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A Simple Method to Separate the Antimicrobial Peptides from Complex Peptic Casein Hydrolysate and Identification of a Novel Antibacterial Domains within the Sequence of Bovine α_s-Casein

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Abstract – *A simple method to separate antimicrobial peptides from complex peptic hydrolysate of bovine casein is proposed. It consists to precipitation of the active peptides under ionic strength and pH conditions. The peptide extract obtained was fractionated using reverse phase high performance liquid chromatography and the actives fractions are analyzed by mass spectrometry (LC-ESI-MS and MADI-TOF). Several peptides derived mostly from α_s-casein are identified. The major component in active fractions are αs_2-CN f(164-179), αs_2-CN f(148-166), αs_2-CN f(180-207), αs_2-CN f(183-207), αs_2-CN f(131-174), αs_1-CN f(153-196), αs_1-CN f(141-187), αs_2-CN f(176-207), αs_2-CN f(164-207), αs_2-CN f(99-163). The minimum inhibitory concentration and mode of action of the peptide extract and purified peptide αs_2-CN f(164-207) were studied against several Gram-positive and Gram negative Bacteria.*

Keywords*: Antimicrobial Peptides, α_s-Casein, Hydrolysis, Precipitation*

Nomenclature

LC-ESI/MS	Liquid Chromatography - Electrospray Ionization/ Mass Spectrometry
RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
DH	Degree of Hydrolysis
MALDI-TOF	Matrix Assisted Laser Desorption Ionization - Time Of Fight
MIC	Minimum Inhibitory Concentration

I. Introduction

The use of food protein as a source of antimicrobial peptides appears among the current voices of research for natural molecules. Indeed, during these last decades, several studies reported the antibacterial properties of some food proteins (lysozyme, lactoperoxydase, lactoferrin,…) [1], [2], as well as the possibility of generating active peptides by enzymatic hydrolysis of the precursor protein in vivo or in vitro [3], [4]. Thus, several antimicrobial peptides were obtained by enzymatic hydrolysis of bovine hemoglobin.

The first peptide obtained by pepsin hydrolysis of this protein corresponded to the fragment α(1-23), active against *Micrococcus luteus* [5]. Thereafter, within the same laboratory thirty active peptides were purified and characterized [6], [7].

In 1998, Ibrahim et al. [8] isolated by acid hydrolysis of the chicken ovotransferrin a peptide f(109-200).

From lysozyme of chicken egg white, the same authors arrived, in 2001, to isolate after hydrolysis with clostripaine a peptide f(98-112) [9]. However, milk proteins showed most interest. Indeed, The biodefensive properties of these proteins have been well described and widely recognized for several decades. The antimicrobial properties of lactoferrin, lysozyme and lactoperoxidase are now well studied [4].

Moreover several antimicrobial peptides were released by enzymatic hydrolysis of major milk protein, inactive in the native state. LAHOV et al., (1971) [10] were the first to identify polypeptides of high molecular weight and basic character after treatment of acidified milk by rennet. In 1974, HII et al. [11] identified the «Isracidin»; antimicrobial peptide corresponding to the N-terminal fragment f(1-23) of α_{s1}-casein. From α_{s2}-casein, Zucht et al., (1995) [12] isolated the «casocidine» I f(165-203), and in 1999, Recio and Visser [13] have isolated two other antimicrobial peptides corresponding to fragments f(183-207) and f(164-179), through pepsin hydrolysis. McCann et al. in 2005 [14], have separated by the action of chymosin other peptides from the same region of the α_{s2}-casein, f(181-207, 180-207, 175-207,172-207, 164-207). In 2006, [15] this team arrived to separate, by peptic hydrolysis of sodium caseinate, the peptide f(183-207) from α_{s2}-casein, already identified and a fragment,

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f(99-109), of α_{s1}-casein. Two antimicrobial peptides were obtained from κ-casein.

The first, called «kappacine», corresponding to the fragment f(138-158), showed an action toward cariogenic bacteria [16]. The second peptide whose sequence corresponds to the fragment f(63-117) was isolated through pepsin hydrolysis of human κ-casein [17]. Hydrolysis of human milk by Lactobacillus helveticus PR4 protease released a peptide corresponding to the fragment f(184-210) of β-casein [18]. Two whey protein, α-lactalbumin and β-lactoglobulin have also generated by enzymatic hydrolysis several antimicrobial peptides [19], [20]. These peptides are mainly active on Gram-positive bacteria. Lactoferricin, corresponding to the fragment f(17-41), is the first antimicrobial peptide released by peptic hydrolysis of lactoferrin, minor milk protein [21]. Other peptides corresponding to the fragment f(277-288), f(267-285), f(267-288), f(268-284) of lactoferrin were also separated [22].

Besides the fact that they represent an interesting source of antimicrobial peptides, the interest in casein is due to their availability and ease of their separation from milk. However, the separation of antimicrobial peptides from enzymatic hydrolysates needs fastidious and expensive techniques and finally without sufficient quantities of active peptides for subsequent characterizations particularly in relation to the application study. So, the aim of our study is revisit the possibility of applying conventional methods such as precipitation under the influence of ionic strength and pH. In the present paper, we try to develop a simple and non expensive method to recover the active peptide from complex bovine casein hydrolyzate by optimizing the separation conditions through ionic strength and pH parameters.

