Extraction, Semi-Quantification, and Fast On-line Identification of Oligopeptides in Grana Padano Cheese by HPLC−MS

STEFANO SFORZA, LINDA FERRONI, GIANNI GALAVERNA,
ARNALDO DOSSENA, AND ROSANGELA MARCHELLI*

Dipartimento di Chimica Organica e Industriale, Università di Parma, Parco Area delle Scienze 17/a,
I-43100, Parma, Italy

Sixteen samples of Grana Padano cheese aged from 2 to 33 months were analyzed by HPLC−MS. The extraction process involved the use of diluted HCl, thus avoiding a strong deproteinizing agent (TCA), and allowing to maintain in solution also very lipophilic peptides. The molecular mass of the most abundant peptides were determined by electrospray ionization (ESI) mass spectrometry. A new method was developed based on the small fragmentation peaks arising from in-source fragmentation and from software analysis of the known casein sequences, which in many cases allowed the direct on-line identification of the oligopeptide sequences. Several new peptides never previously reported were identified, some of which containing bioactive sequences, consistently with what was described in the literature. Semiquantification of peptides at the different stages of aging was also performed by using a suitable internal standard, providing new insights into the evolution of the oligopeptide fraction during aging.

KEYWORDS: Grana Padano cheese; aging process; oligopeptides; HPLC−MS

INTRODUCTION

Grana Padano is an Italian extra-hard cheese, typically produced in the northern part of Italy, made from raw cow milk and matured for at least 8 months. During the aging process, milk curd proteins (namely α_{S1}, α_{S2}, β, and κ caseins) undergo an extensive degradation because of the endoproteases and exoproteases present in raw milk, in the rennet and released by the lactic bacteria present in the natural whey starter (*1*). The nitrogen components of aged cheese consist of a mixture of native caseins, high, medium, and low molecular mass peptides, and free amino acids. Many studies focused on the specificity of plasmin (the main milk protease) (*2*), of chymosin (the main rennet protease) (*3*), and on proteases and peptidases coming from thermophilic lactic bacteria (*4*) *Lactobacillus helveticus* is the dominating species among the thermophilic lactic bacteria in the natural whey starter used for Grana Padano manufacturing (*5*).

In previous papers, Addeo and co-workers used mass spectrometry (MS) to identify in Grana Padano cheese short peptides soluble in 12% trichloroacetic acid (TCA) (*6*), together with phosphopeptides isolated at pH = 4.6 by precipitation with calcium chloride (*7*). In another paper from the same group, the peptide fraction of Grana Padano was more extensively analyzed by suspending the cheese in a citrate buffer at pH = 8.0 and isolating the peptides by subsequent filtrations, including a diafiltration through membranes with a molecular cut-off of

3000 Da. In this way, many oligopeptides with molecular masses ranging from 500 to 2700 Da were identified by Edman degradation and subsequent analysis by fast atom bombardment (FAB) or electrospray ionization (ESI) mass spectrometry (*8*). However, few data concerning the amount of peptides at various stages of aging were reported.

In this paper, we report a HPLC−MS method that allows us to provide a semiquantitative evaluation of the major oligopeptides present in Grana Padano cheese and to correlate their amount with the aging time (from 2 to 33 months). Moreover, we developed a rapid on-line method for the identification of the peptide sequences based on the fragmentation pattern in the mass spectrum of every peptide. By combining these methods (rapid identification and semiquantification), useful insights into the proteolytic processes occurring during aging may be obtained, leading to a better knowledge about the functional features of the end product.

MATERIALS AND METHODS

Materials. Samples of 16 different Grana Padano cheeses (aging time ranging from 2 to 33 months) were obtained from the Istituto Lattiero Caseario (Lodi, Italy). The samples, which came from six different factories, were grated and kept in a closed container under nitrogen at −20 °C until the analyses were performed (usually a few days). All solvents and reagents were HPLC grade and used as commercially available without any further purification.

Sample Preparation. A total of 5 g of finely grated cheese was suspended in 45 mL of 0.1 N HCl. (L,L)-phenylalanylphenylalanine (Phe−Phe) was added as an internal standard (2.25 mL of a 0.1 N solution for samples older than 10 months and 1.25 mL for samples

* Corresponding author. Tel.: ++39-0521-905410. Fax: ++39-0521-905472. E-mail: rosangela.marchelli@unipr.it.

