Peptides Identified during Emmental Cheese Ripening: Origin and Proteolytic Systems Involved

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To determine the proteolytic changes occurring during Emmental cheese ripening, peptides released in cheese aqueous phase were analyzed by reversed-phase HPLC and identified by tandem mass spectrometry sequencing, for which different strategies were illustrated by some examples. Among the 91 peptides identified, most of them arose from α_{s1}- (51) and β-caseins (28), and a few arose from α_{s2}- (9) and κ-caseins (1). An attempt was made to correlate the released peptides with the proteolytic systems potentially involved during Emmental cheese manufacture. Besides the well-known action of plasmin on β- and α_{s2}-caseins, and in the absence of residual fungal coagulant from *Endothia parasitica*, two other proteinases seem to be involved in the hydrolysis of α_{s1}-casein in Emmental cheese: cathepsin D originated from milk and cell-envelope proteinase from thermophilic starters. Moreover, peptidases from starters were also active throughout ripening, presumably like those from nonstarter lactic acid bacteria, in contrast to those from propionic acid bacteria.

Keywords: *Tandem mass spectrometry; Emmental cheese; peptides;, proteinase; peptidase; thermophilic lactic acid bacteria; cathepsin D; plasmin; secondary ripening flora*

INTRODUCTION

Proteolysis is essential for the development of texture and flavor in cheese (*1*). Different proteolytic agents (milk coagulant; endogenous milk proteinases such as plasmin and cathepsin D; starter, nonstarter, and secondary flora proteinases; and peptidases) are involved in producing peptides and free amino acids to a greater or lesser extent depending on the cheese variety. In Swiss-type cheeses, such as Emmental or Gruyere, three flora follow one another during ripening: thermophilic lactic starters, nonstarter lactic acid bacteria, and propionibacteria. It is well-established that plasmin plays an important role in the primary proteolysis of this variety of cheese (*1*). However, the nature of the bacterial enzymes involved in the maturation of such cheese and their mode of action on the caseins have not been well established. This information will be useful in understanding the in situ functional behavior of these enzymes in cheese, as there is increasing evidence that this action mode as observed in solution is affected by the cheese environment (*2*).

In a previous work, it has been shown that the enzymes (especially aminopeptidases of starter bacteria) were released early by lysis in Emmental cheese (*3*) and remained active over a long period of ripening (*4*), contributing greatly to the overall proteolysis in Emmental cheese. A powerful way to gain more information on the proteolytic process in cheeses is to identify the peptides produced throughout the ripening. Such an approach has been used for establishing the functional proteolytic system in Cheddar (*5*), in Parmigiano reggiano (*6*, *7*), and in Grana padano (*8*). Most of these studies have involved the extraction of peptide fractions from cheese and their subsequent analysis by chromatographic methods, especially high-performance liquid chromatography. Peptides were identified by amino acid sequencing combined with automated Edman degradation and/or mass spectrometry. Recent strategies developed in electrospray mass spectrometry have enabled the use of tandem mass to sequencing peptides in complex mixtures based on the sequence tag database search (*9*). Using this method, the aim of the present work was to identify the peptides formed during Emmental cheese ripening. On this basis, an attempt was made to correlate the presence of these peptides with the known specificities of proteolytic agents potentially encountered in Emmental cheese during manufacture.

MATERIALS AND METHODS

Reagents. Urea and β-mercaptoethanol were purchased from Merck (Nogent sur Marne, France); trifluoroacetic acid (TFA) was purchased from Pierce (Touzart et Matignon, Vitry sur Seine, France); and acetonitrile was obtained from Carlo Erba (Nanterre, France). The rest of the reagents were of analytical grade.

Emmental Cheese Making. Emmental cheeses representative of the French industrial Emmental production were taken during ripening from one factory in Brittany. They were manufactured from the same vat according to the usual process of the factory using thermised milk, *Endothia parasitica* (also named *Criphonectria parasitica*) as a coagulant, thermophilic lactic acid bacteria as starters (streptococci and lactobacilli), and propionic acid bacteria added to the milk as a secondary starter. The numeration of the different kinds of flora of the ecosystem present in these cheeses was previously determined and described by Thierry et al. (*10*). The Emmental wheels were ripened according to three distinct periods after pressing and brining of the cheese (day 4): first in a temperate room (at 12−16 °C until day 13), further in a warm room at 23−24 °C until day 36, and finally in a cold room until sale at day

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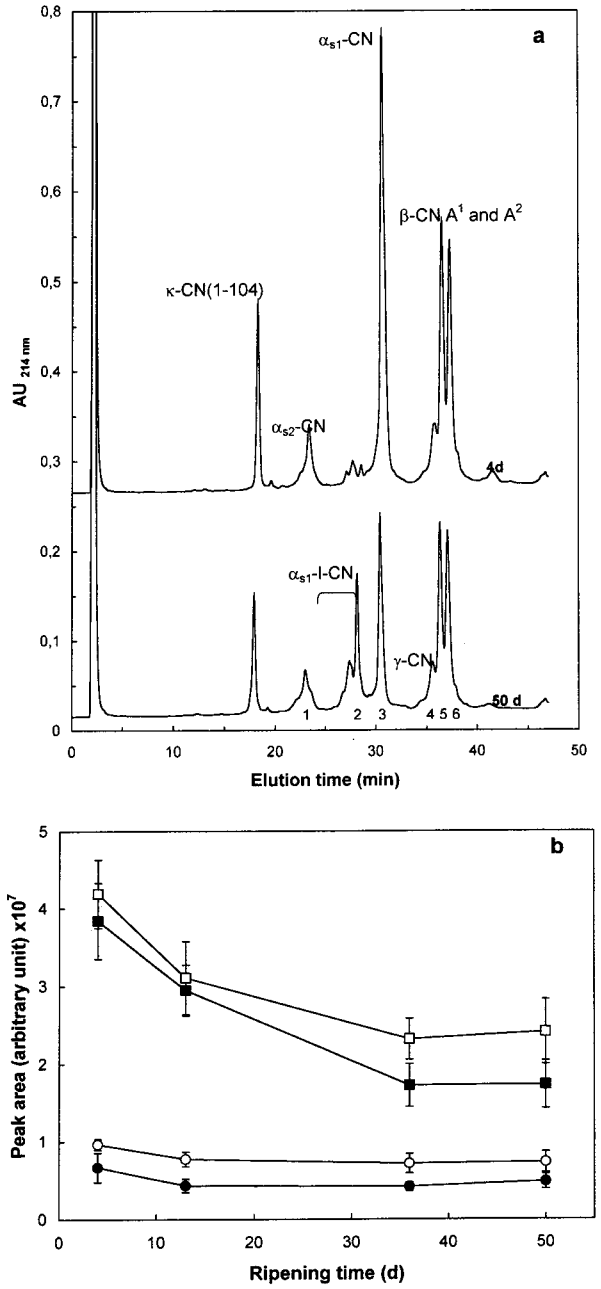

Figure 1. (a) Chromatographic profiles of the caseins present in the residual paracasein matrix during French industrial Emmental cheese ripening. Reversed-phase HPLC analysis on Vydac C_4 column was performed as described in the text. (b) Kinetics of disappearance of the β- (□), α_{s1}-(■), α_{s2}-(●), and para κ-(○) caseins during French industrial Emmental cheese ripening. Peak areas of remaining caseins during ripening were determined from RPHPLC analysis of the residual paracasein matrix as described in the text and represented with standard deviation ($n = 4$). Peak numbers correspond to the fraction collected during RPHPLC analyses and separated by urea PAGE under the conditions described in Figure 2b.

50. A wheel was withdrawn at each ripening period, sliced into 2−2.5 kg sectors corresponding to representative parts of the cheese, and stored under vacuum at −20 °C until use.

