Isolation of Oligopeptides from the Water-Soluble Extract of Goat Cheese and Their Identification by Mass Spectrometry

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A procedure for the separation and identification of small peptides from the water-soluble fraction of a goat cheese was developed. The water-soluble extract was ultrafiltered (1000 Da membrane cutoff), and peptides were isolated by sequential chromatography: size exclusion chromatography (HPLC-grade water), anion exchange chromatography (phosphate buffer gradient), and semipreparative reverse-phase high-performance liquid chromatography (water/acetonitrile gradient). The fractions obtained were analyzed by combined mass spectrometry methods including electrospray ionization, liquid secondary ionization, and tandem mass spectrometry to identify and to confirm the sequences of 28 tri- to octapeptides naturally appearing in goat cheese during ripening. Among these peptides, 26 are produced by degradation of caseins but do not correspond to the known specific cleavages due to chymosin. Only low correlation was found between hydrophobicity of peptides and HPLC elution time with acetonitrile gradient.

Keywords: *Peptides; goat cheese; proteolysis; mass spectrometry; liquid chromatography; HPLC*

INTRODUCTION

Together with lipolysis, proteolysis is one of the major events occurring during cheese ripening (*1*−*3*). Proteolysis of different types of cheeses has been extensively studied, but these studies were mainly focused on the knowledge of maturation process (*4*, *5*). Numerous peptides have been extracted, separated, and identified in several cheeses such as Swiss mountain-cheese (*6*), Cheddar (*4*, *7*−*16*), Parmigiano-Reggiano (*17*, *18*), Comté (*19*−*21*), and Gouda (*22*−*24*). The separation methods commonly used to isolate peptides from cheese were reviewed by McSweeney and Fox (*25*). Most of the identified peptides are large milk casein fragments with a molecular weight (MW) >1000 Da. However, the identification method often based on fast atom bombardment mass spectrometry (FAB-MS) combined with partial Edman degradation was not found to be suitable for small peptides. Chianese et al. (*26*) and Ferranti et al. (*27*) identified more small peptides with MW< 800 Da originated from caseins respectively in sweet and acid cheese whey and in old Grana Padano cheese. A particular interest for the small peptides came from their supposed taste activity (*2*, *28*, *29*). Small polar peptides may be salty, sweet, acid, or umami according to their structure (*30*), although the taste property of small peptides described as umami was recently contested (*31*, *32*). Larger peptides have been shown to be tasteless or bitter (*33*, *34*), and hydrophobic peptides are more likely to develop bitterness. However, a recent study (*35*, *36*) demonstrated that prediction of bitter properties based on hydrophobicity, polarity, or molecular weight of peptides has limited accuracy and little value. Despite the taste properties of the water-soluble fraction of cheeses (*37*, *38*), few studies have dealt with the identification of small peptides naturally occurring in food. These complex fractions generally give rise to very complex reverse phase high-performance liquid chromatography (RP-HPLC) profiles for cheeses (*39*) showing the presence of numerous peptides but globally unsuited for further identification steps, as chromatographic resolution was generally very poor (*11*, *18*). For that reason, only a few small peptides could be identified in Vacherin Mont-d'Or (*40*), Comté (*19*, *20*), and Emmental (*41*). Roturier et al. (*42*) identified several hydrophilic peptides from a model cheese system using FMOC derivation for HPLC analysis. Moreover, their respective contributions to overall or particular tastes in cheese were not determined. Up to now, no direct correlation between peptides and the organoleptic properties of foodstuffs, apart from bitterness, has been clearly demonstrated.

We recently developed an analytical method to purify and characterize the small polar peptides from foodstuffs in order to simplify the complex RP-HPLC profiles otherwise obtained (*43*). On the basis of these preliminary results, we extended the method to the semipreparative level.

In the present study, we describe a preparative multistep procedure used to isolate the major small peptides from the water-soluble fraction of a goat cheese and their subsequent identification by various combined mass spectrometry methods. For the first time we have determined the structure of several small peptides naturally present in goat cheese as a result of ripening, and their origins and properties are discussed.

