]	Protein fragment	Sequence
II	439.3	439.24	β-CN f(73–76)	NIPP
III	529.4	530.28	α_{s2}-CN f(123–126)	LNRE
VI	655.4	655.32	κ-CN f(155–160)	SPPEIN
	761.5	761.40	β-CN f(191–196)	LLYQEP
VII	801.5	801.44	β-CN f(176–182)	KAVPYPQ
VIII	758.4	758.37	β-CN f(183–188)	RDMPIQ
	450.3	450.26	α_{s1}-CN f(90-92)	RYL
IX	622.4	623.26	κ-CN f(149-153)	SPEVI
	689.5	690.33	β-CN f(51–56)	PFAQTQ
	779.6	779.49	β-CN f(169–175)	KVLPVPQ
X	696.4	697.34	α_{s1}-CN f(152–156)	QFYQL
	755.5	755.40	β-CN f(47-52)	DKIHPF
XI	747.4	747.38	β-CN f(193–198)	YQEPVL
	468.3	488.25	κ-CN f(16–18)	RFF
XIII	1015.5	1014.54	β-CN f(192–200)	LYQEPVLGP
XIV	1099.6	1099.60	κ-CN f(73–81)	ILQWQVLSN
	875.5	875.46	α_{s1}-CN f(193–199)	KTTMPLW
XV	897.6	898.48	κ-CN f(157–164)	PEINTVQV

[a] Peak number as indicated in Fig. 2A.
[b] Monoisotopic mass.

Table 2
Casein-derived peptides identified in the M_r 3000 permeate obtained from milk fermented with *L. rhamnosus* and subjected to a hydrolysis process that simulates physiological gastrointestinal digestion

Peak number[a]	Observed mass	Calculated mass[b]	Protein fragment	Sequence	Peptide formation[c]
1	373.3	373.20	β-CN f(72–74)	QNI	3, 4, 5
	387.3	388.20	α_{s1}-CN f(108–110)	QLE	3, 4, 5
2	287.2	287.20	Various fragments	RL	3, 4, 5
3	524.4	524.31	α_{s1}-CN f(80–83)	HIQK	3, 4, 5
	516.5	516.33	β-CN f(29–32)	KIEK	3, 4, 5
4	493.4	493.30	α_{s1}-CN f(4–7)	HPIK	3, 4, 5
5	449.4	450.22	κ-CN f(15–17)	ERF	2, 3, 4, 5
6	548.4	548.30	κ-CN f(61–65)	YAKPA	3, 4, 5
	645.5	645.26	β-Lg f(108–113)	ENSAEP	3, 4, 5
7	653.4	653.31	α_{s1}-CN f(125–130)	EGIHAQ	4, 5
	781.5	781.37	α_{s1}-CN f(125–131)	EGIHAQQ	3, 4, 5
	608.4	608.29	κ-CN f(33–37)	SRYPS	3, 4, 5
8	365.3	365.21	β-CN f(134–136)	HLP	4, 5
	674.4	674.32	β-CN f(1–5)	RELEE	2, 3, 4, 5
9	872.6	872.48	β-CN f(98–105)	VKEAMAPK	3, 4, 5
10	525.4	525.25	α_{s1}-CN f(125–129)	EGIHA	3, 4, 5
	678.5	678.34	α_{s1}-CN f(8–13)	HQGLPQ	3, 4, 5
11	646.5	646.34	α_{s2}-CN f(90–94)	QKFPQ	3, 4, 5
	633.5	633.35	α_{s1}-CN f(104–108)	YKVPQ	4, 5
12	503.3	503.28	β-CN f(179–182)	PYPQ	2, 3, 4, 5
13	603.4	603.29	β-CN f(114–118)	YPVEP	3, 4, 5
14	427.4	427.24	β-CN f(186–189)	PIQA	3, 4, 5
	906.5	906.46	α_{s1}-CN f(8–15)	HQGLPQEV	3, 4, 5
	651.5	651.40	β-CN f(170–175)	VLPVPQ	3, 4, 5
	627.5	627.40	β-Lg f(76–81)	TKIPAV	3, 4, 5
15	512.4	512.27	β-CN f(49-52)	IHPF	3, 4, 5
	747.5	747.36	β-CN f(108–113)	EMPFPK	3, 4, 5
16	644.5	644.32	α_{s1}-CN f(133–138)	EPMIGV	3, 4, 5
18	1000.6	1000.52	β-CN f(193–201)	YQEPVLGPV	3, 4, 5
	750.5	750.36	β-CN f(114–119)	YPVEPF	3, 4, 5
19	680.5	680.36	κ-CN f(103–108)	LSFMAI	3, 4, 5
21	1126.5	1126.71	α-La f(93–102)	KKILDKVGIN	3, 4, 5
22	688.5	688.43	β-CN f(133–138)	HLPLPL	3, 4, 5
23	904.5	904.47	α_{s1}-CN f(24–31)	FVAPFPEV	3, 4, 5
24	897.6	897.54	β-CN f(202–209)	RGPFPIIV	2, 3, 4, 5
25	741.6	741.44	β-CN f(203–209)	GPFPIIV	3, 4, 5