Extraction of Emmental Juice by Hydraulic Press. Emmental juices were expressed from the 2−2.5-kg sectors (thawing for 15−20 h at 4 °C) using a hydraulic press as described by Salvat-Brunaud et al. (*11*), then successively filtered first through Whatman paper 541 (Prolabo, Bruchet Dano, Rennes, France) and second through cellulose acetate membrane filters with 1.2 and 0.45 μm pore size (Sartorius, Palaiseau, France), and stored at −20 °C until use. Total

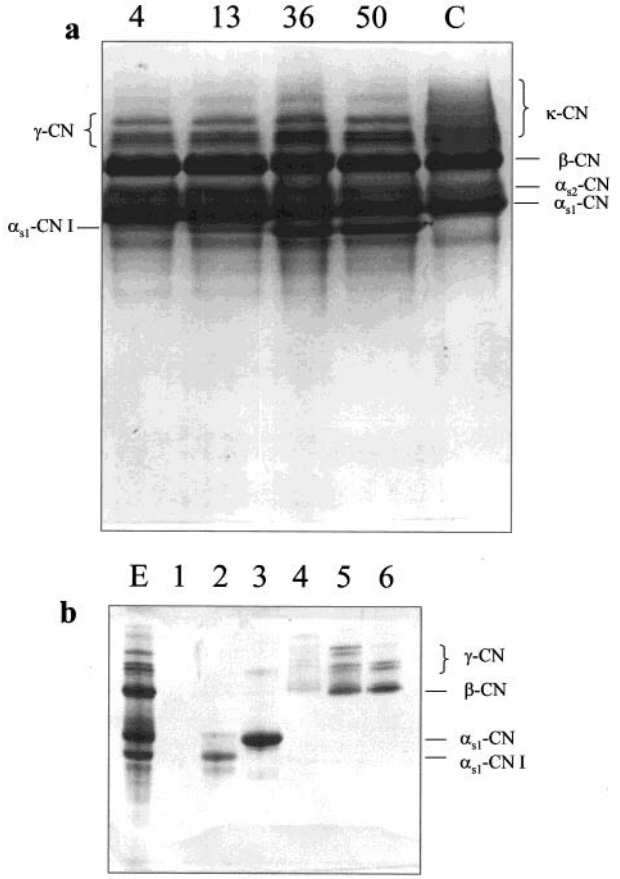

Figure 2. Urea−polyacrylamide gel electrophoresis (18% acrylamide) of (a) bovine sodium caseinate (C) and casein samples from Emmental cheese at 4, 13, 36, and 50 d of ripening; (b) collected fractions from HPLC analysis, Emmental casein fraction after 50 d of ripening (E) and collected fractions with numbers referring to the peaks eluted in the casein fraction from Emmental cheese at 50 d of ripening (Figure 1a).

nitrogen analysis was performed by a micro-Kjeldahl method (*12*), using a factor of 6.38 to convert to milk protein equivalent fraction concentration.

Chromatographic Analyses of Caseins. Caseins were precipitated from a 0.1 M citrate solution of cheese with 1 M HCl according to Gripon et al. (*13*) and were resuspended in 8.75 M urea, then reduced with 16 mM β-mercaptoethanol and incubated for 1 h at 37 °C. Samples were diluted to 1:10 with solvent A (0.106% (v/v) trifluoroacetic acid (TFA) in MilliQ purified water), filtered with 0.45-μm filters before being analyzed (0.1 mL injected) by reversed-phase HPLC on a Spectra Physics SP8800 system (Spectra Physics, San José, CA) with a Vydac C_4 column (150 mm height and 4.6 mm i.d., Touzart et Matignon) using solvent A and solvent B (0.1% TFA (v/v), 80% acetonitrile (v/v), and 20% (v/v) MilliQ purified water) under conditions of a linear gradient from 37 to 57% solvent B in 37.5 min using a flow rate of 1 mL/min, at 40 °C, and a detection at 214 nm (*14*).

The purity of the HPLC peaks corresponding to α_{s2}-, α_{s1}, and β-caseins was checked by urea PAGE according to the conditions of Andrews (*15*). The caseins were identified by the molecular mass as determined by on-line coupling between RPHPLC with C_4 column and electrospray mass spectrometry according to the conditions used by Gagnaire and Léonil (*14*).

Peptide Mapping. Peak with retention time of 27−28 min at ripening time of 50 d was collected after separation on Vydac C_4, dried in a Speed Vac concentrator (Bioblock, Illkirch, France), and solubilized with 60 μL of ammonium acetate buffer (50 mM, pH 7). The sample (40 μL) was hydrolyzed by sequencing grade modified trypsin 0.5 μg/sample (Promega, Charbonnières, France) for 4 h at 37 °C and stored at −20 °C

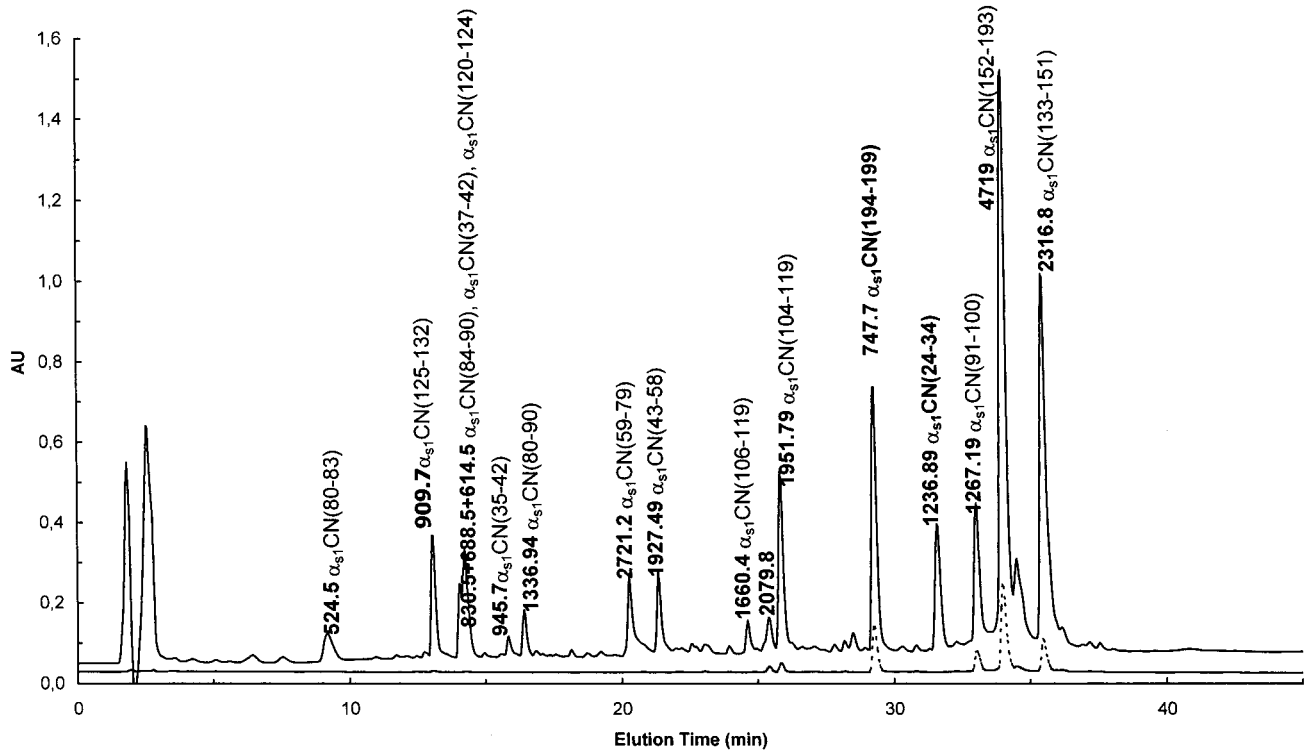

Figure 3. Tryptic peptide mapping of peak 2 collected from casein fraction. The conditions of peak collection and its tryptic hydrolysis are described in the Materials and Methods section. The calculated molecular masses, as well as the corresponding assignments of peptides sequences, are directly noted on the figure. The sequence of the peptides represented in bold corresponds to the N- and C-terminal parts of the casein fragment before hydrolysis. Detection at 216 nm and 280 nm represented by unbroken and dotted lines, respectively.

until direct coupling between RPHPLC and mass spectrometer under the conditions described below.