MATERIALS AND METHODS

Reagents. HPLC grade water was obtained with a Milli-Q water purification system (Millipore Corp., Bedford, MA). All

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of the chemicals used to prepare buffers or reagents were of analytical grade (Merck, Darmstadt, Germany). The solvents used for chromatographic analyses (HPLC grade) were filtered on a 0.45 μm membrane (Filtron Technology Corp., Northborough, MA) and then degassed with helium.

Preparation of Extract. Fifty grams of frozen 3-week-old goat cheese from Lycée agricole de Davayé (France) was grated, homogenized in water, and centrifuged. The supernatant was freeze-dried, redissolved in water, and utrafiltered on a 1000 Da cutoff membrane as described elsewhere (*43*).

For the three purification steps, the absorbance of the eluate was monitored at 214 nm with a Lichrograph L-4000 UV spectrophotometer (Merck) and plotted on an HP3395 integrator (Hewlett-Packard). Data were also collected through an electronic interface by the Coconut acquisition and integration program (P. Mielle and R. B. Almanza, Coconut INRA, 1987−1993) and were then treated with a spreadsheet program.

Size Exclusion Chromatography (SEC). The ultrafiltered extract (5 mL, equivalent to 15 g of cheese) was applied on a Superformance (Merck) column (2.6 × 60 cm) filled with Toyopearl HW-40S (Tosoh Corp., Tokyo, Japan), a copolymer of ethylene glycol and methacrylate resin. Its molecular weight separation range is 100−10000. The elution was realized at room temperature with water as the mobile phase to allow sensory evaluation of the recovered fractions (*44*). A constant flow rate of 2 mL min^{-1} was delivered by a B-100-S-2 pump (Eldex Laboratories Inc., Chicago, IL), with a pressure of 10^6 Pa. One hundred and eighty fractions of ∼2.3 mL each (150 droplets) were collected. According to the profile, 10 fractions were constituted, as described elsewhere (*43*), freeze-dried, and stored at −20 °C until use.

Preparative Anion Exchange Chromatography (AEC). Each of the above fractions was resolubilized in 3 mL of HPLC grade water and filtered on DynaGard ME 0.2 μm mixed ester filters (Microgon Inc., Laguna Hills, CA). One milliliter of resolubilized fraction was applied on a Superformance (Merck) column (1.6 × 60 cm) filled with a weak anion exchange TSK diethylaminoethyl (DEAE)-650 (M) resin (Merck). Due to limited column capacity, the sample was divided into three subsamples and run separately. HPLC pump, injector, and detector were Hewlett-Packard 1050 series (Hewlett-Packard, Waldbronn, Germany). The flow rate was set at 5 mL min^{-1}. Separations were carried out at pH 7 using a ternary gradient of increasing ionic strength. The three eluent solutions were as follows: eluent A, 2 mM phosphate buffer; eluent B, 20 mM phosphate buffer and 0.1 M NaCl; eluent C, 200 mM phosphate buffer and 0.2 M NaCl. The elution was made with 100% eluent A for 30 min, and then the gradient was formed by linearly increasing the concentration of solvents B and C as follows: 30−60 min, 0−10% B; 60−90 min, 10−30% B; 90−150 min, 30−100% B; 150−190 min, 100% B−100% C. The column was then rinsed for 20 min with solvent C and progressively re-equilibrated in 50 min with eluent A, through eluent B. The fractions manually collected in 10 mL polyethylene tubes to avoid undesirable adsorption of peptide material on glass were dried under vacuum using an RC 10.10 centrifugal concentrator (Jouan, St. Herblain, France) and frozen until use.

Semipreparative Reverse Phase Chromatography. The final separations were carried out on an Intersil 10 μm octadecyl bonded phase (C_{18}) 1 × 25 cm Interchrom column (Interchim, Montluçon, France) and precolumn fitted to the same Hewlett-Packard 1050 series HPLC. Eluent A was 0.1% trifluoroacetic acid in HPLC grade water and eluent B was 0.1% trifluoroacetic acid (TFA) in HPLC grade acetonitrile. Separations were conducted at a flow rate of 3 mL min^{-1} with the following binary gradient: 0−45 min, 0−35% B; 45−60 min, 35−70% B; 60−70 min, 70−0% B. Samples from the previous step were dissolved in 2 mL of water, filtered on the DynaGard filters (Microgon Inc.) described above, and injected through a 1 mL injection loop. The homologous fractions manually collected in 10 mL polyethylene tubes from two consecutive chromatographic runs were pooled. The fractions were selected for mass spectrometry analysis if their corresponding peaks were resolved enough or if their absorbance at 214 nm was >0.05 AU The selected fractions were dried in a centrifugal concentrator and frozen until use.