[a] Peak number as indicated in Fig. 2A.
[b] Monoisotopic mass.
[c] 1, undigested product; 2, digestion with pepsin 90 min; 3, digestion with pepsin and Corolase PP 30 min; 4, digestion with pepsin and Corolase PP 120 min; 5, digestion with pepsin and Corolase PP 240 min.

His, Asp, Ile and Leu while less abundant fragment ions occurred when Xxx is Gly or Pro. As an example of Gly–Pro cleavage, peptide YQEPVLGPV (Fig. 3C) contained two Pro residues preceded by Glu and Gly, respectively. Similar intensities were found for y_6, corresponding to cleavage Glu-Pro, and for b_7, corresponding to cleavage Gly–Pro, and none of these two fragment ions showed marked abundancy in the spectrum.

By using *on line* HPLC–MS–MS, most peptides with intensities higher than 5000 units were identified in the M_r 3000 permeates. A total of 15 peptides were identified in the permeate of the undigested fermented milk (Table 1) and 25 peptides in the permeate of the fermented milk subjected to simulated digestion (Table 2). In addition, the formation of peptides during simulated gastrointestinal digestion could be followed by HPLC–MS–MS analysis of the M_r 3000 permeates of the aliquots withdrawn during digestion. None of the peptides identified in the undigested sample survived the simulated digestion process. As shown in Table 2, most peptides appeared after 30 min hydrolysis with Corolase PP, and only four peptides which were formed by peptic hydrolysis survived further hydrolysis with the pancreatic extract. These results explain the behaviour of the ACE-inhibitory activity during simulated gastrointestinal digestion. Analysis by HPLC–MS–MS revealed that the fermented product suffered extensive hydrolysis after 90 min hydrolysis with pepsin (data not shown). These peptic peptides might be responsible for the potent ACE-inhibitory activity observed in the M_r 3000 permeate. However, only few of these sequences survived the enzymatic treatment with Corolase PP and this peptide degradation might explain the gradual loss of ACE-inhibitory activity during hydrolysis with the pancreatic extract.

Among the identified peptides, several have been previously demonstrated to exhibit ACE inhibition. For instance, peptides DKIHPF, YQEPVL and EMPFPK (peptides X, XI and 15 in Tables 1 and 2) have demonstrated ACE-inhibitory activity with IC_{50} values as low as 256, 280 and 423 μM, respectively [3,13]. Other peptide sequences had previously been described as biologically active peptides with various activities. For instance, peptide VKEAMAPK (peptide 9 in Table 2) exhibits antioxidant activity [19]. Moreover, several peptides show a high homology or share important structural features with previously identified bioactive peptides. As an example, peptide NIPP shares the last three C-terminal residues, which play a predominant role in binding to the ACE, with IPP, which is one of the most potent antihypertensive peptides found in food to date [2]. Similarly, peptide KTTMPLW (peptide XIV in Table 1) may exhibit ACE-inhibitory activity as peptide TTMPLW (IC_{50} value of 16 μM [20]), and peptide YQEPVLGPV (peptide 18 in Table 2) may posses immunomodulatory properties as occurs with YQEPVLGPVR [21]. A summary of the biologically active peptides found and potentially new active sequences is shown in Table 3. Some of these peptides are being chemically synthesised and are being used as