Mass Spectrometry. *(i) HPLC/ESI-MS Analysis of the Juice.* Mass spectra were recorded on a PE-Sciex API III plus triple-quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an atmospheric pressure electrospray ionization source. Peptides were separated on a Waters 625 LC system (Waters, Milford, MA) directly interfaced with the mass spectrometer. Emmental juices (50 μL) diluted 1:2 with 8.75 M urea and acidified to pH 2 with TFA 10% (v/v) (50 to 225 μg injected) were analyzed by reversed-phase HPLC on a Vydac C_{18} column (250 mm height and 4.6 mm i.d., Touzart et Matignon) using solvent A (0.106% TFA (v/v) in MilliQ purified water and solvent B (0.1% TFA (v/v) and 80% acetonitrile (v/v) in MilliQ water). A linear gradient from 2 to 70% of solvent B in 90 min was applied for the elution at a flow rate of 0.8 mL/min, at 40 °C and with a detection at 214 nm. A postcolumn flow splitter was used to introduce 1:10 of the HPLC eluate into the mass spectrometer as previously described (*16*).

Ion source voltage (ISV) was set at 4.8 kV and the orifice voltage (OR) was set at 80 V. The quadrupole mass analyzer (in the Q_3 quadrupole mode) was scanned over a *m*/*z* range of 500−2400 Da with a step size of 0.3 Da and a dwell time of 1 ms per step. The molecular masses were determined from these data using an Apple Macintosh computer power PC 80/8100 and the package software Tune 2.5 supplied by Sciex.

(ii) ESI−MS/MS Analysis of the Peptides. The peptides were collected after separation on Vydac C_{18}, dried in a Speed Vac concentrator, and solubilized from 1 to 10 pmol/μL with water/acetonitrile/formic acid solution (49.5/49.5/1, v/v, respectively) just prior to analysis, and infused at 5 μL/min. The ISV was set at 4.8 kV and the OR was set to 60−90 V depending on the charge state of the molecule: in the 70−90 V range for the singly charged state and in the 50−70 V range for the doubly charged state. The quadrupole Q_1 was used in a mass-resolving mode to select the parent ion, and the quadrupole Q_3 was scanned over a range of 60 Da to the mass of the parent ion + 50 Da with a collision step size of 0.3 Da, a dwell time of 2 ms, and a collision gas thickness of 3×10^{15} molecules of Argon/cm^2 according to the conditions used by Mollé et al. (*17*).

Among the strategies used for the identification of the peptides, we have used the searching by peptide sequence tag as developed by Mann and Wilm (*9*) which is included in the software Biotool box supplied by Sciex. The SWISS PROT database was used for the peptide search, performed with the "b" ions and the "y"-type ions. Nomenclature used for designated fragment species is based on Roepstorff notation (*18*). Masses are monoisotopic to 1800 Da unless otherwise noted.

Repeatability. At least three RPHPLC analyses were conducted for each sample, and the means values are reported along with the SD for the experimental data set.

RESULTS

Casein Breakdown and Peptide Profiles in Emmental Juice during Cheese Ripening. Chromatographic profiles showing the separation of proteins present in the water-insoluble fraction at pH 4.6 from French industrial Emmental cheese at different ripening times are presented in Figure 1, and the electrophoretogram is presented in Figure 2a. At the brining step (4 d of ripening), five well-separated major peaks were eluted on the C_4−RP-column. After the samples were collected, they were analyzed by electrospray mass spectrometry and identified as β-A^1 and A^2, α_{s1}-B, and α_{s2}-caseins with 24026.32 ± 3.01, 23986.30 ± 3.38, 23618.53 ± 2.20, and 25230.00 ± 2.10, respectively. The peak with a molecular mass at 12122.50 corresponded to the sequence κ-CN(1−104). This was consistent with the use of *Endothia parasitica* as a coagulant in this cheese making (*19*, *20*). Peak areas of β-, α_{s1}-, and α_{s2}-caseins, as well as para κ-casein, diminished during ripening, but the caseins were far from being completely hydrolyzed after 50 d of ripening. Between the brining

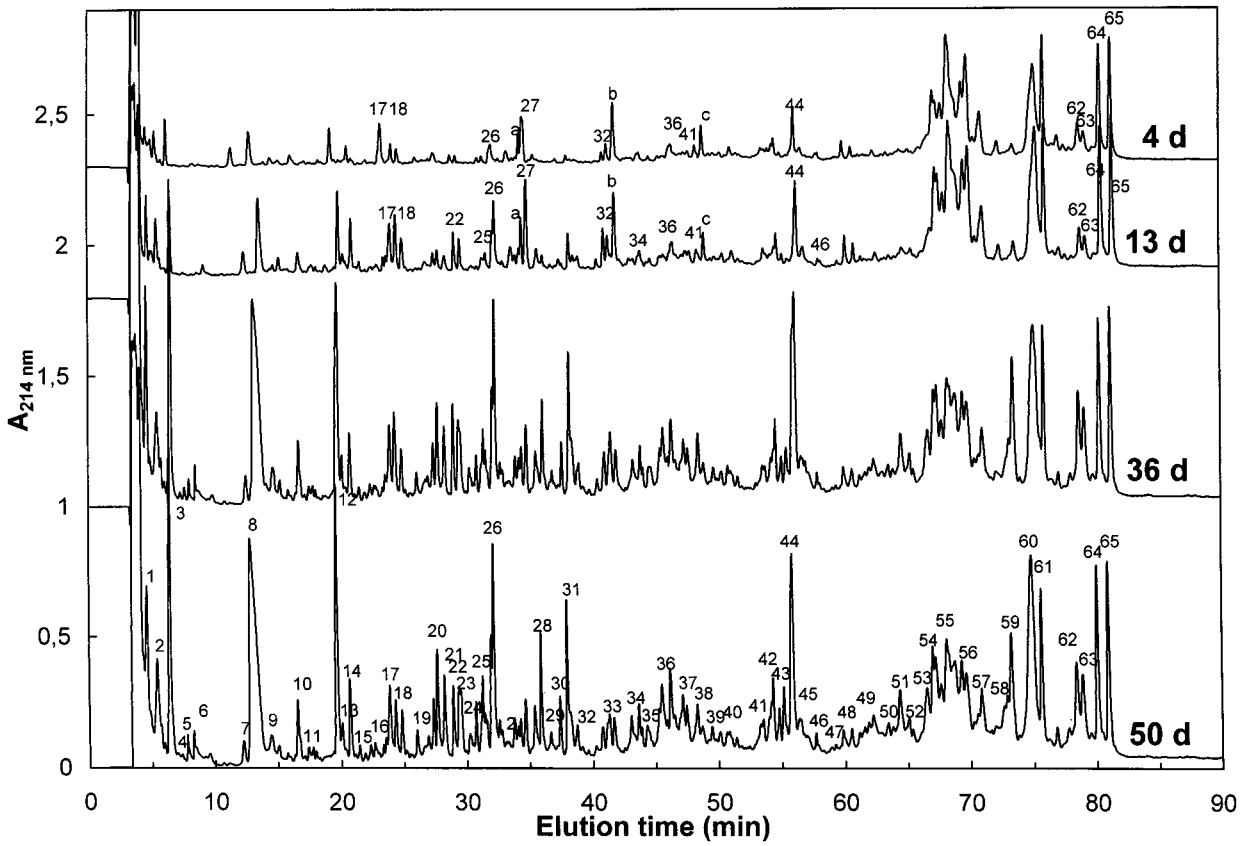

Figure 4. Chromatographic profiles of the peptides present in the juice of French industrial Emmental cheese during ripening. Reversed-phase HPLC analysis on a Vydac C_{18} column was performed as described in the text. The peaks have been collected and analyzed by tandem mass spectrometry. The masses of the peptides identified are reported in Table 1. Peptides noted a, b, and c were identified in juice only at 4 and 13 d of ripening and correspond to β-CN(7−14) (observed M_r = 855.7 Da), β-CN(7−28) (observed M_r = 2708.48 Da), and β-CN(1−28) (observed M_r = 3478.13 Da), respectively.

step and the end of Emmental cheese ripening (50 d), the level of degradation of α_{s1}-casein was higher than those of the other caseins, with 55% of disappearance in comparison with 32% for β-casein, 27% for α_{s2}-casein, and 24% for para κ-casein at the end of cheese ripening (Figure 1b). A significant increase in peak at retention time 27−28 min was also observed (Figure 1a). When this peak was analyzed by urea-PAGE (Figure 2b), the components present within migrated with electrophoretic mobility corresponding to that of α_{s1}-I casein (*1*). This was confirmed by the tryptic peptide mapping which corresponded to α_{s1}-CN(24−199) (Figure 3). A significant increase of 3 bands with electrophoretic mobility lower than that of β-casein was also observed throughout ripening time, which corresponded to γ-caseins (Figure 2b).