Mass Spectrometry. Mass measurement experiments were conducted in the electrospray ionization (ESI) mode on a Trio 2000 quadrupole instrument (Micromass, Manchester, U.K.). The samples were dissolved in 40 μL of the mobile phase water/acetonitrile/formic acid (49:49:2, v/v/v), filtered, and injected (10 μL) at a flow rate of 10 μL min^{-1}. The skimmer cone was set to 35 V. Tandem mass spectrometry analyses were carried out on an Autospec (Micromass) high-resolution magnetic instrument using linked scans (B/E remaining constant) in the positive mode, fitted with either liquid secondary ionization (LSIMS) or electrospray (ESI) ion source. For the LSIMS experiments the cesium gun was set to 30 keV energy and 1 μL of sample was mixed in the tip of the probe with a glycerol/thioglycerol matrix (5:1, v/v) containing 0.2% trichloroacetic acid (TCA). For the ESI experiment the skimmer cone was set to 35 V and 10 μL was injected as described above. The accelerating voltage was 8 kV, and the resolution was 1500 ($m/\Delta m$ at 10% height) with a Hall probe calibration. Daughter ions were formed by collision-induced dissociation (CID). The collision cell in the first field free region (FFR1) was filled with helium so as to decrease the parent ion intensity to 50% of its initial value (which corresponded to a 10 times increase of pressure in the source). For preparation of methylation reagents, 1 mL of anhydrous methanol was cooled on ice and 250 μL of anhydrous acetyl chloride was slowly added dropwise. The reagent was allowed to stand at room temperature for 30 min. The methylation was made by dissolving dried sample with 10 μL of the reagent 15 min before the MS experiment.

Casein Sequences and Database Searches. The determined peptide sequences were compared with the known sequences of the caseins provided by the Swiss-Prot data bank via the ExPASy World Wide Web molecular biology server of the Geneva University Hospital (http://www.expasy.ch/sprot/sprot-top.html). Goat (*Capra hircus*) α_{S1}-casein and variants are referenced as P18626, α_{S2}-casein and variants are P33049, β-casein is P33048, and κ-casein and variants are P02670. For the similarity searches of peptides that did not matched with caseins, we used an automatic electronic mail server called *Blitz* from S. Sturrock and J. Collins, Biocomputing Research Unit, University of Edinburgh, Scotland (http://www.ebi.ac.uk/searches/blitz.html).

RESULTS AND DISCUSSION

Peptide Purification. Peptides are present in a variety of foodstuffs and constitute a fraction that can be responsible for several properties (texture, taste, and biological activities). In the case of processed foodstuffs such as ripened cheese, the peptidic fraction is so rich that the RP-HPLC profiles obtained with the classical methods of purification, that is, SEC and then RP18-HPLC (*11*, *38*, *43*) are by far too complex for direct analysis of peptidic HPLC fractions by mass spectrometry. Figure 1 presents the purification scheme used to purify small peptides from goat cheese for their analysis by MS. The method was based on an analytical method previously published (*43*) and was extended to a semipreparative level. The ultrafiltered water-soluble extract was successively submitted to SEC, AEC, and semipreparative RP18-HPLC.

Preparative AEC. Among the first eight fractions obtained by the SEC step, peptides have been mainly located in the first four fractions (*43*, *45*), and the two first fractions contain the highest quantity of peptides. The profiles obtained for these 4 SEC fractions gave peaks resolved enough to allow a fractionation into 17, 13, 6, and 7 fractions, respectively. However, to have the complete profile pattern of our extract, all of the SEC fractions were chromatographied by AEC.