II. Materials and Methods

II.1. Materials and Bacterial Cultures

Bovine sodium caseinate, pig pepsin (EC 3.4.23.1, 3.200-4.500 units/mg proteins) were purchased from sigma chemical company (Evry, France). Cultures of *Bacillus subtilis* **ATCC 6633** was from the American Type Culture Collection (ATCC) (Rockville, MD, USA) , *Listeria innocua* **LMG 11387**, *Escherichia coli* **JM 109**, *Escherichia coli* **DH5α,** *Staphylococcus aureus* **CIP 4.83,** *E. coli* **CIP 54127**, *L. monocytogenes* **ATCC 3512** (CM/NCTC, UK). Acetonitrile was of HPLC grade. Water was obtained from a Millipore MilliQ system; the resistance was about 18 MV. Common chemicals and solvents of analytical grade were obtained from different commercial sources.

II.2. Digestion of Caseins with Pepsin

Aqueous solution of Bovine casein 5% (w/v) was adjusted to pH 2.5 with 1 M HCl and digested with 1% (w/w of substrate) pig pepsin (EC 3.4.23.1) for 30 min at 37°C. The reaction was terminated by adjusting pH to 7.0 by addition of 1M NaOH and heating at 80 °C for 15 min.

II.3. Determination of the Degrees of Casein Hydrolysis

The degree of hydrolysis (DH) is defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds of protein substrate. DH is determinated by the OPA (o-phthaldialdehyde) method using leucine as standard [23], [24].

A fresh OPA solution was prepared extemporaneously with 25 ml of 100 mM sodium tetrahydroborate, 2.5 ml of 20% SDS solution (w/v), 40 mg of OPA (dissolved in 1 ml of methanol) and 100 mL of β-mercaptoethanol were adjusted to a final volume of 50 ml with distilled water. To assay proteolysis with milk proteins as substrates, an aliquot (usually 10–50 ml containing 5–100 mg proteins) was added to 1.0 ml of OPA reagent in a 1.5 ml quartz cuvette.

The solutions were mixed briefly and incubated for 2 min at room temperature, and then the absorbance at 340 nm was measured.

II.4. Purification of Antibacterial Peptides

II.4.1. Separation of Antimicrobial Peptides from the Peptic Casein Hydrolysate by Precipitation Under Ionic Strength and pH Effect

A quantity of NaCl is added to a fixed volume of hydrolyzate, adjusted to pH 7.5, for a final concentration of 2.0 M of salt. The hydrolyzate was stirred for 30 min at room temperature before being centrifuged at 10,000 × g for 10 min at 4°C.

The pellet was washed twice by centrifugation with phosphate buffer (5 mM, pH 7.5) to remove solubles proteins, then resuspended in phosphate buffer (5 mM, pH 7.5, 100 mM NaCl) to 20 times the original hydrolyzate volume. The pH is adjusted between 1.5 to 2.

After one hour stirring at 7°C, the pellet was centrifuged (10,000 × g, 10 min), and the supernatant is recovered. The peptides concentration is determined using the OPA O-PhthaldiAldehyde methods [18] and the solution was stored at -20°C until performing the antibacterial assay and analysis by Reversed-phase high performance liquid chromatography (RP-HPLC).

II.4.2. Analytical and Preparative HPLC

Reversed-Phase High-performance Liquid Chromatography (RP-HPLC) was performed with an automated HPLC system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and analyze chromatographic data. An analytical XBridge

C_{18} column, (5µm, 4.6 × 250 mm) and preparative XBridge C_{18} column, (5µm, 10 × 250 mm) (Waters, Ireland) were used with a flow rate of 1ml/min and 4ml/min respectively. Gradient elution was carried out with a mixture of solvant A (0.1 %, trifluoroacetic acid (TFA), v/v) in deionized water and solvent B (0.1% TFA in acetonitrile, v/v). Proteins and peptides were eluted as follows; 0-80 min, 40% B; 80-85min, 40- 100% B; 85-90 min, 100% B; 90-100 min, 100-0% B and the HPLC system was equilibrated for 10 min with 100% A.

Elution was performed at room temperature. Samples were filtered through 0.2 µm filters. On-line UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software.

II.5. Peptide Analysis by On-Line LC-ESI-MS

Analysis were performed on a Accela UHPLC system acquired from Fisher Scientific (Thermo Fisher Scientific, Bremen, Germany) that consisted in an autosampler with a column oven and a tray compartment cooler and a quaternary pump with a built-in solvent degasser ; all piloted by Xcalibur software.

