Lait (1997) 77, 683-697

Original article

Combined high resolution chromatographic techniques (FPLC and HPLC) and mass spectrometry-based identification of peptides and proteins in Grana Padano cheese*

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Summary — The 0.2 mol/L trisodium citrate soluble N fraction of 14- and 38-month-old Grana Padano (GP) cheese diafiltered through 3- and 10-kDa cut-off membranes gave two fractions containing peptides with a molecular mass lower than 3 kDa, and higher than 10 kDa. 91 and 38 oligopeptides lower than 3 kDa were identified by using a combination of fast-atom bombardment/mass spectrometry and Edman degradation in the 14- and 38-month-old GP cheese samples respectively. Peptides higher than 10 kDa were submitted to preparative fractionation on Mono Q column with a stepwise ionic strength gradient and then by reverse phase-HPLC on a C4 column. Some native caseins and large-derived peptides were identified by using a combination of electrospray mass spectrometry and Edman degradation.

Grana Padano / casein proteolysis / oligopeptide / mass spectrometry

Résumé – Identification de peptides et protéines dans le fromage Grana Padano à l'aide de techniques chromatographiques haute résolution (FPLC et HPLC) couplées à la spectrométrie de masse. La matière azotée soluble dans le tampon citrate 0,2 mol/L à pH 8,0 de deux échantillons de 14 et 38 mois de Grana Padano, un fromage italien à pâte dure, a été diafiltrée sur membranes à porosité de 3 et 10 kDa. Deux fractions ont été recueillies, contenant des peptides de masse moléculaire inférieure à 3 kDa et supérieure à 10 kDa. La fraction inférieure à 3 kDa a été fractionnée par chromatographie liquide haute performance (CLHP) en phase inverse sur colonne C18 et les peptides identifiés en combinant la spectrométrie de masse à bombardement rapide et la dégradation d'Edman. La plupart des 91 peptides identifiés dans cette fraction A du fromage de 14 mois provenaient des

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* Presented as an oral communication at the IDF Symposium 'Ripening and Quality of Cheeses', Besançon, France, February 26–28, 1996.

caséines α_{s1} (44 peptides) et β (32 peptides). La majorité de ces derniers présentaient une origine commune dans la région 1–105/107 de la caséine β. Trente peptides résultaient de la dégradation du peptide α_{s1}-CN (f1-79) formé par action de la plasmine sur la caséine α_{s1}. Treize peptides provenaient de la caséine α_{s2} et un seul de la para-κ-caséine. Trente-huit peptides ont été identifiés dans la fraction inférieure à 3 kDa du fromage de 38 mois (dont 21 peptides identifiés dans le fromage jeune) : 8 issus de la caséine β, 27 de la caséine α_{s1}, 1 de la caséine α_{s2} et 2 de la para-κ-CN. La fraction supérieure à 10 kDa a été soumise à un fractionnement sur colonne Mono Q, puis à une séparation par CLHP en phase inverse sur colonne C4. Les peptides ont été identifiés en combinant la spectrométrie de masse à l'électrospray et la dégradation d'Edman. Les résultats concernant une fraction chromatographique sont présentés : les variants B et C de la caséine α_{s1} et plusieurs fragments de grosse taille tels que le peptide α_{s1}-CN-I ont été identifiés. Les résultats montrent que les différentes techniques de spectrométrie de masse permettent de caractériser, mieux que d'autres techniques, la nature des peptides issus des caséines, quelle que soit leur taille.

Grana Padano / protéolyse/ peptide/ spectrométrie de masse

INTRODUCTION

The ripening of a hard cheese such as the Italian AOC Grana Padano (GP) cheese requires at least 1 year during which, among other modifications, the extensive enzymatic hydrolysis of caseins takes place. A general mechanism of casein degradation in cheese has been suggested, implying the intervention of starter and milk proteinases and rennet in the formation of primary peptides, and peptidases in the refining of the primary products into decreasing-size peptides and free amino acids (FAAs) (Grappin et al, 1985). The N components of cheese consist of pH 4.6 insoluble native caseins and derived high-molecular mass (HMM) peptides, soluble medium and low molecular mass peptides, and free amino acids. Knowledge has been accumulated on the specificity of plasmin (Andrews, 1978a, b ; Le Bars and Gripon, 1989, 1993), chymosin (Visser, 1993; McSweeney et al, 1993), whereas little information is available on proteinases and peptidases from thermophilic lactic acid bacteria (Pritchard and Colbear, 1993) such as *L helveticus*, the species dominating in the natural 'whey' starter used for GP manufacture (Bottazzi et al, 1996). The cleavage sites in β-CN by the cell envelope proteinase from *L helveticus* were the same as those hydrolyzed by the lactococcal P_I-type proteinase (Zevaco and Gripon, 1988); others were different, although there appear to be several different specificity types among the proteinases from this species (Khalid et al, 1991). The prevailing knowledge on the soluble peptides of hard cheeses is limited to the 12% trichloracetic soluble and insoluble peptides of Parmigiano Reggiano cheese (Addeo et al, 1992, 1994), the phosphopeptides from Comté cheese (Roudot-Algaron et al, 1994), and the water soluble peptides of Cheddar cheese (Singh et al, 1995).

We are attempting to describe the proteolysis of GP cheese on a molecular basis. Our present knowledge concerns a variety of HMM-plasmin-mediated peptides from β-, and to a lesser extent α_{s1}-CN, whose origin has been established either by immunoblotting with polyclonal antibodies against single casein fractions or by direct sequencing of a few isolated peptides (Addeo et al, 1995). In addition to this, in GP cheese there were a lot of pH 4.6 soluble phosphopeptide (CPP) components which were degraded into shorter peptides (Ferranti et al, 1997). However, since CPPs represent only a secondary fraction among the wider pH 4.6 soluble N cheese fraction, containing the non-phosphorylated peptides components, a

procedure was assayed to isolate and characterize the main peptides.