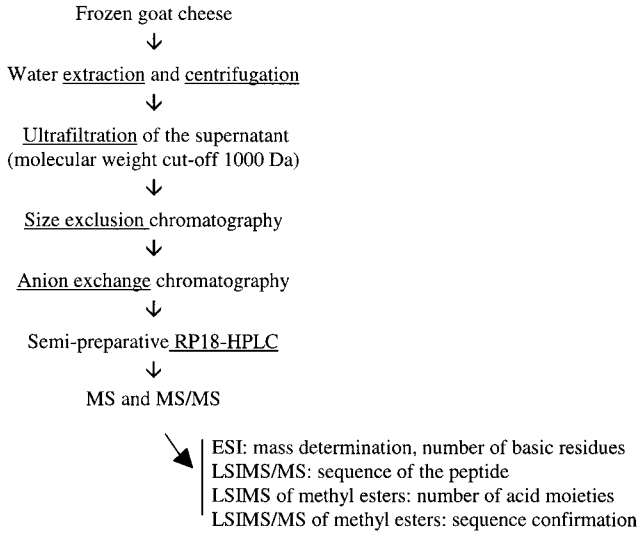

Figure 1. Purification scheme of small peptides from goat cheese by preparative methods and identification procedure.

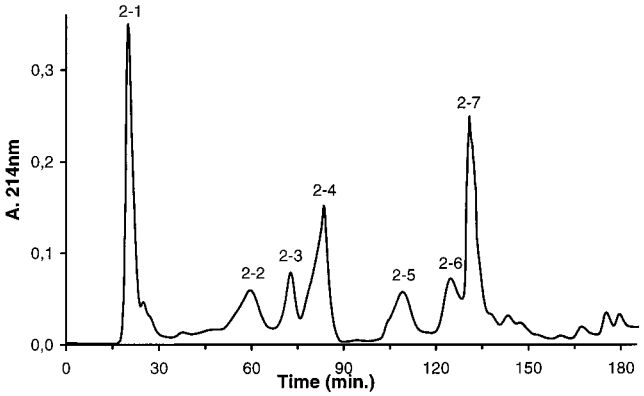

Figure 2. Chromatogram of the second SEC fraction (SEC2) obtained by AEC. Elution was made at 3 mL/min by increasing pH 7 phosphate buffer concentration from 2 to 200 mM and NaCl concentration from 0 to 0.2 M.

The profile obtained by AEC of the second SEC fraction (SEC2) is presented in Figure 2. This fraction gave the most complex AEC profile, whereas the fifth fraction (SEC5) and subsequent ones gave simpler profiles with a lower absorbance than the fractions containing the peptides. A good repeatability was obtained with a variation of the retention times of 5 min as a maximum between the three injections made for a 3-h analysis. An acute point was to re-equilibrate slowly the column with the eluent A.

Semipreparative RP-HPLC. The first goal of that step was to eliminate the buffer salts from the AEC fractions as the presence of salts can cause ion suppression in fast atom bombardment mass spectrometry (FAB-MS) or LSIMS (*46*) and to a lower extent (from $\sim 10^{-2}$ M) in ESI (*47*). The second goal was to reach an efficient separation of the peptides contained in each AEC fraction. Sommerer et al. (*43*) have demonstrated this method to be effective at an analytical scale.

Only the 42 AEC fractions originating from the first 4 SEC fractions containing peptides were analyzed by RP-HPLC. The profiles were dependent upon the AEC fraction analyzed as the amount of salts injected was very different from one fraction to another. The first four AEC fractions containing the rather nonpolar compounds, cations, and weak anions gave generally richer RP-HPLC profiles than the AEC fractions eluted later.

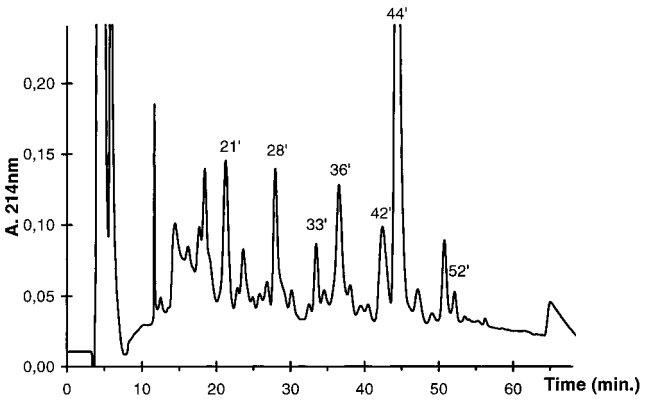

Figure 3. Chromatogram of the second fraction (AEC2) from the SEC2 fraction (2−2 in Figure 2) obtained by semipreparative RP-HPLC. Sample was separated at 3 mL/min using an HPLC grade acetonitrile/trifluoroacetic acid gradient in HPLC grade water.