The Accela UHPLC system was coupled to an Orbitrap mass spectrometry system (Thermo Fisher Scientific, Bremen, Germany) equipped to ESI ion source. HPLC analyses were performed with an analytical C_{18} column (Xbridge, 5µm, 4.6 × 250 mm). HPLC gradient is the same previously described (section *II.4.2*). A split was applied to divide the flow at 300 µL/min. ESI inlet conditions were : Nitrogen used both as a sheath and auxiliary at arbitrary flow of 20 and 10 respectively. Capillary temperature was set at 275°C. In the positive mode, capillary voltage was set to 93.4 V, source voltage to 3.3 kV and the source current 0 to 8µA. Spectra were recorded over the masse/charge (m/z) range 400 to 2000.

II.6. Peptides Identification by Mass Spectrometry

Data were acquired using an UltraFlex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam ™ laser. Direct profiling data were performed in positive reflectron mode, for a mass range from *m/z* 500 to 6000 or from *m/z* 3000 to 20000. A total of 1000 spectra were acquired at each spot at a laser frequency of 100 Hz.

FlexImaging ™ 2.1 software was used to record the data. Standards for spectral calibration consisted in a mixed solution of peptides ranging between *m/z* 900 to 3500 or a mixed solution of protein ranging between m/z 3000-9000.

Two matrices were used (UV absorber) : the first consisted of a solution of 10 mg/ml cyano-4-hydroxycinamic acid (HCCA) in a mixture of water and acetonitrile (30/70 ; v/v) with 0.1% TFA, and the second corresponded to sinapinic acid (SA) solution 10 mg/ml prepared in a mixture 25/75 (v/v) of water and acetonitrile with 0.1% TFA.

Deposition of the sample on the target can be achieved by the method of the thin layer : 1 µl of matrix and 1 µl of each HPLC fraction were deposited. The samples were dried before being analyzed. 1000 laser shots at each target have generally been accumulated to produce the final mass spectrum. Processing software of the spectra has allowed for each peak detected and determined the corresponding masses in positive mode. The accuracy in the mass is 0.01%.

II.7. Assays for Antibacterial Activity

II.7.1. Agar Diffusion Method

Antibacterial activities of the different peptides were determinated by a plate diffusion assay. *L. innocua, Bacillus subtilis* and *Escherichia coli* **JM 109** grown in Brain Heart Broth were used as the indicator organism. 20µl of sample were applied to Műller-Hinton agar (containing 5g glucose per litre) seedes with strain (approximately 1×10^6 CFU/ml). The plates were incubated at 37°C for 18-20 h and examined for zones of growth inhibition on the resultant bacterial lawn.

II.7.2. Minimum Inhibitory Concentration Determination of Antibacterial Peptides

The Minimum inhibitory concentration **(**MIC) assays were performed in sterile 96-well microplates (Costar 3799, Corning Incorporated, United Kingdom). In each well containing 190µl of Műller-Hinton medium were added 50 µl of peptide and 10 ul of the strain tested prediluted in Muller-Hinton medium for a final bacterial load at 2-8 × 10^4 CFU/ml. The absence of bacterial growth is revealed by measuring absorbance at 630 nm on a microplate reader (MRX II, Dynex Technologies, microcicer company) after 18 h of incubation at 37°C. Peptides were tested in triplicate. Absorbance of wells at concentrations corresponding decays of the peptide is compared to that of the wells of a negative control consisting of Muller-Hinton medium and a control culture.

The MIC was defined as the lowest concentration of peptide that resulted in no increase of absorbance at 630 nm after incubation at 37°C for 18 h without shaking.

III. Results

III.1. Peptic Hydrolysis of Bovine Casein

Bovine casein was hydrolysed by pepsin at pH 2.5 (37°C) and kinetics studies were followed at 2.5, 5, 10, 15, 30, 45 min and from one to six hours of hydrolysis taking a sample each hour and finally a last sampling at 24h. The resulting hydrolysates were analyzed in one step by reversed phase HPLC on a C18-column according to a method described in section (II.4.2).

Fig. 1 shows the chromatograms of peptic hydrolysates of bovine casein according to the degree of hydro-

lysis. Examination of the profiles obtained indicates the occurrence, in the initial stage of hydrolysis, of several large fragments, with elution times close to those of the substrate. As the evolution of the hydrolysis, the rate of the initial components decreases in favor of those of smaller peptides, which appear in small amounts to the top of the chromatograms and continued to increase during the hydrolysis. This change occurs while a part of the substrate remains intact. This suggests that pepsin acts on casein as a mechanism namely one by one which characterizing enzymatic reactions when the reaction mixture was composed of intact substrate and final product of small size. This means that the protease cleaves one molecule of protein substrate at a time. However, the appearance also of intermediate peptides of the beginning of hydrolysis, characteristic of the zipper mechanism indicates that the action of pepsin on casein follows an intermediate mechanism between the two described [25].