Concomitantly, the amount of soluble nitrogen matter, including peptides and free amino acids, gradually increased in the juice of Emmental cheese during ripening, with total nitrogen content varying from 16 TN/kg of juice at the brining stage (4 d) to 60 TN/kg of juice at the end of the ripening period (50 d). The highest increase in nitrogen content was observed during the warm room period, which is in agreement with other results on Emmental (*10, 11*). The RPHPLC profiles of the peptides present in the juice (Figure 4) showed the same pattern from the brining step up to the end of ripening. The peak intensity strongly increased during the warm room period (between 13 and 36 d of ripening), which is specific of the Emmental cheese manufacture (i.e., 22−23 °C for 23 d in our study). Between this period and the end of ripening, the intensity of the different peaks did not vary significantly due to storage at 4 °C until sale (Table 1).

Identification Strategies of the Peptides Released in Emmental Juice. More than 100 molecular masses were determined in Emmental juice at 50 d of ripening by using on-line HPLC−MS (Figure 4). Among them, several masses corresponded to those of whole proteins such as α_{s1}-casein, β-casein A^1 and A^2, α-lactalbumin, and β-lactoglobulin A and B (see highest masses reported in Table 1). However, the quantity of soluble α-lactalbumin, β-lactoglobulin, and caseins in juice was minor and represented less than 2% of the TN of the cheese at the brining stage.

The collected peptide fraction, as shown in Figure 4, was identified by sequencing using tandem mass spectrometry. The peptides identified in Emmental juices at different times (4, 13, and 50 d) of ripening are listed in Table 1. The identification of these peptides has been achieved by using several strategies that are illustrated in Figure 5. All of the strategies used database searches to assign sequence information. Figure 5a shows the collisional activated dissociation (CAD) spectrum of the singly charged ion $[M+H]^+$, m/z = 668.3. A full series of *y*-type (y^6 to y^1) and *b*-type ions (b^6 to b^1) allowed unambiguous assignment of the sequence as (L/I)PP-(L/I)T(Q/K). Because (L/I) and (Q/K) have the same nominal molecular weight, they cannot be distinguished by collision at low energy. As the sequence of the caseins are well-known, the sequence assignment only matched with the theoretical sequence (74−79, Mr 667.38, peak n*o*. 27) of β-casein. In other cases, when only a partial amino sequence was found, peptides were identified by

Table 1. Assignment of the Peptides Identified in the Emmental Juice

peak no.[a]	mass (Da)[b] observed M_r	mass (Da)[b] calculated M_r	sequence assignment of the peptides[c]	content of peptides (expressed as area of peak in chromatogram, AU*min)[d] day 4	day 13	day 36	day 50
3	702.20	702.34	α_{s2}-CN(184−188)	0.0489	0.1045	0.2856	0.3307
4	488.10	488.22	**α_{s2}-CN(16−19)**				
	524.40$	524.30	α_{s1}-CN(6−9)				
	616.20	616.35	α_{s1}-CN(34−38)				
	694.37	694.28	κ-CN(20−24)				
7	388.40*$	388.19	β-CN(29−31)	0.0396	0.0403	0.0338	0.0349
	427.40	427.25	β-CN(199−202)				
8	493.40*$	493.29	α_{s1}-CN(4−7)	0.0501	0.1079	0.5124	0.6134
	758.24	758.41	α_{s1}-CN(4−9)				
9	886.64	886.51	α_{s1}-CN(3−9)			0.0555	0.0519
10	536.40	536.26	β-CN(64−68)		0.0250	0.0597	0.0618
	623.40	623.29	β-CN(64−69)				
	713.40	713.44	α_{s2}-CN(194−199)				
11	422.40	422.22	β-CN(64−67)				0.0225
	479.40	479.23	α_{s1}-CN(32−35)				
	587.40	587.28	α_{s1}-CN(15−19)				
	1067.40	1067.34	**β-CN(18−25)**				
12	1139.84	1139.66	α_{s1}-CN(1−9)		0.0575	0.2258	0.1191
13	1011.59	1011.61	α_{s}-CN(1−8)		0.0247	0.0394	0.1522
14	874.64	874.55	α_{s1}-CN(1−7)		0.0492	0.0511	0.0521
15	449.40	449.26	α_{s1}-CN(31−34)				0.0106
	488.40$	488.24	β-CN(11−14)				
	1347.30	1347.43	**β-CN(16−25)**				
16	542.40	542.27	α_{s1}-CN(10−14)				0.0216
	578.40	578.31	α_{s1}-CN(31−35)				
	678.30	678.35	α_{s1}-CN(8−13)				
	806.40	806.44	α_{s1}-CN(7−13)				
17	670.50	670.32	α_{s1}-CN(9−14)	0.0400	0.0449	0.1129	0.1220
	746.40*$	746.45	α_{s1}-CN(1−6)				
18	807.30*$	807.38	α_{s1}-CN(8−14)	0.01080	0.0290	0.0418	0.0412
	935.39$	935.47	α_{s1}-CN(7−14)				
	1244.70	1244.63	α_{s2}-CN(153−162)				
	1434.59	1434.46	**β-CN(15−25) (−1P)**				
19	890.39	890.48	β-CN(95−102)			0.0225	0.0203
	1452.00	1451.72	β-CN(95−107)				
20	919.64	919.52	α_{s1}-CN(6−13)			0.0721	0.0770
	1048.64	1048.56	α_{s1}-CN(6−14)				
	1153.64	1153.63	α_{s1}-CN(4−13)				
21	1283.54	1283.45	α_{s1}-CN(4−14)			0.0556	0.0547
	1411.60	1411.62	α_{s1}-CN(3−14)				
22	1535.39$	1534.88	α_{s1}-CN(1−13)		0.0234	0.0599	0.0592
23	1664.78	1664.93	α_{s1}-CN(1−14)			0.0888	0.0856
24	1703.39	1703.52	**β-CN(16−28)**			0.0254	0.0298
25	697.60	697.40	α_{s2}-CN(116−122)		0.0243	0.0751	0.0845
	1870.04$	1870.57	**β-CN(15−28)**				
	2006.00	2006.70	**α_{s2}-CN(7−21)**				
26	2143.64*$	2143.84	α_{s1}-CN(6−21)	0.0252	0.0759	0.1974	0.1893
	2273.24$	2272.96	α_{s1}-CN(5−21)				
	2528.73$	2529.23	α_{s1}-CN(6−24)				
	2657.58$	2658.38	α_{s1}-CN(5−24)				
a	855.7*	855.68	**β-CN(7−14)**	0.0203	0.0410		
27	667.39	667.38	β-CN(74−79)	0.0467	0.0734	0.0532	0.0359
	787.40*	787.40	β-CN(1−6)				
	2018.84	2018.87	**β-CN(13−28) (−1P)**				
	2789.43	2789.54	**α_{s2}-CN(35−66)**				
	2747.28	2747.51	**α_{s2}-CN(1−21)**				
	3133.08	3132.96	**α_{s2}-CN(1−24)**				
28	658.33	658.32	α_{s1}-CN(25−30)		0.0212	0.0641	0.0768
	2211.48$	2212.01	**β-CN(12−28)**				
29	2131.68	2132.03	**β-CN(12−28) (−1P)**			0.0298	0.0251
30	870.49	870.48	α_{s1}-CN(16−22)			0.0379	0.0378
	2469.78	2469.27	***β*-CN(11−29)**				
31	2341.53	2341.12	**β-CN(11−28)**		0.0232	0.1439	0.1315
32	714.50	714.38	α_{s1}-CN(16−21)	0.0144	0.0112	0.0408	0.0341
	1148.49	1147.64	α_{s1}-CN(7−16)				
	2594.98*$	2594.42	**β-CN(8−28)**				
	2839.23	2839.74	**β-CN(11−32?)**				
b	2708.48*	2708.50	**β-CN(7−28)**	0.0502	0.0754		
33	1877.48	1877.22	α_{s1}-CN(1−16)			0.0712	0.0725
34	754.50*$	754.41	α_{s1}-CN(10−16)		0.0198	0.0475	0.0450
	813.50	813.45	α_{s1}-CN(15−21)				
	965.00	965.54	β-CN(74−82)				
35	2392.38	ND					0.0976