The RP-HPLC profiles obtained with the semipreparative column were somewhat different from those previously obtained with the narrow-bore column (2.1 mm i.d.) (*43*). Nevertheless, the separation of the AEC fractions was efficient, and the resolution was good enough for the collection of fractions corresponding to each peak. The RP-HPLC profile of the second EAC fraction (AEC2) is presented in Figure 3 as an example. A large number of RP-HPLC fractions were collected, and 97 were selected for MS analysis.

Mass Spectrometry and Peptide Identifications. The selected fractions were submitted to electrospray ionization (ESI) to determine the molecular weights of the compounds contained in these fractions. The positive ionization mode led to the production of the protonated molecular ion of the compounds $(M + H)^+$. For peptides containing a basic amino acid (i.e., arginine, lysine, or histidine) we unambiguously detected the doubly charged and doubly protonated compound $(M + 2H)^{2+}$ by observing the isotopic peak at half a mass unit above the mass measured.

Linked scan experiments (constant B/E) in the LSIMS mode allowed us to determine the sequences of 28 peptides (Table 1).

These sequences were confirmed by methylating the free carboxylic acid moieties of the peptides. The molecular masses of the methylated peptides were first measured. A mass 14 units (i.e. $-CH_2-$) above the mass detected in the previous step meant that only one acidic moiety was present (at the C terminus of the peptide). Multiples of 14 mass units indicated the presence of free acidic moieties in the peptides (mainly glutamic acid, which represents 75% of the acidic amino acids in goat milk caseins). According to the fragmentation pattern and nomenclature of peptides (*48*), B/E experiments on those methylated peptides gave shifted Y series daughter ions and unmodified masses for the B series fragments. The results obtained unambiguously confirmed the sequences previously determined in the linked scanning of the purified peptides.

Using this procedure, we identified 28 peptides ranging from tripeptide to octapeptide with molecular masses from *m/z* 375 to 902 g/mol for the $(M + H)^+$ ion (Table 1). Of the 97 RP-HPLC fractions analyzed by mass spectrometry, the majority contained material that did not give a signal corresponding to peptides but to amino acids or other organic compounds (data not shown).

Table 1. Small Peptides from the Water-Soluble Extract of Goat Cheese Purified by Successive LC Methods and Identified by MS and MS/MS

peptide[a]	mass[b] (g/mol)	HPLC fraction[c]	caprine milk casein fragment (*C. hircus*)	occurrence of the sequences of ovine caseins (*O. aries*)	occurrence of the sequences of bovine caseins (*B. taurus*)	Q^d (kcal/mol)
GLSPE	502 (1)	2−2 28	α_{S1} 10−14	yes	no	**1.12**
VLNENL	701 (1)	2−2 36	α_{S1} 15−20[e]	yes	yes	**1.12**
		2−3 37				
ENLL	488 (1)	2−3 41	α_{S1} 18−21	yes	yes	1.34
ENL	375 (1)	2−3 28	α_{S1} 18−20	yes	yes	**0.99**
VVAPFPEV	857 (1)	2−2 52	α_{S1} 24−31	yes	no	1.69
		2−3 52				
VVAPFPE	758 (1)	2−2 44	α_{S1} 24−30	yes	no	1.69
		2−3 44				
		3−3 44				
		3−4 44				
VAPFPE	659 (1)	2−2 42	α_{S1} 25−30	yes	yes	1.69
		2−3 42				
		3−3 42				
		3−4 42				
RKENINE	902 (2) (dc)	2−1 27	α_{S1} 33−39	yes	no	**0.90**
NINEL	602 (1)	2−4 37	α_{S1} 36−40	yes	no	**1.18**
NINE	489 (1)	2−4 22	α_{S1} 36−39	yes	no	**0.87**
INEL	488 (1)	2−3 36	α_{S1} 37−40	yes	no	1.48
DAKQM	592 (1) (dc)	2−1 22	α_{S1} 56−60	yes	no	**0.66**
EQL	389 (1)	2−3 29	α_{S1} 96−98	yes	yes	**0.96**
			118−120	yes		
			α_{S2} 127−129	yes	yes	
NVPQL	570	2−1 31	α_{S1} 105−109	yes	no	1.32
		3−1 39				
EIVPK	585 (1) (dc)	2−1 28	α_{S1} 110−114	yes	no	1.86
QEL	389 (1)	2−3 29	α_{S1} 140−142	no	yes	**0.96**
ITVDDK	690 (2)	2−2 21	α_{S2} 72−77	yes	yes	**1.28**
NVVG	388	2−1 20	β 7−10	yes	no	**0.84**
KIEK	517 (1) (dc)	2−1 20	β 29−32	yes	yes	1.63
VLPVPQ	652	3−1 39	β 170−175	yes	yes	1.82
RDMPIQA	830 (1) (dc)	2−1 32	β 181−187	yes	yes	**1.16**
DMPIQ	603 (1)	2−2 33	β 182−186	yes	yes	1.46
FDDKIA	708 (2) (dc)	2−2 42	κ 18−23	yes	no	1.38
		2−3 42				
STPTTE	635 (1)	1−4 17	κ 134−139	yes	yes	**0.75**
AIVN	416	3−1 26	κ 140−143	no	no	**1.18**
SIASA	448	2−1 20	κ 154−158	yes	no	**0.64**
KTVEPN	687 (1) (dc)	2−1 21		no	no	**1.13**
QDLNNR	759 (1) (dc)	2−1 31		no	no	**0.59**