Our analytical approach is based on: i) the use of a preliminary separation of the N cheese components according to the molecular size by diafiltration through membranes with cut-off of 3 and 10 kDa; ii) the further preparative fractionation by HPLC or FPLC to obtain single pure components or a mixture of components; and iii) the identification of peptides by mass spectrometry. In this work, oligopeptides with a molecular mass lower than 3 kDa in 14- and 38-month-old GP cheese samples have been characterized with a few HMM-peptides from the 14-month-old sample, by using a combination of fast atom bombardment (FAB)/ or electrospray(ES)/mass spectrometry (MS) and Edman degradation.

MATERIALS AND METHODS

Cheese samples and fractionation of cheese nitrogen

Two samples of Grana Padano cheese (GP) after 14 and 38 months of ripening supplied by the 'Consorzio di Tutela del formaggio Grana Padano' (Milan, Italy) were used in this study. Grated cheese was freeze-dried and extracted with diethyl ether in a Soxhlet apparatus. Cheese powder (1 g) was suspended in 20 mL 0.2 mol/L trisodium citrate buffer (pH 8), then kept in an ultrasonic bath (Mod T 460, Carlo Erba Reagenti, Milan) for 15 min, then shaken for an additional 30 min and left to stand 1 h at 4 °C. After centrifugation at room temperature for 20 min at 4000 *g*, the fat layer was carefully removed with a spatula. The resulting soluble N cheese fraction was filtered on Whatman paper 3 MM and then ultrafiltered using 3- and 10-kDa cut-off membranes (Centripep, Amicon Division, Beverly, MA, USA). Each retentate was diluted with 20 mL citrate solution and ultrafiltered again. The operation was repeated three times giving rise, after pooling, to three fractions containing peptides with a molecular mass lower than 3 kDa, between 3 and 10 kDa and higher than 10 kDa. Each fraction was concentrated three times in a centrifuge operating under vacuum (SpeedVac, Concentrator SVC 100H, SVPT srl, Rome, Italy) and stored at -20 °C until further analysis.

Reverse phase HPLC of peptides with a molecular mass lower than 3 kDa

The permeate containing peptides with a molecular mass lower than 3 kDa was fractionated by reverse phase (RP)-HPLC on a Kontron equipment (Kontron Instruments, Milan, Italy) consisting of two Model 420 pumps, a Rheodyne sample injector (250 µL loop) and a Model 491 solvent programmer. Peptide detection was carried out at 220 nm using a Kontron variable-wavelength detector (Model 430). Reverse phase columns µ-Bondapak C18, 250 × 4.6 mm and 250 × 10 mm (Waters Associates, Milford, MA, USA) were used for analytical and semi-preparative purposes. Solvent A was 0.1% trifluoroacetic acid (TFA) in water. Solvent B was 0.1% TFA in acetonitrile. The flow was of 1 mL/min. The gradient was: t=0 min, %B 0; t=5 min %B, 0; t=34 min, %B 29; t=35 min, %B 38; t= 43 min, %B 48. 250 mL-volumes were injected into the column and the peptide fractions corresponding to each chromatographic peak collected, pooled and dried by centrifugation under vacuum. The operation was repeated ten times to obtain enough peptide material to characterize peptides by FAB/MS.

Identification of peptides by fast atom bombardment mass spectrometry (FAB/MS)

FAB mass spectra were recorded on a VG Analytical ZAB 2 SE double-focusing mass spectrometer (VG Instruments, Manchester, UK) fitted with a VG cesium gun operating at 25 keV (2 µA) in the mass range 300–3000 Da. The spectra were recorded on UV direct print paper and counted manually. The dried HPLC fractions were dissolved in 1 mol/L-HCl and loaded onto a glycerol-thioglycerol-coated probe tip. The peptide components were identified on the basis of the molecular mass value and the N-terminal sequence as described previously (Addeo et al, 1992), taking into account the amino acid sequences of α_{s1}-, β-, and α_{s2}-casein reported by Swaisgood (1993) taking into account the corrections made by different authors.

Chromatographic fractionation of peptides with molecular mass above 10 kDa

The fraction containing the oligopeptides with a molecular mass higher than 10 kDa was further fractionated by a fast protein liquid chromatography on a Mono Q (HR 10/10, Pharmacia, Uppsala, Sweden) anion exchange column equilibrated in the 20 mmol/L monosodium phosphate, pH 7.0 buffer and operating at room temperature at 1.0 mL/min. A linear gradient from 0–0.32 mol/L NaCl was used. Fractions corresponding to each peak were pooled, dialyzed against distilled water and freeze dried. Single fractions were further separated by HPLC on a C4 column according to the procedure of Ferranti et al (1995).

Identification of peptides by electrospray mass spectrometry (ES/MS)

The identity of the single peptides was established by a combination of electrospray (ES)-mass spectrometry (MS) and Edman degradation. In brief, molecular masses were assigned to the corresponding peptides along the sequence of the four casein fractions by using a suitable computer program developed in our laboratory (F Barone, unpublished data). The assignments were confirmed by submitting the peptide fractions to at least two steps of manual Edman degradation, followed by ES/MS analysis of the truncated species, in order to obtain the necessary N-terminal sequence information.

RESULTS

HPLC of Grana Padano low molecular mass peptides

A fraction containing soluble low molecular mass N forms was isolated by diafiltration through a 3-kDa cut-off membrane starting from a citrate buffer solution of freeze-dried and defatted GP cheese. The RP-HPLC profiles of the 14- and 38-month-old cheese samples are shown in figure 1. They were

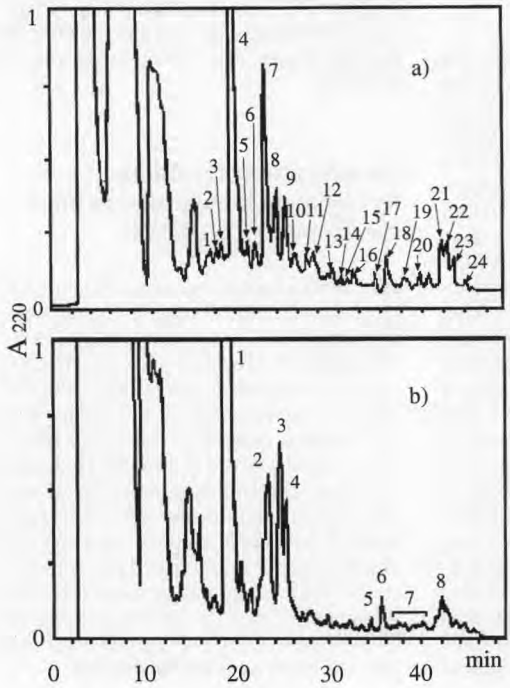

Fig 1. Reversed-phase HPLC profile of oligopeptides with molecular mass lower than 3 kDa of 14- (**a**) and 38-month-old (**b**) Grana Padano cheese, showing the peak or group of peaks collected and identified.