Oligopeptides in Grana Padano Cheese

J. Agric. Food Chem., Vol. 51, No. 8, 2003 **2131**

younger than 10 months). The suspension was homogeneized for 1 min by Ultra Turrax T50 (Janke and Hunkel Italabortechnik) and then centrifuged at 6000 rpm for 30 min at 4 °C by an ALC 4237R centrifuge. The solution was filtered through paper filters (pore dimensions 15−20 μm) and then extracted four times with 70 mL of ethyl ether. The solution was filtered again with a Millipore 46 mm Steril Aseptic system through 0.45 μm HVLP Millipore filters. A total of 2.5 mL of the resulting solution were dried and redissolved in 2.0 mL of a formic acid solution (pH = 3). The solution was diafiltered through Millipore PLGC filters (nominal molecular cut-off 10 000 Da) and then through Amicon Ultrafilters YM3 (nominal molecular cut-off 3000 Da) by using, in both cases, an Amicon Micropartition system MPS-1. The filtrate was dried under nitrogen, redissolved in H_2O (0.2% CH_3CN and 0.1% HCOOH), and analyzed by HPLC.

HPLC−PDA−MS Analysis. The HPLC equipment consisted of a Waters Alliance 2690 separation module connected with a Waters 996 PDA detector and a Micromass ZMD mass spectrometer.

HPLC conditions: Jupiter (Phenomenex) C18 column, 5 μm, 300 Å, 250 × 4.6 mm. Eluent A: H_2O (0.2% CH_3CN and 0.1% HCOOH); eluent B: H_2O/CH_3CN 65:35 (0.1% HCOOH). Elution: 0−15 min isocratic 99% A, 15−60 min linear gradient from 99% A to 10% A, 60−70 min isocratic 10% A. Flow rate: 1 mL/min. PDA conditions: range of 210−300 nm, 1.2 nm resolution, 1 spectrum/s. MS conditions: ESI interface with 90% splitting of the column flow, positive ions, single quadrupole analyzer. Capillary voltage 3500 V, cone voltage 30 V, source temperature 100 °C, desolvation temperature 150 °C, cone gas (N_2) 60 L/h, desolvation gas (N_2) 450 L/h. Acquisition in total ion mode (150−1500 Da), scan time 2.9 s, inter-scan delay 0.1 s.

RESULTS

Sample Preparations. To isolate the oligopeptide fraction, we first applied the method most commonly employed in the literature (*9*), by extracting with water and then with a 12% TCA solution. The fractions thus obtained were analyzed by HPLC−MS. In the TCA soluble fractions, only peaks at low retention time (<45 min) ascribable to small polar peptides (<500 Da) and phosphopeptides (<1000 Da) were detected in the chromatograms, indicating that bigger and/or apolar oligopeptides were precipitated with this method. To maintain in solution higher molecular mass (>1000 Da) and/or more lipophilic oligopeptides, we decided to avoid TCA as a precipitating agent and to suspend the cheese samples in 0.1 N HCl. Being the acidic water extract obtained in this way was very rich in nitrogen compounds, we decided to perform different filtrations and ultrafiltrations to avoid problems related to column clogging. The difference in the oligopeptide extraction efficiency between the TCA and the HCl extraction procedures may be seen in **Figure 1**, which shows two chromatograms obtained from the same sample prepared in either way (PDA detector, 214 nm wavelength).

It is possible to observe that, while the first part of the chromatogram (20−45 min) shows no dramatic changes in peak distribution and intensity, the second part (45−65 min) is definitely richer in peptides for the sample prepared using aqueous HCl. The same result was obtained for all the samples analyzed, thus confirming that the present method allows a more complete analysis of the peptide fraction, particularly as far as the more lipophilic/high molecular mass peptides are concerned.

Sixteen different samples coming from six different factories and aged from 2 to 33 months were then prepared using 0.1 N HCl and by performing filtrations and ultrafiltrations with 10 and 3 kDa cut-off filters to obtain the MW range of our interest (details in Materials and Methods). Every cheese sample was independently prepared twice.