III.2. Fractionation of ACTIF Peptic Hydrolysate

Several studies indicate the possibility of generating antimicrobial peptides by pepsin hydrolysis of bovine casein [13]-[15]. Antimicrobial activity was investigated on all hydrolysates taken at different intervals of hydrolysis. Hydrolysates were tested *against L. innocua* LMG 11387, *B. subtilis* ATCC 6633 and *E coli* JM109 which were used as the test strain. A clear zone of growth inhibition were observed starting from 15 min of hydrolysis (Table I). Thus, to be able to isolate a actives peptides and there precursors, analysis was carried out on sample from 30 min of hydrolysis which showed activity against all tested strain. This hydrolysate is fractionated using preparative RP-HPLC and the resulting fractions were collected at 1 min intervals.

TABLE I
ANTIBACTERIAL ACTIVITY OF PEPTIC CASEIN HYDROLYSATES

Hydrolysats	Test strain		
	L. innocua LMG 11387	*B.subtilis* ATCC 6633	*E.coli* JM109
T0	–	–	–
2.5 min	–	–	–
5 min	–	–	–
10 min	–	–	–
15 min	+	–	+
30 min	+	+	+
45 min	+	+	+
1 h	+	+	+
2 h	+	+	+
3 h	+	+	+
4 h	+	–	+
5 h	+	–	+
6 h	+	–	+
24 h	+	–	+

Assessment of each fraction, for antibacterial activity, identified the fractions with retention times of 61, 66, and 69 min as having inhibitory activity against *L. innocua* (Fig. 2(a)).

III.3. Separation of Antimicrobial Peptides from the Peptic Casein Hydrolysate by Precipitation Under Ionic Strength and pH Effect

The total hydrolysate contains, beside the active peptides, a large number of peptides without antimicrobial activity. Thus, in order to separate the active peptides from this heterogeneous hydrolysate, we tried to separate them by precipitation under the ionic strength by adding salt (NaCl) and maintaining the pH of the hydrolysate in neutral zone. However, under these conditions different inactive peptides are also precipitated.

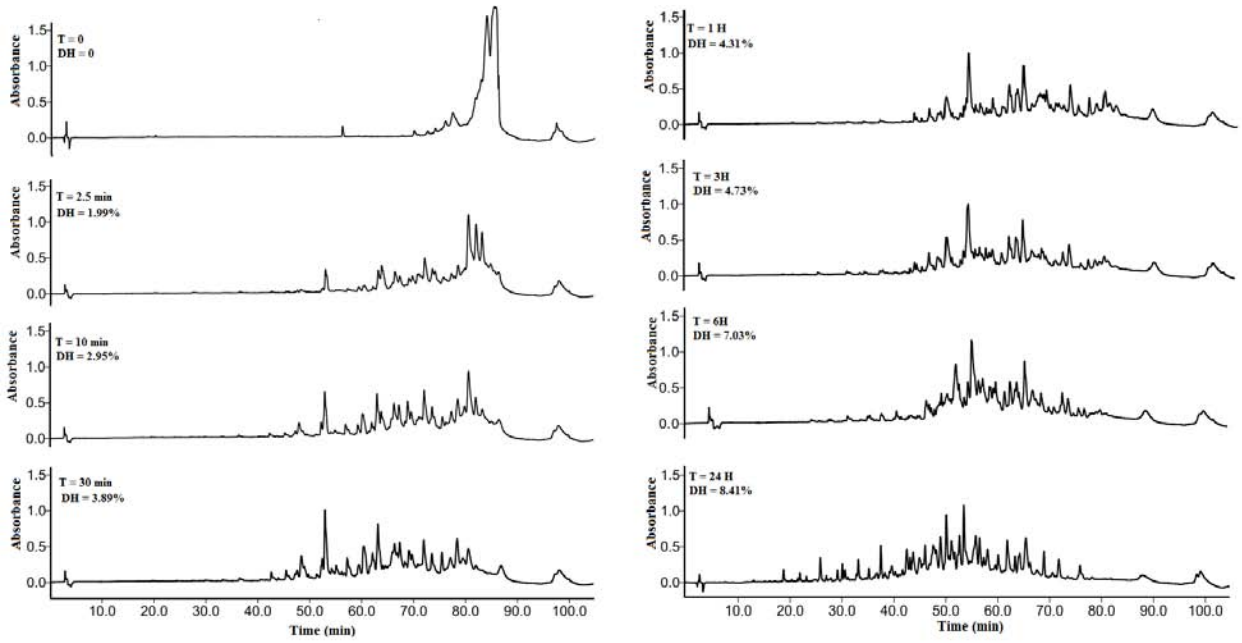


Fig. 1. Reverse-phase HPLC profiles of bovine casein hydrolysates obtained at different times of hydrolysis

Therefore another step of solubilization at low pH was required to separate the active peptides. The latter are recovered after 1 h of stirring at 4°C by centrifugation at 10,000 × g. The peptide extract thus obtained showed antimicrobial activity against *L. innocua* indicating the presence of active peptides. The chromatographic profile of the peptide extract obtained is shown in Fig. 2(b).

Several fractions separated have a retention time similar to those of the active fractions collected from the total hydrolysate as described in the previous section (fig. 2(a))

However, to identify the peptides present in this solution and responsible for the antimicrobial activity, all fractions (numbered 1 to 18 of the chromatogram, Fig. 2(b)) were collected by RP-HPLC on a preparative C18 under the conditions indicated in section II.4.2. The antimicrobial activity of collected fractions was determined primarily on *L. innocua*. Thereafter other strains were tested on fractions that showed clear inhibition zones toward *L. innocua*. The results obtained are presented in Table II.