*Profil CLHP en phase inverse des peptides de masse moléculaire inférieure à 2 kDa issus de Grana Padano de 14 (**a**) et 38 mois (**b**), montrant les pics, ou groupes de pics, collectés et identifiés.*

similar to those of Parmigiano-Reggiano (PR) cheese (Addeo et al, 1992; Fox and McSweeney, 1994), also belonging to the same class of AOC hard cheese. The group of peaks eluted in the final part of the chromatogram was quantitatively more important in the cheese of lower age. The two samples of 14- and 38-month-old GP cheese were examined in detail for structural characterization.

The nature of oligopeptides in 14-month-old Grana Padano cheese

About 30 peaks were collected from the fractionation by HPLC of the 14-month-old cheese sample, as shown in figure 1a. After drying, peptides were subjected to molecular mass determination by FAB/MS. By using a suitable software program for the identification of peptides through the molecular mass, many possible peptides were found for each experimental mass value, as shown in table I, concerning the peak *n* 9 of the HPLC separation shown in figure 1a. The results indicated that the molecular mass determination alone did not allow peptides singly or in mixture be unambiguously identified. Therefore, in order to improve the procedure of identification, additional structural information was searched through the N-terminal residue determination; however, a mixture of N-terminal residues was obtained owing to the presence of several peptides in the mixture. This made the automated sequencing of peptides in mixture ineffective for the peptide identification. This problem was overcome through the determination of the molecular mass by FAB/MS on the truncated peptides in mixture after each consecutive Edman degradation step. However, the determination of the N-terminal residue only reduced the number of the possible peptides among those having similar molecular mass, as reported in table I. A reliable identification of the peptide components in mixture was obtained through the determination of two or three N-terminal residues in addition to the molecular mass of the native peptide. Following this procedure, the oligopeptides having

Table I. Identification steps by FAB/MS and Edman degradation of the peptides occurring in the HPLC peak number 9 relative to 14-month-old Grana Padano cheese.
Étapes d'identification par FAB/MS et dégradation d'Edman des peptides apparaissant dans le pic 9 issus d'un Grana Padano de 14 mois.

Molecular mass of single peptides, m/z	*Number of casein peptides having similar mol mass* [a]	*Molecular mass after a first Edman cycle* [b]	*Number of peptides having similar mol mass and N-terminal residue*	*Molecular mass after the second Edman cycle* [a] *Number of peptides having same*	*mol mass and two N-terminal residues Identity of peptides*	
662	10	548 (-Asn)	1	435 (-Leu)	1	α_{s1}(19–23)
758	12	644 (-Asn)	2	515 (-Glu)	1	α_{s1}(17–22)
879	19	792 (-Ser)	2	764 (-Tyr)	1	κ(37–43)
979	21	850 (-Glu)	2	737 (-Leu)	1	β(44–51)
1054	13	955 (-Val)	2	841 (-Asn)	1	α_{s1}(37–45) 1P
1059	19	972 (-Ser)	3	901 (-Ala)	1	α_{s1}(115–123)

[a] Identification by a computer program; [b] the molecular mass difference corresponds to the amino acid residue in parenthesis.

[a] *Identification effectuée par un programme informatique ;* [b] *la différence de masse moléculaire correspond au résidu d'acide aminé entre parenthèses.*

Table II. Oligopeptide components from a 14-month-old Grana Padano cheese sample found in the fractions separated by HPLC on a C18 column (fig 1a).
Peptides provenant d'un échantillon de Grana Padano de 14 mois, identifiés dans les fractions obtenues en CLHP sur colonne C18 (fig 1a).