[a] Peptides are sorted by their position in goat caseins. [b] The first number indicates the mass of the protonated compound $(M + H)^+$ detected in LSIMS, the second number, in parentheses, indicates the number of acidic amino acids (detected after the methyl esterification); (dc) indicates that the doubly charged $(M + 2H)^{2+}$ was detected in ESI. [c] The first figure indicates the number of the SEC fraction, the second indicates the number of the anion exchange fraction, the third indicates the retention time in minutes of the RP-HPLC step. [d] Hydrophobicity value calculated according to the method of Ney (*59*). The bold values are <1.3 kcal/mol. [e] α_{S1} variant A goat casein is characterized by two single point mutations, $P_{16}\rightarrow L_{16}$ and $E_{77}\rightarrow Q_{77}$.

Among these 28 peptides, 11 contained proline, which is a result of the large amount of proline contained in caprine caseins (*49*). Those proline-containing peptides gave characteristic fragmentation profiles, and the Y ion resulting from the breakdown of the bond adjacent to the proline residue dominated the spectrum (Figure 4). One explanation could be the tension of the five-bond ring, which may weaken the amide bond. Another hypothesis or complementary reason could be that the Z and C ions are very unlikely to appear as they would need the simultaneous cleavage of two bonds of the ring. Nevertheless, we could detect the C3 ion despite its very low intensity (Figure 4). Once the peptidic bond is broken, the nucleophilic nitrogen may be protonated easily and forms a Y ion rather than a B ion as the proton affinity of the tertiary amine is superior to the proton affinity of the oxygen of the carbonyl group (*50*). However, we could observe the corresponding B ions albeit of weaker intensity.

Another particular example is the fragmentation profile of the peptide RKENINE (Figure 5). Usually the internal energy in the gas phase is sufficient to protonate not only the most basic site but also other sites. This distribution of protonated amide nitrogens leads to a distribution of amide cleavages and fragment ions. This was described as the "mobile proton" model (*51*). For the peptide RKENINE, the only detected ions are the ones containing the N-terminal extremity of the peptide (i.e., A, B, C, and D ions). This is explained by the two basic residues arginine and lysine side by side at the N terminus that fixed the proton and thus the positive charge. Despite the fact that the cleavages between the carbonyl and the nitrogen leading to the B ions are usually the most frequent cleavage path, the most abundant ions detected for this particular peptide were the C and D ones.