III.4. Identification of Antibacterial Peptides

The peptide fractions collected from the active extract were analyzed by LC/MS and MALDI-TOF under the conditions indicated above (section *II.5* and *II.6*). By the use of online proteomics programs, it was possible to determine the peptide sequences corresponding to the masses obtained. This analysis was conducted at the Ex-Pasy website (ExPASy; SIB bioinformatics resource portal, (http://us.expasy.org/tools/). The research was conducted on the actives fractions of bovine caseins by specifying the conditions of hydrolysis (pH > 2.5), the type of protease used (porcine pepsin A) and that the masses are treated as mono-isotopic.

In addition, the sequences determined were compared to those generated by hydrolysis of bovine casein reported in different studies.

The results obtained are given in Table III. The peptides identified corresponded mainly of fragments generated from the hydrolysis of bovine α_{s2}-casein and α_{s1}-casein. Fraction 4 contains two major component of molecular mass of 2012 and 2348 Da (Fig. 3(a)). The first corresponds to antibacterial peptide αs_2-CN f(164-179) reported by Recio and Visser (1999)[13].

The second corresponded to amino acid residues αs_2-CN f(148-166). Antimicrobial activity of fraction 10 is due to the presence of two peptides of mass 3459 and 3115 Da corresponded, respectively, to amino acid residues f(180–207) and f(183–207) of bovine α_{s2}-casein (Fig. 3(b)). The fraction 13 includes four majority fragments with mass of 5430, 4921, 4463.5 and 4080.8 Da correspond, respectively, to peptides f(131-174, 153-196, 99-137) of the α_{s2}-casein and peptide f(20-55) of α_{s1}-casein (Fig. 3(c)).

The antimicrobial activity of these peptides has not been previously reported. Three majority fragments are included in the fraction 14, those with a mass equal to 5470 and 3793 correspond to peptides αs_1-CN f(141-147) and αs_1-CN f(161-195) while that identified to have a molecular mass of 3957 Da correspond to αs_2-CN f (176-207) (Fig. 3(d)). Mass analysis by MALDI-TOF indicates that the fraction 15 is pure and contains a single peptide with a molecular mass of 5254 Da (Fig. 3(e)). The purity of this fraction is confirmed by analysis on LC-MS (Fig. 4).

This peptide is identified as αs_2-CN f (164-207). Fraction 17 is characterized by the presence of peptide with high molecular weight. Fragments of mass equal to 7562,7415, 7185, 5714 Da were identified as peptides f(99-163/100-164), f(99-162), f(28-90), f(138-184) of α_{s2}-casein (Fig. 3(f)), respectively. The peptide of mass equal to 6714 correspond to fragments f(9-68) of α_{s1}-casein. This fraction showed antimicrobial activity toward the Gram-positive and Gram-negative bacteria tested (Table II).

III.5. MIC Determination and Bacteriostatic or Bactericidal Effect of Antibacterial Peptide

Enough material of the most abundant peptide αs_2-CN f(164-207) on peptide extract was collected from the preparative HPLC system to determine the MIC against various microorganisms.

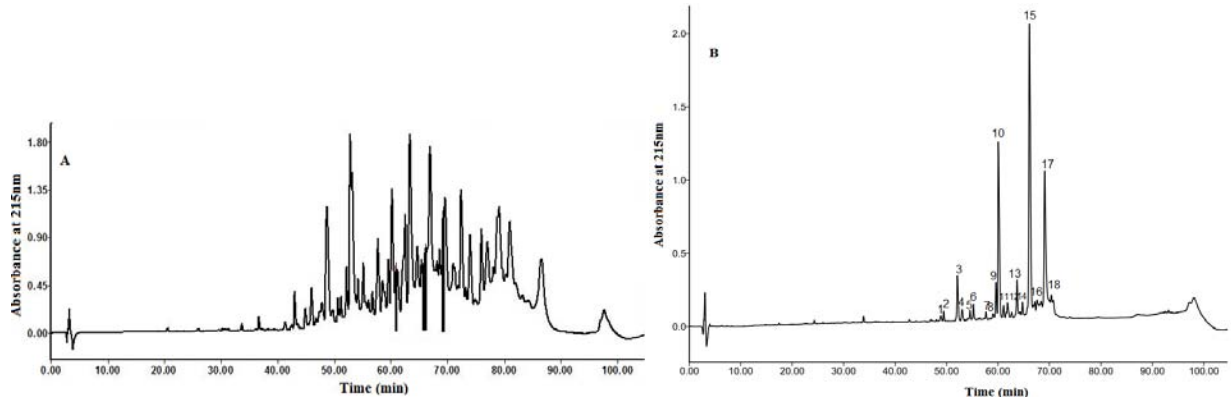
In addition, the peptide extract was also assessed for its MIC.