Table 1 (Continued)

peak no.[a]	mass (Da)[b] observed M_r	mass (Da)[b] calculated M_r	sequence assignment of the peptides[c]	content of peptides (expressed as area of peak in chromatogram, AU*min)[d] day 4	day 13	day 36	day 50
36	805.60*$	805.39	α_{s1}-CN(24−30)	0.056	0.0855	0.2636	0.3083
	942.70$	942.49	α_{s1}-CN(14−21)				
	2556.53	2556.57	**β-CN(33−52)**				
37	661.60	661.38	α_{s1}-CN(19−23)			0.0381	0.0392
	904.60	904.47	α_{s1}-CN(17−23)				
38	2616.68	2617.05	α_{s1}-CN(1−22)			0.0801	0.0724
40	1017.74	1017.55	α_{s1}-CN(16−23)		0.0134	0.0310	0.0217
	2460.83	2460.86	α_{s1}-CN(1−21)				
41	904.90	904.47	α_{s1}-CN(17−23)	0.0167	0.0254	0.0482	0.0418
	1116.89	1116.62	α_{s1}-CN(15−23)				
c	3478.63	3479.40	**β-CN(1−28)**	0.0212	0.0219		
42	1245.89	1245.66	α_{s1}-CN(14−23)			0.0944	0.0931
43	904.60*$	904.47	α_{s1}-CN(25−32)			0.0444	0.0507
44	2764.58*$	2764.23	α_{s1}-CN(1−23)	0.0464	0.0875	0.2766	0.2796
45	2035.53	2035.33	α_{s1}-CN(7−23)			0.0867	0.0431
46	769.70	769.47	β-CN(82−88)			0.0127	0.0119
	1906.79$	1907.15	α_{s1}-CN(8−23)				
48	1641.29	1641.88	α_{s1}-CN(10−23)			0.0303	0.0276
50	1881.40	1881.24	β-CN(193−209)			0.0299	0.0347
60	23618.53 ± 2.20	23615.79	α_{s1}-casein B	0.02275	0.3297	0.4056	0.3187
61	14180.05 ± 4.06	14176.13	α-lac	0.1084	0.1242	0.1235	0.1465
62	24026.32 ± 3.01*$	24023.31	β-casein A^1	0.0325	0.0392	0.1275	0.1485
63	23986.30 ± 3.38*$	23983.28	β-casein A^2	0.0233	0.0249	0.1135	0.1287
64	18278.61 ± 1.97*$	18278.27	β-Lg B	0.1155	0.1362	0.1871	0.1670
65	18366.91 ± 1.29*$	18363.38	β-Lg A	0.1144	0.1409	0.1781	0.1352

[a] Peptide peaks noted a, b, and c were observed only at 4 and 13 d of ripening. [b] Molecular masses presented in the table corresponded to masses observed at 50 d of ripening; and those noted * and $ represent masses already observed at 4 and 13 d of ripening, respectively. [c] Peptides written in bold correspond to phosphopeptides. Abbreviations: α-lac, α-lactalbumin; β-Lg B, β-Lactoglobulin variant B; β-Lg A, β-Lactoglobulin variant A. [d] The peak areas were estimated to lie within 17.3% for 0.00 <area < 0.05, 14.1% for 0.05 < area < 0.1, and 13.0% for area > 0.1.

using the peptide sequence tag as proposed by Mann and Wilm (*9*). For example, the CAD mass spectrum of the doubly charged ion $[M+2H]^{2+}$ at $m/z = 941.6$ from the ion parent (Mr = 1881.4) produced abundant cleavage ions at *m/z* 1591.2, 1461.9, 1364.7, 1265.7, 1152.6, and 1095.6. This was consistent with the partial sequence G(L/I)VPE (Figure 5b). This information, together with the determined peptide mass (1881.4), the type of ions (*y*), and the lowest and highest mass (1095.6 and 1591.2) of the ion series, was assembled by PeptideSearch software into a peptide sequence tag and uniquely retrieved β-casein for the sequence β-CN(193−209) (peak n*o*. 50).

The third type of peptides present in the cheeses was phosphopeptides (Figure 5c). In this case, examination of the CAD spectrum revealed notable characteristics: prominent ions were formed by the loss of 98 or 49 Da from the singly or doubly charged parent ion, respectively, due to the β-elimination reaction during the CAD fragmentation process (*21*). Hence, MS/MS spectra of phosphopeptides displayed signals corresponding to both phosphate fragmentation pattern and peptide backbone fragmentation. Thus, fragment ions 471.2 and 520.1 corresponded to the doubly charged fragment ions with a loss of three and two phosphates, respectively. The partial sequence obtained, together with the knowledge of the molecular mass of the peptide and the number of the phosphoserine residues present within (3 in this example), was sufficient to unambiguously identify the peptide β-CN(15−25)-1P (peak n*o*. 18) by the sequence tag.

Peptides Produced during Emmental Cheese Ripening. Among the 91 peptides reported in Table 1, those originated from α_{s1}- and β-caseins dominated the degradation products, with 52 and 29 peptides arising from both caseins, respectively, and only 9 arising from α_{s2}-casein and 1 arising from κ-casein. An intensive degradation from the N-terminal half of α_{s1}-casein was already observed at the brining step (Figure 4 and Table 1), on one hand by the splitting of the Phe_{23}−Phe_{24} bond leading to the formation of α_{s1}-CN(1−23) released in the juice and of α_{s1}-CN(24−199) remaining in the casein fraction, and on the other hand by the formation of peptides α_{s1}-CN(1−8), α_{s1}-CN(1−9), α_{s1}-CN(8−14), α_{s1}-CN(6−21), α_{s1}-CN(10−16), α_{s1}-CN(24−30), and α_{s1}-CN(25−32). This degradation of the N-terminal part of the α_{s1}-casein continued throughout ripening with production of 45 peptides out of the 52 with splitting bonds comprised between Arg_1 and Phe_{23} (i.e., Ile_6−Lys_7, His_8−Gln_9, Gln_9−Gly_{10}, Gln_{13}−Glu_{14}, Leu_{21}−Arg_{22}, Arg_{22}−Phe_{23}, and Phe_{23}−Phe_{24}), whereas the C-terminal part of the molecule, i.e., α_{s1}-CN(24−199), was almost not hydrolyzed. Moreover, the pattern of these released peptides showed that they were themselves progressively degraded with the recurrent loss of one or more residues from their N-terminal end.

In contrast to α_{s1}-casein, β-casein was degraded more evenly over the whole sequence, except the region between Pro_{110} and Leu_{192}, from which no peptide was released. Thirteen out of the 29 released peptides arose from the splitting of Lys_{28}−Lys_{29} and Lys_{29}−Ile_{30}. Peptide β-CN(1−28) was already produced at brining stage and was completely hydrolyzed at the end of ripening time into peptides with different lengths resulting from different kinds of cleavage. As described for α_{s1}-casein, a progressive degradation of the N-terminal end of those peptides was observed. Moreover, a recurrent degradation pattern was observed from the C-terminal end as shown by the formation of β-CN(64−69), β-CN(64−68), and β-CN(64−67).