HPLC peak	*MH⁺*	*MH⁺ after Edman cycle*			*N-terminal sequence*	*Peptide*
		I	*II*	*III*		
1	746	689	590		Gly-Val	β(94–100)
2	1048	881	782		SerP-Val	α_{s1}(75–82)1P
3	921	754	683		SerP-Ala	α_{s1}(115–121)1P
	609	494	423		Asp-Ala	α_{s1}(157–162)
4	659	546	489		Ile-Gly	α_{s1}(136–141)
	609	494	366		Asp-Lys	**β(47–51)**
	589	460	361		Glu-Val	**α_{s2}(68–72)**
5	593	464	349		Glu-Asp	α_{s1}(50–54)
6	920	757	700		Tyr-Leu	κ(38–47)
	763	634	519		Glu-Asp	**α_{s1}(55–60)**
	761	632	519		Glu-Leu	**α_{s1}(39–45)**
	720	720	720		— -	**β(46–51)**
7	850	737	609		Leu-Gln	β(45–51)
	841	712	599		Glu-Leu	**α_{s1}(39–45)1P**
8	955	840	712		Asp-Lys	β(47–54)
	758	644	515		Asn-Glu	α_{s1}(17–22)
	698	627	528		Ala-Val	α_{s2}(116–122)
9	1059	972	901		Ser-Ala	α_{s1}(115–123)
	1054	955	841		Val-Asn	α_{s1}(37–45)1P
	979	850(985)	737(872)		Glu-Leu	**β(44–51)**
	879	792(927)	764		Ser-Tyr	κ(37–43)
	758	644	515		Asn-Glu	α_{s1}(17–22)
	662	548	435		Asn-Leu	α_{s1}(19–23)
10	1015	887(1022)	759		Gln-Lys	α_{s1}(131–139)
	856	742	643		Asn-Val	**β(7–14)**
11	856	742	643		Asn-Val	**β(7–14)**
	786	623(758)	645		Tyr-Leu	α_{s2}(179–184)
	632	503	390		Glu-Leu	β(2–6)
	584	456	357		Lys-Val	α_{s1}(105–109)
12	975	874(1009)	746		Thr-Lys	**α_{s2}(198–205)**
	937	836	708		Thr-Gln	β(78–86)
	788	632	503		Arg-Glu	**β(1–6)**
	746	659	602		Ser-Gly	α_{s1}(161–166)
13	869	812	699		Gln-Leu	α_{s1}(10–17)
	831	703	575		Gln-Lys	α_{s2}(172–178)
	703	575	428		Lys-Phe	**α_{s2}(173–178)**
	520	423	295		Pro-Gln	α_{s2}(177–180)/
14	1150	994(1129)	831(966)		Arg-Tyr	α_{s2}(170–178)
	933	862(997)	749(884)		Ala-Leu	α_{s2}(175–182)
15	1126	997(1132)	884(1019)		Glu-Leu	**β(44–52)**
	1020	883	755		His-Gln	α_{s1}(8–16)
	998	941	828		Gly-Leu	α_{s1}(10–18)
	965	852	755		Ile-Pro	β(74–82)
16	1227	1098	969		Glu-Glu	β(4–14)
	1126	997(1132)	884(1019)		Glu-Leu	β(44–52)

	1099	970	871		Glu-Val	α_{s1}(14–22)
	1079	965	852		Asn-Leu	β(73–82)
	755	698	585		Gly-Leu	**α_{s1}(10–16)**
17	1427	1299(1434)	1136(1271)		Lys-Tyr	**α_{s1}(103–114)**
	1162	1033(1168)	904(1039)		Glu-Glu	α_{s2}(156–164)
	913	784	628		Glu-Arg	α_{s1}(89–95)
	865	778	691		Ser-Ser	β(17–22)
	806	659	560		Phe-Val	**α_{s1}(24–30)**
	771	642	514		Glu-Gln	α_{s1}(96–101)
18	1810	1681	1610		Glu-Ala	α_{s1}(61–74)4P
	905	791	662		Asn-Glu	**α_{s1}(17–23)**
	791	662	548		Glu-Asn	**α_{s1}(18–23)**
19	1340	1253	1140		Ser-Leu	**β(57–68)**
	1251	1150(1285)	1022		Thr-Lys	**α_{s2}(198–207)**
	1219	1120	1049		Val-Ala	**α_{s1}(25–35)**
	1090	991(1126)	920		Val-Ala	**α_{s1}(25–34)**
	983	905	791	662	Asn-Glu	α_{s1}(17–23)
	735	622	523		Leu-Val	α_{s1}(58–63)
20	1765	1678	1565		Ser-Leu	β(57–72)
	1678	1565	1466		Leu-Val	**β(58–72)**
	1494	1423	1292		Ala-Met	α_{s1}(53–65)
	1283	1154	998		Glu-Arg	α_{s1}(89–98)
	1117	1018	905		Val-Leu	α_{s1}(15–23)
	1022	923	810		Val-Ile	**α_{s2}(200–207)**
21	2186	2099	1986	1887	Ser-Leu–Val	**β(57–76)**
	2099	1986	1887	1724	Leu–Val-Tyr	β(58–76)
	1949	1802(1937)	1838	1767	Phe-Val–Ala	α_{s1}(24–40)
	1725	1638	1525	1426	Ser-Leu-Val	β(57–72,Pro^{67})
	1551	1450	1322	1221	Thr-Gln-Thr	β(78–91)
	1366	1219(1354)	1120	1049	Phe-Val-Ala	α_{s1}(24–35)
	1237	1090(1225)	991	920	Phe-Val-Ala	**α_{s1}(24–34)**
	905	806	735	638	Val-Ala-Pro	α_{s1}(25–32)
	770	671	572	473	Val-Val-Val	β(82–88)
22	1854	1707	1560		Phe-Phe	α_{s1}(23–38)
	1641	1494	1347		Phe-Phe	α_{s1}(23–36)
	1052	905	806		Phe-Val	**α_{s1}(24–32)**
	809	695	582		Asn-Leu	α_{s1}(19–24)
23	2510	2423	2310		Ser-Leu	β(69–91)
	2299	2212	2099		Ser-Leu	β(57–77)
	2043	1956	1843		Ser-Leu	β(69–87)
	1880	1717	1589		Tyr-Gln	**β(193–209)**
	1781	1680	1552		Thr-Gln	β(78–93)
	1717	1589	1460		Gln-Glu	β(194–209)
	1641	1584	1471		Gly-Leu	**α_{s1}(10–23)**
	1522	1423	1267		Glu-Arg	α_{s1}(89–100)
	1396	1267	1111		Glu-Arg	**α_{s1}(89–99)**
	1052	905	806		Phe-Val	α_{s1}(24–32)
24	2740	2653	2540		Ser-Leu	**β(69–93)**
	2316	2201			Asp-	κ(20–28)
	1700	1700			Gln-Glu	β(194–209)*

*Gln^{194} cyclyzed to pyroglutamic acid. Figures of MH^+ in parentheses represent a 135-molecular mass increase due to the phenylisothiocyanate coupling with the ε-NH_2 group of each lysine residue.

**Gln*^{194} *cyclisée en acide pyroglutamique. Les valeurs de* MH^+ *entre parenthèses représentent une augmentation de 135 unités de masse, due à la fixation de phenylisothiocyanate sur les groupement* ε-NH_2 *de chaque résidu lysine.*

molecular mass lower than 3 kDa were identified in the 14-month-old GP cheese sample as shown in table II. It was not possible to record mass values for the material recovered from HPLC separation before peak 1 (fig 1a).