The purified peptide was tested at an initial concentration of 0.624 mg/ml while the peptide extract was tested at an initial concentration of 1.929 mg/ml. Both sample showed activity against Gram-positive and Gram-negative bacteria.

TABLE II
ANTIBACTERIAL ACTIVITY OF PEPTIDE FRACTIONS COLLECTED FROM THE PEPTIDE EXTRACT

Fraction[a]	Diameters inhibition (mm)			
	L.innocua LMG1138	*E.coli JM109*	*S.aureus* CIP 4,83	*B.subtilis* ATCC6633
Fraction 4	5	—	—	—
Fraction 10	5	5	—	—
Fraction 13	8	8	—	7
Fraction 14	10	8	—	7
Fraction 15	11	11	8	11
Fraction 17	11	11	8	11

Note : — indicates absence of inhibition zone
[a]Refer to the RP-HPLC in Fig. 2(b)



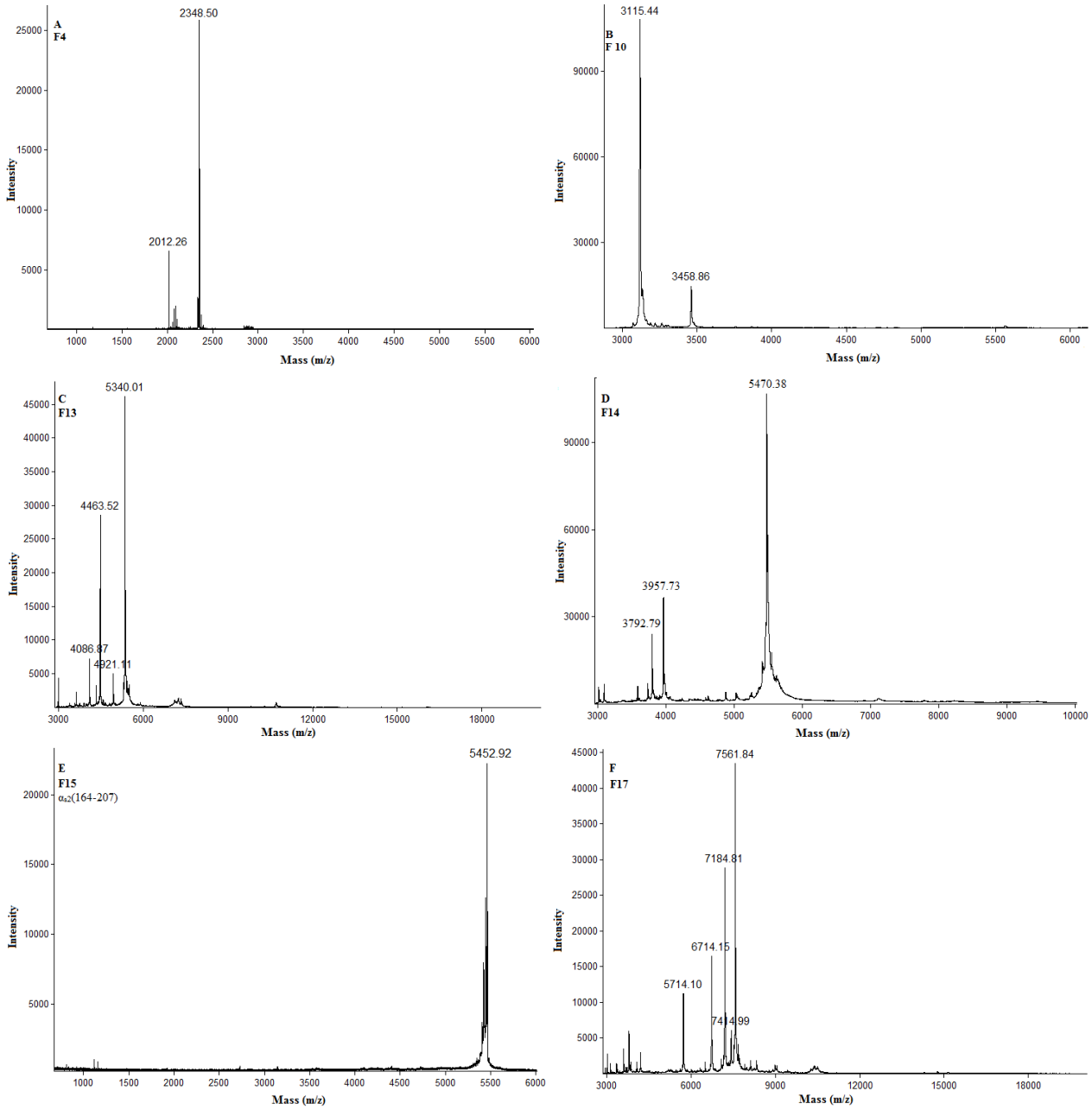
Figs. 2. Reverse-phase HPLC profile of bovine casein hydrolysate for 30 min of peptic hydrolysis, fractions which exhibited antibacterial activity are black filled (a). Reverse-phase HPLC profile of peptide extract prepared by precipitation from casein hydrolysate with 2M of NaCl and at pH 7.5 (b).