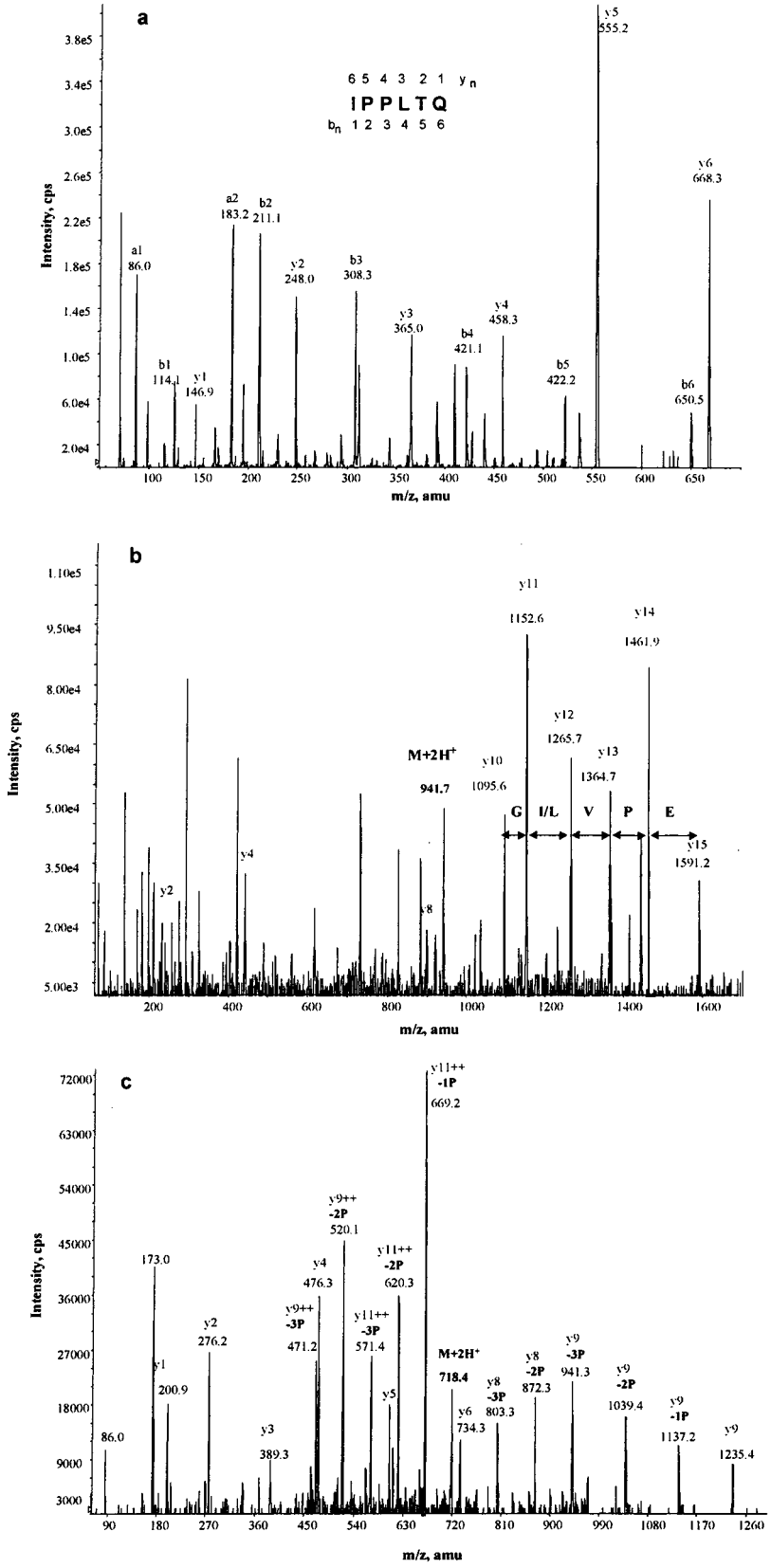

Figure 5. Full scan of a collisional activated dissociation (CAD) of (a) a singly charged peptide derived from β-casein with m/z = 668.4. Conditions of collision are described in the Materials and Methods section; (b) a peptide present in Emmental juice of measured mass of 1881.4 Da. Subtraction of the masses of the major ion peaks results in the partial sequence shown. The information needed for the peptide sequence tag in SWISS PROT database is described as follows: measured peptide molecular mass, 1881.40 Da; run of sequence ions, 1591.2, 1461.9, 1364.7, 1265.7, 1152.6, and 1095.6; type of ion series, *y* series; partial sequence, G(L/I)VP(E/K); mass of region 1, 1095.6; mass of region 2, 1591.2; search string entered into peptide search, [1095.6]G-(L/I)VP(E/K)[1591.2]. In addition, scan CAD spectrum presents "*y*" fragment ions of the MS/MS spectrum verifying the peptide β-CN(193−209) found in the database; and (c) a phosphopeptide present in Emmental juice of measured mass of 1434.59 Da with "*y*" and "*b*" fragment ions of the MS/MS spectrum verifying the peptide β-CN(15−28)-1P found in the database.

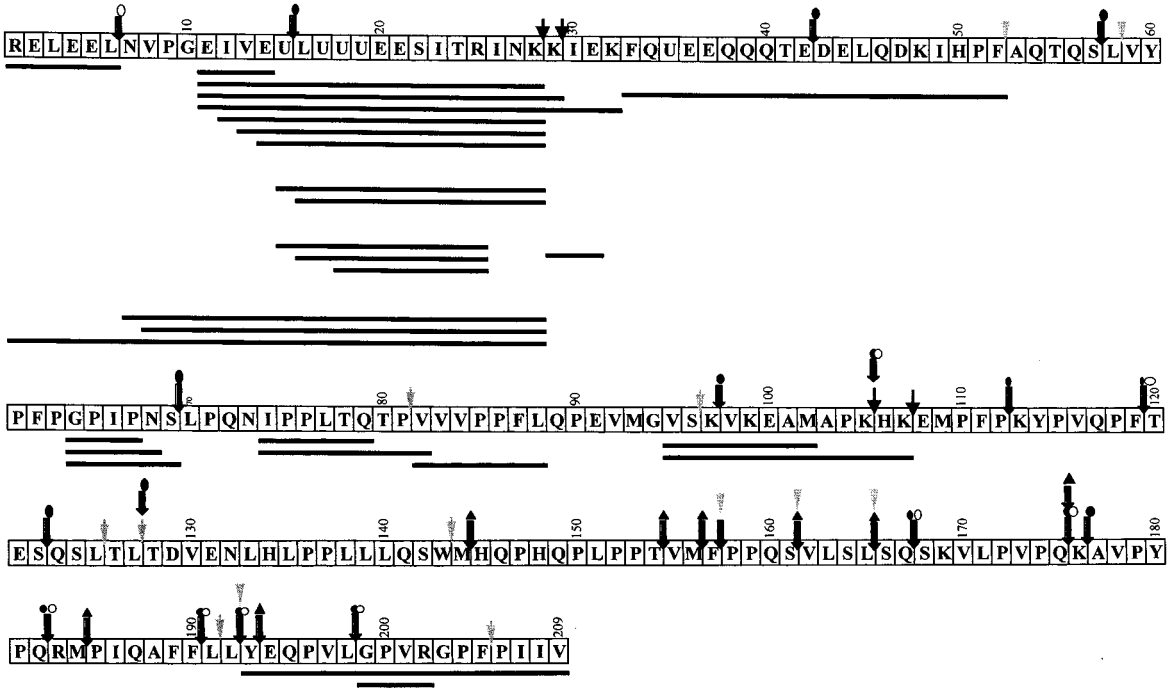

Figure 6. Localization of identified peptides from β-casein released in Emmental juice at the end of the ripening. Arrows designate the splitting bonds found under in vitro conditions and cleaved by the following: (↓, down arrow) plasmin (*26*), (shaded down arrow) cathepsin D (*27*, *35*), and from bacterial origin like the cell-envelope proteinase of (arrow topped by open circle) *Lactobacillus helveticus* CNRZ 303 (*36*), (arrow topped by closed circle) *Lb. helveticus* CP790 (*37*), and (arrow topped by closed triangle) *Lb. delbrueckii* subsp. *lactis* ACA-DC 178 (*38*).

Only 9 peptides were released from α_{s2}-casein, with 5 out of the 9 released peptides arising from the N-terminal part of the molecule. The low number of identified peptides from this casein can be attributed to its low content in the caseins and therefore a higher difficulty in detecting peptides arising from it. This can also be the case of the peptides from para κ-casein, as only one was identified in juice (κ-CN (20−24)), unless this molecule was rather resistant to proteolysis as shown in other types of cheese (*1*, *6*, *7*).