Thirty-two peptides originating from β-, 44 from α_{s1}-, 13 from α_{s2}-CN, and three from para-κ-CN, were readily identified by using this procedure.

Peptides in 38-month-old Grana Padano cheese

The HPLC peaks of the 38-month-old cheese sample (fig 1b) were collected single or in groups and the relative peptide components identified by the same analytical procedure indicated above. In table III the list of the peptide components occurring in the 38-month-old GP cheese sample is reported.

Eight peptides were from β-, twenty-seven from α_{s1}-, one from α_{s2}-, and two from para-κ-CN. Some peptide components were common to both the younger and the older cheese. The 38-month-old GP cheese sample contained 15 α_{s1}- , five β- novel peptides, and one κ-CN-novel peptide. It must be underlined that the number of peptides of the aged cheese was apparently lower than that of the younger cheese, and that the peptides from the older cheese were generally shorter forms of the components detected in the younger cheese.

In the 38-month-old cheese sample only one precursor of the β-CM7 was detected, the β-CN(f60-72) component, which means that no one of the seven bioactive-precursor peptides containing the sequence 60–66 detected in the 14-month-old cheese resisted to the cheese enzymes. Therefore, one might consider that peptide 60–72 is the terminal product of all the β-CM precursors of the 14-month-old cheese.

Seven closely-related α_{s1}-CN-CPPs differing either for the N-terminal extension 63/64/65/67–74 or the level of phosphorylation eg 4P to 1P were found (table III).

Together with (61–74)4P and (62–74)4P peptides, the same components were found in a CPP fraction specifically isolated from the pH 4.6 soluble fraction of the 14-month-old cheese sample (P Ferranti, personal communication). However, the mixture lacked the less phosphorylated (65–74)3P peptide which was found only in the 38-month-old cheese sample. N-terminal Ser^{115} was found either phosphorylated or unphosphorylated.

Identification of peptides with molecular mass above 10 kDa in the 14-month-old Grana Padano cheese

A preparative fractionation of the peptides having a molecular mass above 10 kDa carried out by chromatography on MonoQ column showed the profile which in figure 2 is similar to that previously obtained for a 24-month PR cheese sample (Addeo et al, 1995). Here also, the protein material eluted under each chromatographic peak was a mixture of components. As example, the last peak eluted from the MonoQ column analyzed by gel electrophoresis at alkaline pH presented three main bands (fig 3), α_{s1}-CN being the most important whereas α_{s1}-I-CN and unidentified components in lower amounts. In order to further fractionate this material, a HPLC chromatography was carried out on a C4 column, as shown in figure 4. Thirteen peaks were collected which were submitted to analysis by ES/MS. Since each peak was constituted by a mixture of components, a combination of ES/MS for the molecular mass determination and manual Edman degradation for the N-terminal sequence determination was used for the identification of the HMM-peptides. The determination of at least two N-terminal residues together with the molecular mass of the native peptides was necessary to unambiguously identify HMM-peptides in mix-

Table III. Oligopeptide components from a 38-month-old Grana Padano cheese found in the fractions separated by HPLC (fig 1b).
Peptides provenant de la fraction de masse moléculaire inférieure à 3 kDa d'un Grana Padano de 38 mois identifiés dans les fractions obtenues en CLHP (fig 1b).

HPLC peak	*MH⁺*	*MH⁺ after Edman cycle*			*N-terminal sequence*	*Peptide*
		I	*II*	*III*		
1	921	754	683		SerP-Ala	α_{s1}(115–121)1P
	841	712	599		Glu-Leu	**α_{s1}(39–45)1P**
	589	460	361		Glu-Val	**α_{s2}(68–72)**
2	720	720	720		no residue	**β(46-51)**
	761	632	519		Glu-Leu	**α_{s1}(39–45)**
	763	634	519		Glu-Asp	**α_{s1}(55–60)**
	954	867	700		Ser-SerP	α_{s1}(64–74)1P
	1016	917(1052)	939		Val-Leu	κ(78–87)
	1034	867	700		SerP-SerP	α_{s1}(67–74)2P
3	731	602	474		Glu-Lys	α_{s1}(35–40)
4	1610	1481	1314		Glu-SerP	α_{s1}(63–74)4P
	1481	1314	1201		SerP-Ile	α_{s1}(64–74)4P
	1314	1201	1034		Ile-SerP	α_{s1}(65–74)3P
	1244	1187	1088(1223)		Gly-Val	β(94–105)
	1187	1100	1029		Ser-Ala	α_{s1}(115–124)
5	758	644	515		Asn-Glu	α_{s1}(17–22)
	995	908(1043)	780		Ser-Lys	β(168–176)
	1139	972	901		SerP-Ala	α_{s1}(115–123)1P
	1401	1314	1201		Ser-Ile	α_{s1}(64–74)3P
	1530	1401	1314		Glu-Ser	α_{s1}(63–74)3P
6	1427	1299(1434)	1136		Lys-Tyr	**α_{s1}(103–114)**
	806	659	560		Phe-Val	**α_{s1}(24–30)**
7	1466	1303	1206		Tyr-Pro	β(60–72)
	1299	1136(1271)	1008		Tyr-Lys	α_{s1}(104–114)
	905	791	662		Asn-Glu	**α_{s1}(17–23)**
	791	662	558		Glu-Asn	**α_{s1}(18–23)**
	489	376	279		Leu-Pro	κ(56–59)
8	1022	923	810		Val-Ile	α_{s2}(200–207)
	1117	1018	905		Val-Leu	α_{s1}(15–23)
	1237	1090(1225)	991	920	Phe-Val-Ala	**α_{s1}(24–34)**
	1246	1117	1018		Glu-Val	α_{s1}(14–23)
	1366	1219(1354)	1120	1049	Phe-Val–Ala	α_{s1}(24–35)
	1322	1221	1124		Thr-Pro	β(80–91)
	1494	1347	1248		Phe-Val	α_{s1}(24–36)
	1707	1560	1461		Phe-Val	α_{s1}(24–38)
	1396	1267	1111		Glu-Arg	α_{s1}(89–99)
	1641	1584	1471		Gly-Leu	**α_{s1}(10–23)**
	1717	1589	1460		Gln-Glu	β(194–209)
	1680	1552	1451		Gln-Thr	β(79–93)
	1880	1717	1589		Tyr-Gln	**β(193–209)**
	1552	1423	1267		Glu-Arg	α_{s1}(89–100)

Figures of MH^+ in parenthesis represent a 135-molecular mass increase due to the phenylisothiocyanate coupling with the ε-NH_2 group of each lysine residue of peptides.