Table 3
Sequences identified in the M_r 3000 permeates from the digested or undigested fermented milk that share structure homology with previously described bioactive peptides

Peak no.[a]	Sequence	Previously described sequence	Activity[b]	Ref.
II	NIPP	IPP	Antihypertensive (IC_{50}5 μM)	[2]
VII	KAVPYPQ	AVPYPQR	Citomodulatory and antioxidant	[19,22]
IX	KVLPVPQ	SKVLPVPQ	Antihypertensive (IC_{50}39 μM)	[14]
		VLPVPQK	Antioxidant	[19]
X	**DKIHPF**	**DKIHPF**	ACE-inhibitory (IC_{50}256.8 μM)	[3]
XI	**YQEPVL**	**YQEPVL**	ACE-inhibitory (IC_{50}280.0 μM)	[13]
XIV	KTTMPLW	TTMPLW	ACE-inhibitory (IC_{50}16 μM)	[20]
			Immunomodulatory	[23]
8	HLP	LHLP	ACE-inhibitory (IC_{50}210 μM)	[24]
9	**VKEAMAPK**	**VKEAMAPK**	Antioxidant	[19]
11	YKVPQ	KKYKVPQ	ACE-inhibitory (IC_{50} nr)	[5]
12	PYPQ	AVPYPQR	Citomodulatory	[22]
14	VLPVPQ	SKVLPVPQ	Antihypertensive (IC_{50}39 μM)	[14]
		VLPVPQK	Antioxidant	[19]
15	IHPF	DKIHPF	ACE-inhibitory (IC_{50}193.9 μM)	[3]
15	**EMPFPK**	**EMPFPK**	ACE-inhibitory (IC_{50}423 μM)	[13]
22	HLPLPL	HLPLP	ACE-inhibitory (IC_{50}2336 μM)	[24]
24	RGPFPIIV	LLYQQPVLGPVRGPFPIIV	ACE-inhibitory (IC_{50}21 μM)	[14]
25	GPFPIIV	LLYQQPVLGPVRGPFPIIV	ACE-inhibitory (IC_{50}21 μM)	[14]

The sequences in bold letters correspond exactly to previously found bioactive peptides; nr, non reported.

[a] Peak no as shown in Fig. 2A.

[b] The activity was referred to as antihypertensive when the effect was demonstrated in laboratory animals or humans. If the activity was only demonstrated in vitro the activity was referred to as ACE-inhibitory.

the starting point for further studies on biologically active peptides derived from food proteins.

4. Conclusions

This report presents an alternative procedure in the search for biologically active peptides derived from food proteins. The formation of bioactive peptides by fermentation or by the action of gastrointestinal enzymes on food proteins 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, antioxidant and immunomodulatory activity in the M_r 3000 permeate of the fermented milk. Moreover, based on the known structure-activity relationships, several putative active sequences could be selected to be tested for different biological activities. It has to be stressed that the identified peptides were originated by enzymatic hydrolysis and they have not been just predicted by computing simulation. Therefore, these peptides are likely produced in vivo after consumption of fermented milks. The successful strategy of rapid peptide identification by HPLC–MS–MS may initiate future studies on biologically active peptides derived from food proteins avoiding the tedious steps of purification and isolation.

Acknowledgement

This work has received financial support from the project CAL01-046-C2. The authors would like to thank Röhm Enzyme Gmbh for providing the enzymatic preparation (Corolase PP). B.H.-L. was the recipient of a fellowship from Instituto Danone, Spain.

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