Among the peptides produced, we also observed phosphopeptides which came from β- and α_{s2}-caseins with 12 and 5 phosphopeptides, respectively. We can notice that no phosphopeptide derived from α_{s1}-casein was observed in Emmental juice, as previously reported for Comté (*22*). However, for cheeses such as Parmigiano Reggiano (*6*) and Grana Padano (*8*, *23*), for which the ripening time is longer and casein proteolysis more extended, 16 phosphopeptides from α_{s1}-casein were identified. In addition, some phosphopeptides were dephosphorylated: β-CN(12−28) (−1P), β-CN(13−28) (−1P), β-CN(15−28) (−1P), and β-CN(15−25) (−1P). We observed that the dephosphorylation occurred on the isolated phosphoserine residue Ser_{15}, but not on the phosphorylated Ser involved in a cluster of three residues (Ser_{17}−Ser_{18}−Ser_{19}). This phenomenon was in complete agreement with the finding of Ferranti et al. (*23*) in Grana padano cheese. The results also show that only the dephosphorylated peptides were further degraded through the recurrent loss of their N-terminal amino acids, as shown by the formation of β-CN(16−25) and β-CN(18−25).

DISCUSSION

Identification of the released peptides in different kinds of cheese has been established as a way to determine the specificity of the enzymes involved in cheese ripening, and through the cleavage sites to provide insights on parts of the caseins in which they are active (*1*). This study corresponds to the first large view of the proteolytic systems involved in the degradation process of the caseins within Emmental cheese. Such an approach was based on the knowledge of the specificities determined in solution of different proteinases potentially implicated in the Emmental manufacturing as reported on Figures 6−8 for β-, α_{s1}-, and α_{s2}-caseins, respectively.

Because of the inactivation of residual coagulant by high cooking temperature during Swiss-type cheese making, and more drastically observed when *Endothia parasitica* is used (*24*), plasmin has a strong contribution to primary proteolysis (*25*). In Emmental, its high activity toward β-casein was easily recognizable by cleavage sites at Lys_{28}−Lys_{29} and Lys_{29}−Ile_{30} (*26*), the early release of peptide β-CN(1−28), and complementary large- size γ-caseins (Figure 2). Evidence of the action of plasmin was also found on the breakdown pattern of α_{s2}-casein: Lys_{21}−Gln_{22}, Lys_{24}−Asn_{25}, Lys_{152}−Leu_{153}, Lys_{188}−Asp_{189}, and Lys_{199}−Val_{200} (Figure 8), and to a lesser extent, on that of α_{s1}-casein with splitting at Lys_3−His_4, Lys_7−His_8, and Arg_{22}−Phe_{23} (Figure 7). Our result was consistent with those found in Italian hard cooked cheeses such as Parmigiano Reggiano (*6*, *7*) and Grana Padano (*8*).

An extensive proteolysis of the N-terminal part of α_{s1}-casein was already observed at the brining step. As shown in Figure 7, this degradation pattern could proceed with the action of two kinds of proteinases potentially present in the Emmental cheese: cathepsin D and cell-envelope proteinase of starter bacteria, especially from Lactobacilli species. Both enzymes are able to hydrolyze α_{s1}-casein at Phe_{23}−Phe_{24} and to produce throughout ripening α_{s1}-CN(1−23) in juice and

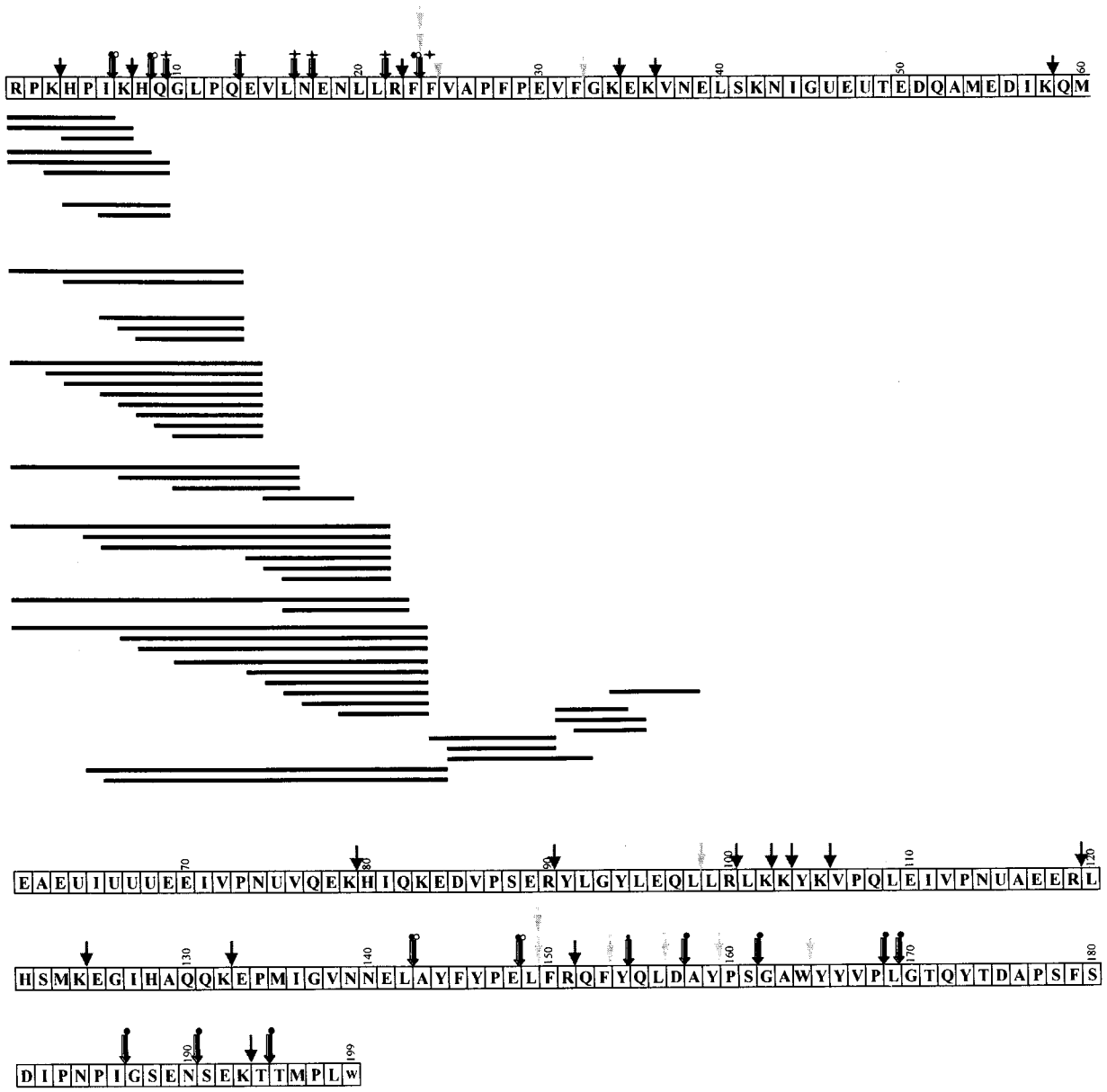

Figure 7. Localization of identified peptides from α_{s1}-casein released in Emmental juice at the end of the ripening. The arrows designate the potential splitting bonds found under in vitro conditions and cleaved by the following: (↓, down arrow) plasmin (*39*, *40*), (shaded down arrow) cathepsin D (*27*, *35*), and from bacterial origin like the cell-envelope proteinase of (arrow topped by open circle) *Lactobacillus helveticus* CNRZ 303. (*36*), (arrow topped by closed circle) *Lb. helveticus* CP790 (*37*), and (arrow topped by plus sign) *Lb. helveticus* L89 (*29*).

α_{s1}-CN(24−199) in the casein fraction, as well as β-casein at Leu_{192}−Tyr_{193} to form β-CN(193−209) (*27*). However, it is difficult to directly evaluate the contribution of each of the proteinases to primary proteolysis independently of each other.