Les valeurs de MH^+ entre parenthèses représentent une augmentation de 135 unités de masse, due à la fixation de phenylisothiocyanate sur les groupements ε-NH_2 de chaque résidu lysine des peptides.

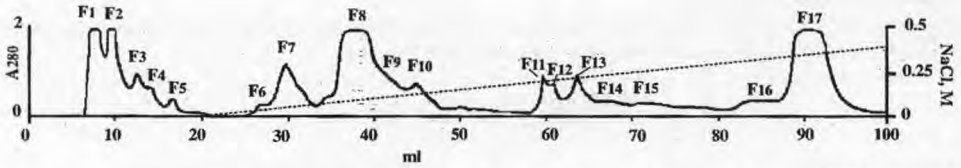

Fig 2. Chromatographic separation by FPLC on Mono Q column of the retentate on 10 kDa cut-off membrane (100 mg) of 14-month-old Grana Padano cheese using a linear gradient of 0–0.32 mol/L NaCl. Fractions corresponding to each peak were pooled, dialysed against distilled water and freeze dried. Peak 17 was used both for gel electrophoretic analysis and fractionation by HPLC.

Séparation chromatographique des peptides de masse moléculaire supérieure à 10 kDa (100 mg) issus d'un Grana Padano de 14 mois, sur colonne Mono Q avec un gradient linéaire de 3 à 0,32 mol/L de NaCl. Les fractions correspondant à chaque pic ont été rassemblées, dialysées contre de l'eau distillée et lyophilisées. Le pic 17 a été utilisé pour l'électrophorèse et le fractionnement par CLHP.

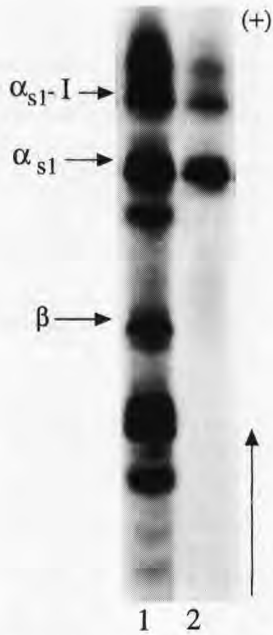

Fig 3. Polyacrylamide gel disc-electrophoresis at pH 8.6 of the retentate on a 10-kDa cut-off membrane (fraction B) from a 14-month-old Grana Padano cheese (lane 1) and the most retained protein components on the MonoQ column (lane 2).

Électrophorèse en gel de polyacrylamide à pH 8,6 des peptides de masse moléculaire supérieure à 10 kDa (fraction B) issus d'un Grana Padano de 14 mois (puits 1) et de la fraction protéique la plus retenue sur colonne MonoQ (puits 2).

ture. The peptides identified in the seven HPLC peaks (7 to 13) are reported (table IV).

The high number of components identified means that neither electrophoresis nor HPLC was able to reveal the complexity of the casein composition as ES/MS did. A number of protein species was found consisting of either intact α_{s1}-, α_{s2}-, and β-CN and of derived peptides. Native α_{s1}-CN B genetic variant was found to be a more complex mixture of differently phosphorylated protein chains containing 5 to 9P instead of just two phosphorylation levels, eg, 8 and 9P, as reported in literature (Swaisgood, 1993). Only the α_{s1}-CN C-8P form was detected in HPLC fraction 9, although other differently phosphorylated forms can occur in other, not yet examined chromatographic fractions. Similarly, two α_{s2}-CN(1–207)8P and 9P were detected among the α_{s2}-CN components never reported to occur in bovine casein. We do not know if they were native or new-formed partially dephosphorylated components. Surprisingly, the occurrence of α_{s1}-CN-derived peptides 24–199 and 25–199, at different levels of phosphorylation, derived from both the α_{s1}-CN B and C variants, confirms the susceptibility of the 23–24 and 24–25 bonds. Both the 1–22 and 1–23 peptides were identified either in the GP cheese whey or the in vitro hydrolyzate of α_{s1}-CN by rennet (Chianese et al,

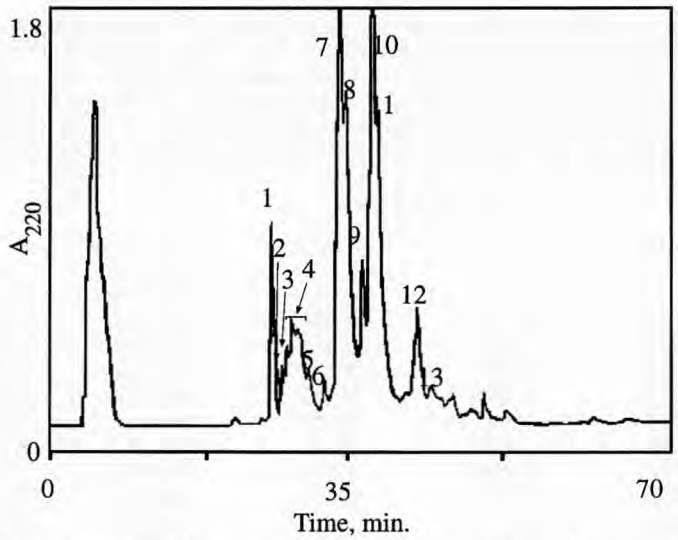

Fig 4. Reversed-phase HPLC profile of the most retained fraction on MonoQ column (lane 2, fig 3) isolated from the fraction B containing peptides with a molecular mass higher than 10 kDa. C4 column; 5 mg of peptides injected. Solvent A was 1 mL trifluoracetic acid/L distilled water; solvent B, 1 mL trifluoroacetic acid/L acetonitrile. The gradient was: t=0, %B, 35; t=10 %B, 35; t=80, %B, 100; t=90, %B, 100; t= 90.1, %B, 35; t=100, %B, 35. The flow was maintained at 2 mL/min and absorbance of eluate was followed at 220 nm.