As summarized in Table 1, the majority of the peptides originated from the SEC2 fraction, seven from SEC3 fraction, and only one from the SEC1 fraction. It is noteworthy that, in each case, the first AEC fractions contained the largest parts of the peptides and that the AEC fractions eluted later contained fewer peptides, which is confirmed by the very simple RP-HPLC chromatograms obtained for these later eluted AEC frac-

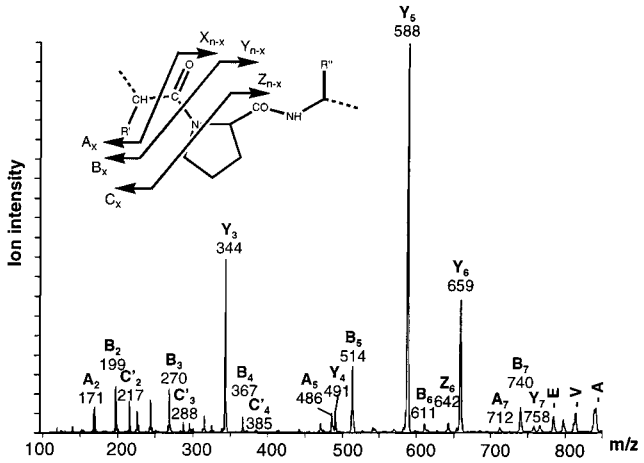

Figure 4. LSIMS B/E daughter ion mass spectrum of a proline-containing peptide identified in goat cheese as VVAPF-PEV (MH^+ 857 g/mol). The Y ions after the spectrum are the most intense ones. *n* indicates the number of residues of the peptide (eight for this peptide), and *x* represents the position of a particular amino acid in the chain from the N terminus.

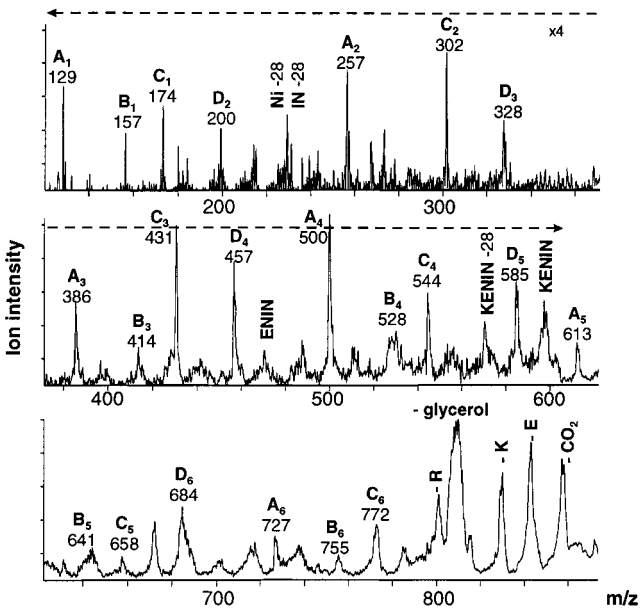

Figure 5. LSIMS B/E daughter ion mass spectrum of a peptide identified in goat cheese as RKENINE (MH^+ 902 g/mol). The two basic residues at the N terminus induced a fixed charge fragmentation thus leading to B series ions only.

tions. These fractions may contain stronger anionic compounds, such as free amino acids.

The largest number of peptides came from the degradation of α_{S1}-casein, although it represents a minor casein fraction compared to β- and γ-casein fractions. As for bovine casein (*52*) it may be due to a better exposition to proteolytic agents.

When compared with goat milk casein sequences, only two peptides (KTVEPN and QDLNNR) were related neither with goat milk caseins nor with other known milk proteins. Their origin may be autolysis of cheese microflora.

Some identified peptides presented only slight differences such as the addition or deletion of one or two amino acids at one extremity of the chain. They may apparently come from the same casein subsequence (i.e., for VLNENL, ENLL, ENL, or VVAPFPEV, VVAPFPE, VAPFPE or RKENINE, NINEL, NINE, INEL or RDMPIQA, or DMPIQ).

The sequences of the peptides identified were compared with the sequences of caseins from goat, ewe, or cow milk. Only 2 peptides were not part of the sequences of the ewe (*Ovis aries*) milk caseins, whereas 14 peptides were not part of the sequences of bovine (*Bos taurus*) milk caseins. This can be easily explained by the higher degree of sequence homology between ewe and goat milk caseins than between cow and goat ones.