The potential involvement of cathepsin D was supported by additional specific cleavages occurring at Phe_{24}−Val_{25} and Phe_{32}−Gly_{33} on α_{s1}-casein and at Phe_{52}−Ala_{53} and Pro_{81}−Val_{82} on β-casein (Figure 6), corresponding to the release of peptides α_{s1}-CN(25−32), β-CN(33−52), and β-CN(82−88), respectively. Replacement of chymosin by a more thermolabile coagulant arising from *Endothia parasitica* would indicate that cathepsin D could play an important role in the hydrolysis of α_{s1}-casein and β-casein in Emmental cheese. Moreover, such an activity is suitable with the acidic pH encountered in Emmental cheese, i.e., pH 5.2−5.4 (*27*). Besides cathepsin D, the potential involvement of the cell-envelope proteinases (CEP) arising from thermophilic lactobacilli species was also supported through formation of peptides such as β-CN(1−6) and β-CN(7−28) (Figure 6), present in the juice at the brining step, and by the splitting of bonds Ser_{69}−Leu_{70} and Leu_{198}−Gly_{199} on β-casein. These specificities are considered typical of the CEP action from *Lb. helveticus* on β-casein substrate in solution (*28*). In the same way, the formation of peptides α_{s1}-CN(1−6), α_{s1}-CN(8−14), α_{s1}-CN(7−14), α_{s1}-CN(6−21), and α_{s1}-CN(10−16) could correspond to the action of CEP from *Lb. helveticus* L89 CEP on fragment α_{s1}-CN(1−23) as described by Martin Hernandez et al. (*29*). This set of peptides was produced early and accumulated throughout ripening, especially during the warm-room period in relation to the increase in α_{s1}-CN(1−23). These data suggest that both cathepsin D and CEP could act competitively in casein degradation during Emmental cheese ripening.

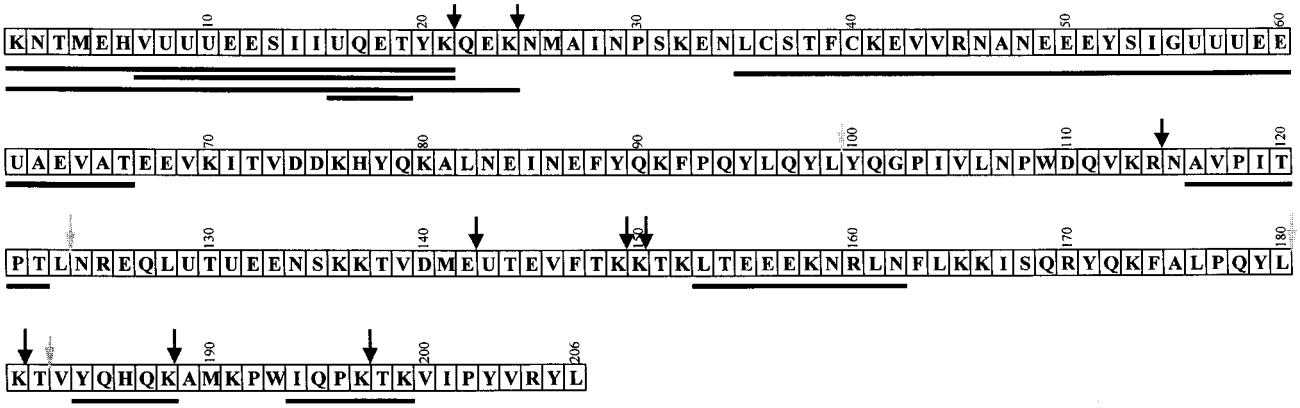

Figure 8. Localization of identified peptides from α_{s2}-casein released in Emmental juice at the end of the ripening. The arrows designate the potential splitting bonds found under in vitro conditions and cleaved by the following: (↓) plasmin (*41*), and (shaded down arrow) cathepsin D (*27*, *35*).

Data obtained in the present study clearly indicate that, besides the action of the indigenous milk proteinases, the thermophilic lactobacilli play a significant role in the Emmental proteolysis through the action of their CEP. In addition to this action, thermophilic lactobacilli are considered the more active bacterial species during ripening, not only because of higher proteolytic activities, but also because of autolytic activities demonstrated both under in vitro conditions (*30*, *31*) and in Emmental cheese (*3*). Effectively, a great number of peptides of different lengths, with the loss of one or more residues from the N- or C-terminal end, showed aminopeptidase and carboxypeptidase activity in agreement with the presence of a bacterial aminopeptidase of large specificity such as PepN and PepC (for example. peptides α_{s1}-CN(1/3/4/6/7/8/9/10−14) or β-CN(11/12/13/15/16−28)) and in agreement with previous works (*6*−*8*). In addition, specific hydrolysis after the dipeptides Arg−Pro and His−Pro in the N-terminal part of the α_{s1}-casein can be related to the action of PepX. These kinds of activities have previously been shown in Emmental juice (*4*). In the same way, the carboxypeptidase action was illustrated by the liberation of peptides β-CN(64−69), β-CN(64−68), and β-CN(64−67). If carboxypeptidase activity has been clearly shown in different types of cheeses (*5*, *7*, *8*, *23*), only a few enzymes were found, or even partially characterized, in lactobacilli (*28*).

Among the thermophilic starters used in Emmental cheese, our results failed to show the contribution of *Streptococcus thermophilus* because studies on the specificity of the proteinase and peptidases from this species are not available on the caseins. However, the formation of some released peptides, which cannot be explained on the basis of the known specificities of plasmin, cathepsin D, and lactobacilli CEP on β- and α_{s1}-caseins, does not preclude that the thermophilic streptococci may also participate, to a lesser extent, in the proteolytic breakdown of the caseins. Thus, the observed splitting bonds at Gly_{10}−Glu_{11}, Pro_{63}−Gly_{64}, Asn_{73}−Val_{74}, Gln_{79}−Thr_{80}, Leu_{88}−Gln_{89},Gly_{94}−Val_{95}, and Met_{102}−Ala_{103} on β-casein, and at Glu_{14}−Val_{15}, Asn_{19}−Leu_{20}, Glu_{30}−Val_{31}, and Asn_{38}−Glu_{39} on α_{s1}-casein could result from the action of potential streptococci endopeptidase.

Our results give some insights on the contribution of the other flora present in Emmental cheese during ripening: propionic acid bacteria (PAB) and nonstarter lactic acid bacteria (NSLAB) mainly represented by *Propionibacterium freudenreichii* and *Lactobacillus paracasei* (*10*). Actually, the most striking result was to observe that the peptide profile was changed quantitatively but not qualitatively during warm-room ripening, while adventitious NSLAB have finished their growth at the beginning of the warm room and PAB are growing during warm room, as shown on this cheese by Thierry et al. (*10*). For the PAB, it is known that their proteolytic activity is low in cheese because of a weak cell-envelope proteinase activity (*32*) and a low ability to undergo lysis in Emmental cheese (*3*). Recently, we have shown that this bacterial species weakly contributes to secondary proteolysis in a model system, as they were shown to consume peptides instead of producing them (*33*). Data obtained in the present study confirm this. Concerning NSLAB, their influence would be more significant because (i) this flora has cell-envelope proteinases and peptidases similar to that of thermophilic lactobacilli (*28*) and their ability to lyse has been demonstrated in vitro and in cheese (*34*); and (ii) a slight increase in secondary proteolysis was observed in Emmental juice compared to the enzymes of starters (*33*). Nevertheless, no change was seen on the peptide pattern, suggesting that either their contribution is insignificant or their enzyme specificities are close of that of thermophilic lactobacilli.

Finally, it was observed that some regions of the different caseins (Figures 6−8) were not hydrolyzed, although potential splitting sites of the different proteinases were shown under in vitro conditions of hydrolysis. Thus, plasmin was active only on 5 out of the 17 sites on α_{s1}-casein and 5 out of the 9 on α_{s2}-casein. Cathepsin D was active on 3 out of the 13 sites on β-casein, on 3 out of the 9 on α_{s1}-casein, and 2 out of the 3 on α_{s2}-casein. Lactobacilli cell-envelope proteinase was active on only 6 out of the 26 sites on β-casein and on 8 out of the 18 potential sites on α_{s1}-casein. This can be related to (i) the modulation of the specificity of the proteolytic enzymes in cheese by the physicochemical conditions (pH, water activity, and mineral and salt content) (*2*) and (ii) the accessibility of the substrate itself, due to structural rearrangement during formation of the casein matrix and environmental conditions in cheese.

ABBREVIATIONS USED

ESI, electrospray ionization source; MS, mass spectrometry; MS/MS, tandem mass spectrometry; CAD, collisional activated dissociation; *m*/*z*, mass-to-charge

ratio; urea PAGE, polyacrylamide gel electrophoresis in the presence of urea; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; RPHPLC, reversed-phase high-performance liquid chromatography; TN, total nitrogen; CN, casein; CEP, cell-envelope proteinase; −1P, phosphopeptide with one removed phosphorylated residue; NSLAB, nonstarter lactic acid bacteria; PAB, propionic acid bacteria.

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