TABLE III
MOLECULAR MASS, PRIMARY STRUCTURE OF PEPTIDES RELEASED FROM BOVINE CASEIN HYDROLYSATE WITH PEPSIN CONTAINED IN ACTIVES FRACTION

Fraction[a]	Weight (Da)	Sequence position[b]	Amino acid sequence
4	2012.20	α_{s2} 164-179	LNFLKKISQRYQKFALPQY
	2348.58	α_{s2} 148-166	TKKTKLTEEEKNRLNFLKK
10	3458.86	α_{s2} 180-207	LKTVYQHQKAMKPWIQPKTKVIPYVRYL
	3115.44	α_{s2} 183-207	VYQHQKAMKPWIQPKTKVIPYVRYL
13	5340.01	α_{s2} 131-174	SEENSKKTVDMESTEVFTKKTKLTEEEKNRLNFLKKISQRYQKF
	4921.11	α_{s1} 153-196	FYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTM
	4463.52	α_{s2} 99-137	LYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKK
	4086.87	α_{s2} 62-95	AEVATEEVKITVDDKHYQKALNEINQFYQKFPQYL
		α_{s1} 20-55	LLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAME
14	5470.38	α_{s1} 141-187	LRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMESFSDIPNPIG
	3957.73	α_{s2} 176-207	LPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL
	3792.79	α_{s1} 161-195	SKDIGSESTEDQAMESFSDIPNPIGSENSEKTT
15	5452.92	α_{s2} 164-207	LKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL
17	7561.84	α_{s2}99-163 /α_{s2}100-164	L/YQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVFTKK KLTEEEKNRLNF/L
	7414.99	α_{s2} 99-162	QVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVFTKKTKLTEEEKNRLN
	7184.81	α_{s2} 28-90	INPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDKHYQK ALNEINQFY
		α_{s1} 9-72	QGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAE SISSSEEIV
	6714.15	α_{s1} 9-68	SSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVA TEE
	5714.10	α_{s2} 138-184	TVDMESTEVFTKKTKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVY
		α_{s1} 26-77	MAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDKH

Note: [a] Refer to the RP-HPLC in Fig. 2(b)
[b] Corresponding site in bovine αs-casein sequence

However, The MIC of αs_2-CN f(164-207) and peptide extract against the Gram-positive bacteria, *L. innocua* LMG1138 , *L. monocytogenes* ATCC 3512 and *B. subtilis* ATCC 6633 was considerably lower than the MIC against the Gram-negative bacteria, *E. coli* JM109, *E. coli* CIP 54127 and *E. coli* DH5α (Table IV). Nevertheless, the peptides tested seems not very active against *S. aureus* CIP 4.83 (MIC > 624.2 µg/ml for αs_2-CN f(164-207) and MIC >1929 µg/ml for peptide extract). Although when the peptides were tested on these bacteria by a plate diffusion assay, zones of inhibition were observed, however, with the presence of some colonies. Thus, rates of peptides higher than those used on these bacteria must be tested to determine their MIC. In order to determine whether the MIC correspond to the Minimal Bactericidal Concentration (CMB) the wells corresponding to the MIC were used to determine if the peptide has bactericidal or bacteriostatic characteristics properties. For that, each well containing the MIC is used to count the survival cells. The cells concentration obtained without addition of peptides are also enumerated to determine the percentage of cell death. After incubation at 37◦C for 18 h, we counted the number of CFU (Colony Forming Units) per Petri dish, taking care to count plates containing 30–300 colonies (Table V). The bactericidal effect can be attributed to a molecule when the latter induces a higher percentage of cell death or equal to 99.99% when it use at a concentration near to its MIC (MBC/MIC ratio near 1). As shown in Table V, cell death measured for these peptides do not reach 99.99%, so the peptides are not bactericidal but bacteriostatic when used at a concentration corresponding to the MIC. Nevertheless it is necessary to note that although these peptides show a high MIC toward gram negative bacteria, they exhibit, against a higher mortality rate compared with that observed on Gram-positive bacteria.



Figs. 3. Mass spectra of fractions 4,10,13,14,15 and 17, obtained by MALDI-TOF

TABLE IV
MIC VALUES OF THE ANTIBACTERIAL PEPTIDES. THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF THE PEPTIDE WAS DETERMINED IN A MICROTITER PLATE ASSAY SYSTEM AFTER 18 H INCUBATION AT 37°C

	Minimum inhibitory concentration (MIC)							
	Peptide extract		α_{s2}-CN f(164-207)		Tétracycline		Nisine	
	µg/ml	µM	µg/ml	µM	µg/ml	µM	µg/ml	µM
Gram-positive bacteria								
L. innocua LMG1138	30.14	NA	9.754	1.788	0.25	0.000520	31,25	8.928
B. subtilis ATCC6633	60.28	NA	19.50	3.577	0.50	0.001040	15,625	4.462
L. monocytogenes ATCC3512	60.28	NA	—		—		62,5	17.857
S. aureus CIP4.83	>1929	NA	>624.2	> 114.49	0.488	0.001015	15,625	4.464
Gram-negative bacteria								
E. coli JM109	241.12	NA	78.02	14.311	15.62	0.032481	—	
E. coli DH5á	964.50	NA	312.10	57.245	3.90	0.008110	—	
E. coli CIP 54127	482,25	NA	—		—		—	

Note : NA indicates not applicable as sample contains multiple peptides;
— indicates not tested.