Lots of large peptides have been described in the literature, but few small ones have been identified. To our knowledge, only peptide KIEK was already found as a tryptic peptide of cow β-casein and evaluated as nonbitter (*53*) despite its high *Q* value (1.63 kcal/mol). We previously identified the peptide ITVDDK in the water-soluble extract of goat cheese (*43*), and this peptide could be related to a tryptic fragment of α_{S2}-casein. Among the 28 peptides identified, 26 do not correspond to specific cleavage of caprine α_{S1}-casein by chymosin (*54*, *55*) and have not been previously described for cow, ewe, or goat cheese. To our knowledge, this was the first time that such a large number of small peptides naturally occurring in cheese and not found in hydrolysates of purified caseins (*56*) by selected enzymes were identified.

By treating purified casein (*56*) and soybean protein (*57*) with specific enzymes (trypsin), bitter peptides were produced but few peptides produced in this way were found in foodstuffs as the natural enzymatic degradation pathways are different. Other studies on purified caseins (*53*) showed peptides with modified amino acids or peptides having sequences that not correspond to any known casein fragment, probably due to chemical modifications occurring during the experiment.

Properties of Peptides. Maeno et al. (*58*) identified the peptide KVLPVPQ as a degradation product of β-casein by a proteinase from *Lactobacillus helveticus* CP790. This peptide differs from the peptide VLPVPQ we identified only by the presence of a lysine at the N terminus. These authors showed that the peptide KVLPVPQ had strong antihypertensive properties after oral administration from 0.5 mg of peptide/kg of body weight on spontaneously hypertensive rats. It should be interesting to test this biological activity on the peptide VLPVPQ naturally occurring in goat cheese.

The taste of the purified tryptic casein peptides has been largely studied (*53*), but the role of those peptides in the taste of cheese may not be of great importance as we identified in our extract 26 peptides (among 28) that were not directly produced by a specific rennet enzyme.

It has been assumed for a long time that the prediction of the bitterness of peptides is partly related to their global hydrophobicity (*Q* value) (*59*). The majority of the identified peptides (17) have a *Q* value of <1.3 kcal/mol, which is the limit given in the literature for bitterness. Eight peptides have a *Q* value >1.4 kcal/mol, and three peptides have a *Q* value between 1.3 and 1.4 kcal/mol. However, a peptidic fraction of this goat cheese isolated by nanofiltration and containing all of the peptides identified in this study diluted in water solutions or incorporated in a cheese model (*37*) were evaluated by trained panelists. Neither any direct taste properties nor synergistic effects with any other molecules was detected (*43*, *60*, *61*); putative bitterness of the peptides with a *Q* value >1.3 kcal/mol was not perceived, probably due to their too low concentration in the

extract. The bitterness of this cheese seems mainly due to divalent cations (*60*, *61*).

It is also noteworthy that it is not possible to predict accurately the hydrophobicity of the peptide by the retention time in RP-HPLC. We observed a correlation value (R^2) of 0.32 when plotting the retention time against the calculated hydrophobicity of our 28 peptides (data not shown). This result is partly contradictory with the current knowledge (*62*), but the hydrophobicity of the amino acids and peptides in predictive models (*63*) was not determined by the same methods as the hydrophobicity values we used (*59*). This last one is claimed to correlate the hydrophobicity of the peptides calculated from thermodynamic characteristics of the side chain of the amino acid residues to the ability for the considered peptide to have a bitter taste. However, our results show that it seems to be impossible to predict bitterness of small peptides only on the basis of chromatographic parameters, and they are in accordance with Lee and Warthesen (*35*), who did not find any relationship between the bitterness intensity of isolated HPLC peaks, the hydrophobicity of peptides, and the peak elution order of cheese peptides with a molecular weight between 500 and 3000 Da.

The purification method described here, that is, successive SEC, AEC, and RP-HPLC, has been demonstrated to be a reliable method to separate small peptides from a complex mixture such as the water-soluble extract of cheese. It allows their total sequencing by combination of mass spectrometry methods. Work on the identification, quantification, and flavor properties of the water-soluble components of cheeses is continuing.

ABBREVIATIONS USED

AEC, anion exchange chromatography; CID, collision-induced dissociation; ESI, electrospray ionization; FAB, fast atom bombardment; RP-HPLC, reverse phase high-performance liquid chromatography; LSIMS, liquid secondary ionization mass spectrometry; MSG, monosodium L-glutamate; SEC, size exclusion chromatography.

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