Profil CLHP en phase inverse de la fraction protéique la plus retenue sur colonne MonoQ cellulose (puits 2, fig 3) issus de la fraction B, qui contient les peptides de masse moléculaire supérieure à 10 kDa. (Colonne C4 ; 5 mg de peptides injectés. Tampon A : acide trifluoroacétique de 1 mL/L d'eau distillée ; tampon B : acide trifluoroacétique à 1 mL/L d'acétonitrile. Gradient : t = 0,35 % B ; t = 10,35 % B ; t = 80, 100 % B ; t = 90, 100 % B ; t = 90,1, 35 % B ; t = 100, 35 % B. Débit maintenu à 2 mL/min et absorption à 220 nm).

1997). One may hypothesize that the fragment 24–199 α_{s1}-CN chymosin-derived and that 25–199 was the product with the N-terminal residue-depleted by a cheese aminopeptidase. The fragment 1–143 also seems to come from the hydrolysis of α_{s1}-CN by chymosin although neither Pélissier et al (1974) nor McSweeney et al (1993) reported the cleavage of the 142–143 bond by chymosin in solution. Similarly, the four closely-related β-CN peptides (f7/8/9/11–209) corresponded either to site cleavages not previously reported to be derived from endoproteases at known specificity or to intermediate transient peptides produced by cheese aminopeptidases by action on a parent HMM-peptide.

DISCUSSION

An efficacious fractionation process and an advanced strategy of identification

Singh et al (1995) pointed out that diafiltration gave very reproducible results on the N form separation and believed that no artifacts were formed during the fractionation procedure, given both the short time needed for the operation and the low temperature at which ultrafiltration occurs in the presence of preservative agents. Preliminary assays on three consecutively diafiltered fractions by determining identity of the HPLC peak components through FAB/MS,

Table IV. Oligopeptides identified under the HPLC peaks 7–13, as shown in figure 4, occurred in the most retained fraction on Mono Q column (lane 2, fig 3) from fraction B containing peptides at a molecular mass higher than 10 kDa of a 14-month-old Grana Padano cheese sample.
Peptides identifiés dans les pics CLHP 7 à 13 (fig 4) issus de la fraction la plus retenue sur la colonne Mono Q (puits 2, fig 3) après injection de la fraction B, qui contient les peptides de masse moléculaire supérieure à 10 kDa, d'un échantillon de Grana Padano de 14 mois.

HPLC peak	*MH⁺*	*MH⁺ after Edman*		*N-terminal sequence*	*Peptide*
		Cycle I	*Cycle II*		
7	20880	20735	20662	Phe-Val	α_{s1}C(24–199)8P
	20787	20635	20535	Phe-Val	α_{s1}B(24–199)7P
	20733	20634	20563	Val-Ala	α_{s1}C(25–199)8P
	17213	17059	16960	Arg-Pro	α_{s1}(1–143)8P
	17059	16965	16832	Pro-Lys	α_{s1}(2–143)8P
8	20732	20633	20561	Val-Ala	α_{s1}B(25–199)8P
	20782	20638	20536	Phe-Val	α_{s1}B(24–199)7P
	20876	20729	20657	Phe-Val	α_{s1}C(24–199)8P
9	23685	nd		Arg-Pro	α_{s1}C8P
10	23611	23451	23362	Arg-Pro	α_{s1}B(1–199)8P
	23531	23378	23281	Arg-Pro	α_{s1}B(1–199)7P
11	23611	23458	23361	Arg-Pro	α_{s1}B(1–199)8P
	23531	23375	23278	Arg-Pro	α_{s1}B(1–199)7P
12	25100	nd			α_{s2}9P
	25020	nd			α_{s2}8P
	22438	nd		Arg-Pro	α_{s1}B6P
	22358	nd		Arg-Pro	α_{s1}B5P
	22845	nd			βA^2(11–207)
	23116	nd			βA^2(7–207)
13	23989	nd		Arg-Glu	$\beta A^2$5P
	24574	nd			α_{s2}(9–209)
	24744	nd			α_{s2}(8–209)

nd, not determined.
nd, non déterminé.

did not show any processing of our peptides. However, as pointed out by Singh et al (1995), hydrophilic peptides can accumulate in the permeate as the polysulphone membranes tend to reject hydrophobic peptides. Such a mechanism was actually observed by us during diafiltration of in vitro hydrolysate of whole casein by rennet. Peptides such as α_{s1} (f1–22) and α_{s1}(f1–23) having both a nominal molecular mass below 3000 kDa, eg, 2617 and 2764, respectively were found to concentrate in the retentate (Chianese et al, 1997). This means that these hydrophobic oligopeptides would accumulate in the fraction containing components at molecular mass in the intermediate range between 3 and 10 kDa, not analyzed within this work. Concerning the strategy of peptide identification, the use of FAB/MS and ES/MS has allowed us to directly analyze peptides, without any necessary derivatization reaction, which represents an advance in comparison with the old time-consuming procedures requiring a preliminary derivatization step. However, the molecular mass determination alone was insufficient to allow the identification of peptides. For this, it was necessary to submit

the peptide mixture to a manual Edman degradation step followed by FAB/ or ES/MS re-examination of the truncated peptide mixture. The mass signals shifted according to the mass value of the N-terminal residue and this procedure repeated once or twice allowed to identify unambiguously the peptides. Since a partial suppression of the signals taking place in FAB analysis (Naylor et al, 1986), having as upper detection limit about 3000 Da, ES/MS was used, especially for large peptides and proteins. These techniques were of utmost importance for identifying cheese protein and peptides characterized by a microheterogeneity such as the discrete phosphorylation and the simultaneous presence of closely related differently long peptides.