TABLE V
CELL DEATH CAUSED BY PEPTIDES WHEN USED AT A CONCENTRATION CORRESPONDING TO THE MINIMUM INHIBITORY CONCENTRATION

	Mortality evaluation (%)	
	Peptide extract	α_{s2}-CN f(164-207)
Gram-positive bacteria		
L .innocua LMG1138	99.87	99.97
B. subtilis ATCC6633	99.97	99.98
Gram-negative bacteria		
E. coli JM109	99.99	99.99
E. coli DH5α	99.99	99.92

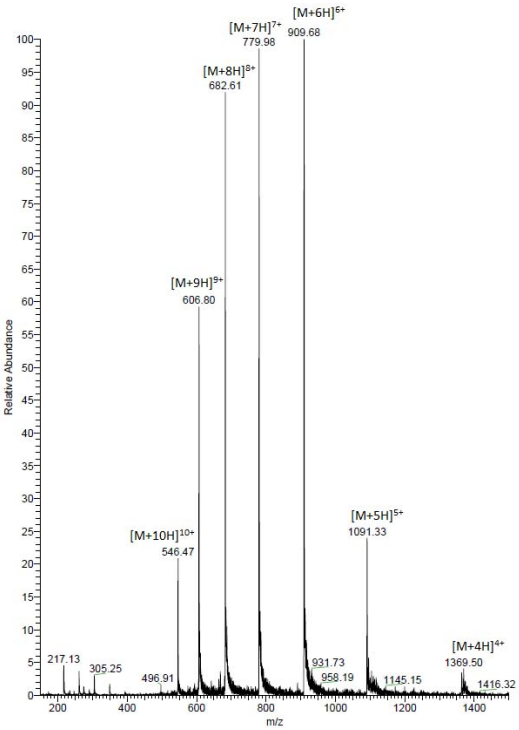


Fig. 4. Mass spectra of peptide αs_2-CN f (164-207) analysed par ESI/MS

IV. Discussion

McCann et al. [14] reported that centrifugation at pH 3.0 of the chymosin digest of sodium caseinate removed the insoluble protein and peptide material without affecting the antibacterial activity, indicating that antibacterial peptides were soluble at pH 3.0. However, when adjusting the pH to 7.0 of such treated hydrolysate, we observed the formation of a turbid. Thus, using base to increase pH of the pepsin digest create salts in the solution causing peptides to precipitate out of solution. Thus we chose to study the effect of ionic strength and pH on the separation of active peptides. The treatment of peptic hydrolysate as described in Section II.4.1., made it possible to separate several antimicrobial peptides. Among the fragments identified some are already reported by previous works [15], [16], [24]. The latter revealed the release of several active peptides from the C-terminal regions of the α_{s2}-casein such as the peptide f(164-179, 181-207, 180-207, 175-207,172-207, 183-207, 164-207) [13]-[15]. However, the peptide extract obtained in this work is characterized therein by the presence of the peptide αs_2-CN f(183-207) and peptide αs_2-CN f(164-207).

The latter, present in considerable quantities in the peptide extract, showed a broad spectrum of activity by acting on both gram-positive and gram-negative bacteria. Indeed, McCann et al [14] have already indicated that this fragment was the most active compared to other peptides separated in the same region of the αs_2-casein. In addition, we identified new active fragments, from different regions of two casein fractions. This is the case of peptides included in fractions 13 and 17 such as peptides f(99-163/100-164), f(99-162), f(99-137), f(131-174), f(153-196) of α_{s2}-casein. However, some antimicrobial peptides already deferred are included in the sequence of these fragments or share common segments. This is the case of f(150-188) αs_2-casein peptide identified by Zucht et al, (1996) [12] allows us to suggest that some of these identified fragments are precursors of active peptides.

V. Conclusion

Although antimicrobial peptides issue from bovine caseins showed a broad spectrum of action, their use, in particularly in food industry, remains limited at the stage of research. There is no much works treating the study of antibacterial activity of peptides in the food medium.

Added to skimmed milk and to the carrot juice, the peptides resulting from α_{s2}-casein hydrolysis have showed substantial loss of activity.

The latter seems to be influenced by the presence of metals cations [26]. So, studies on the factors influencing the activity of these peptides and appropriate methods for efficient applications are necessary. However, this requires sufficient availability of these peptides and this was the aim of our work. Effectively, it was possible to obtain a peptide extract from casein hydrolysate containing several active antimicrobial peptides some of which are already reported by previous work and new peptides from αs_2-casein specially. These latter must be separated and purified to be characterized.

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