The mechanisms of casein degradation in Grana Padano cheese

The proposed β-CN derived peptides identified in the 14-month-old GP cheese sample, originated mainly from the N-terminal half protein, perhaps from the soluble peptides 1–105/107 (the protease-peptones complementary to γ^2- and γ^3-CN). There were five components coming from the β-CN(f1–28)4P-plasmin mediated peptide, 23 from the peptide region between 44 and 100 residues and two from the C-terminal region of β-CN as a consequence of the peptide bond cleavage between residues 192/193 and 193/194. These last two are cleavage sites of the *Lactococcus* cell envelope proteinase (Singh et al, 1995). The formation of soluble peptides from the N-terminal region of β-CN is consistent with our previous results showing an increase of γ-CN during GP ripening (Addeo et al, 1996). This increase is linked to a parallel increase of the soluble proteose-peptones such as 1–28, 1–105, 1–107, 29–105, and 29–107 from the plasmin action on β-CN (Andrews, 1978a, b).

Two peptides, differing only for the amino acid substitution His^{67} –>Pro^{67} were detected, which indicated that cheese milk consisted of a mixture of β-CN variants such as A^1 , B, and C and A^2 and A^3, having His^{67} and Pro^{67} as marker-residues respectively. The amino acid sequence 60–66 corresponding to the β-casomorphin 7(β-CM7) (Brantl, 1979) was found in eight peptides which represent actual precursors of bioactive peptides. No peptide containing residues between 23 and 43 was isolated from the 14-month-old cheese. This means that, if formed, these peptides were degraded by the cheese exopeptidases into free amino acids within the first months of ripening. An aminopeptidase activity was clearly deduced from the simultaneous occurrence in the cheese of four peptides in addition to the parent β-CN(44–52), having one to three N-terminal amino acid residues less. Similarly, pairs of peptides such as 1–6 and 2–6, 58–77 and 57–77, 58–76 and 57–76 are to be considered also the degradation result of an active aminopeptidase which removes the N-terminal residues, one by one, from the parent peptides. The pair of peptides 58–77 and 58–76, 44–52 and 44–51 also seems indicative of a carboxypeptidase activity in cheese which is known to remove one by one the C-terminal residues of peptides. Finally, the two pairs of peptides 78–93 and 78–91 and 69–93 and 69–91, were indicative of a possible oligoendopeptidase activity converting parent peptides into two-residue shortened at the C-terminal end.

Among the 44 α_{s1}-CN-derived peptides of the 14-month-old GP cheese sample, 30 were from the 1–79 region, and the others from the peptide bond cleavage within the residues 89 and 166. No peptide started with a residue beyond this site, meaning that the α_{s1}-CN(f1–79) component, formerly named α_{s1}-PL1, which originated in cheese by the plasmin action on α_{s1}-CN (Addeo et al, 1995), could be the parent peptide. From this soluble peptide, by action of cheese

endopeptidases, a number of shorter peptides could take origin further processed by cheese exopeptidases. This was also verified by examining the 10–16/17/18 and 19–23/24 and 15/17/18–23, and 17–22 and 14–22, which differed for one or more N- and/or C-terminal amino acid residues. An additional set of peptides consisting of the components 24–40, 24–38, 24–36, 24–35, 25–35 and 24–34 and 25–34 and 24–32 and 25–32 and 24–30 is also a complex mixture of peptides formed from an unknown parent peptide which was degraded by cheese endopeptidases and exopeptidases into a number of individual components differing by one or more amino acid residues.

Of the 13 α_{s2}-CN peptides identified in the 14-month-old GP cheese sample, ten originated from the C-terminal region. The components constituting the peptide set 170/172/173–178, 175–182, 177–180, and 179–184 were closely-related to each other, indicating a common origin from a parent soluble peptide. However, the peptides 198–205, 198–207 and 200–207 could originate from the chymosin activity on α_{s2}-CN since both the components α_{s2}(f198–207) and α_{s2}(f200–207) were generated, among other peptides, during the in vitro action of rennet on whole casein (Chianese et al, 1997). Only three peptides were isolated from the para-κ-casein part, indicating that this peptide was also sensitive to protease attack even if to a lesser extent than the other casein fractions.

Although only two samples of different ages were analyzed in detail, the changes occurring in the oligopeptide fraction of the 14– and 38-month-old cheese samples gave some precise indications on the production of peptides. The α_{s1}-CN-derived peptides of the permeate containing peptides molecular mass with than 3 kDa dominated, both in young and old GP cheese, over those derived from β-CN. This was consistent with the results of previous studies concerning the degradation of β- and α_{s1}-casein which continued throughout the ripening process (Addeo et al, 1996) by action of plasmin mainly. After about 12 months, β-casein was entirely hydrolyzed whereas about 50% initial α_{s1}-casein residue in cheese (Addeo et al, 1996). Thus, the contribution of β- and α_{s1}-casein to form oligopeptides in GP cheese was unequal, the former being more important. Some β-CN-derived peptides might be also more susceptible to exopeptidases than the α_{s1}-CN peptides becoming hydrolyzed in mature cheese. Therefore, a degradation scheme of peptides in GP cheese necessarily includes a concerted action of endopeptidases, and exopeptidases. Soluble peptides are the result of the intervention: of i) endopeptidases such as plasmin and others from bacterial origin; and ii) aminopeptidases, and carboxypeptidases, both removing one by one amino acid residues from the terminal ends of soluble peptides. Important enzyme activities are those associated with the cell-envelope or the cytoplasm of *L helveticus* species dominating in GP cheese. These enzymes seem to produce a casein hydrolysate containing flavor-significant oligopeptides from which free amino acids, particularly glutamate, the most represented in GP cheese, giving a brothy taste to mature GP cheese, are formed.

ACKNOWLEDGMENT

This work was supported by the Italian National Research Council (CNR), RAISA special project, sub-project No 4.

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