Molecular Mass of Peptides. The peptides traceable in all samples that gave rise to strong signals with both MS and PDA
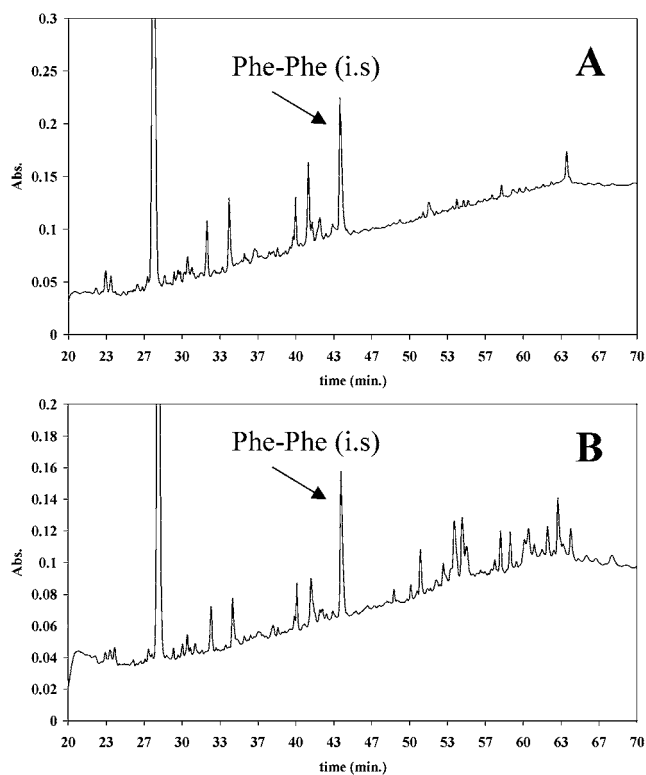
Figure 1. Chromatograms (214 nm wavelength detection) obtained from the same sample prepared with TCA (above, A) and HCl (below, B) as deproteinizing agent.
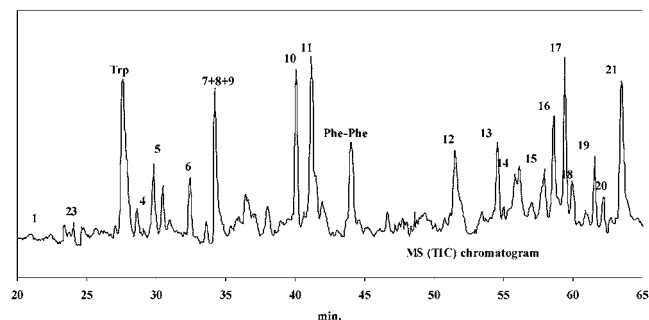
Figure 2. Typical MS chromatogram of a cheese sample. The peaks considered to be identified and semiquantified are numbered from 1 to 21.

detection were analyzed to determine their molecular mass, which was easily deduced from the mass spectra associated to every peak: the small peptides were usually identified by their protonated mass peak (MH+), whereas oligopeptides gave rise to a pattern of multicharged ions, which allowed the determination of the reconstructed molecular mass. In **Figure 2**, a typical MS chromatogram (full scan aquisition) of a cheese sample is reported; the peaks to be identified are numbered from 1 to 21.

The characteristic mass peaks detected for every peptide are reported in **Table 1**, together with the assigned molecular weights. Although many other peptides are present in the cheese samples, as it can be seen in the chromatogram, we decided to concentrate on those giving the most intense signals in the different samples.

It is worth noting that, despite the last diafiltration with a molecular cut-off of 3000 Da, some of the peptides detected have higher molecular masses, ranging from 260 to over 4000 Da. Being the results are very reproducible (average RSD is

Table 1. Identification of the Molecular Masses of the Most Significant Peptides Detected in Grana Padano Cheese

peak number	t_r (min)	characteristic mass peaks (m/z)	ext. MW (Da)
1	19	978 (MH⁺)	977
2	23	311 (MH⁺)	310
3	24	227 (MH⁺)	226
4	28	261 (MH⁺)	260
5	30	261 (MH⁺)	260
6	32	295 (MH⁺)	294
7	34	1330 (MH⁺), 665 (MH₂²⁺)	1329
8	34	1320 (MH⁺), 661 (MH₂²⁺)	1319
9	34	1349 (MH⁺), 675 (MH₂²⁺)	1348
10	40	852 (MH₂²⁺), 569 (MH₃³⁺)	1703
11	41	1001 (MH₂²⁺), 668 (MH₃³⁺)	2000
12	51	692 (MH₄⁴⁺), 554 (MH₅⁵⁺)	2764
13	54	855 (MH₂²⁺), 570 (MH₃³⁺)	1708
14	56	1047 (MH₄⁴⁺), 837 (MH₅⁵⁺), 697 (MH₆⁶⁺), 598 (MH₇⁷⁺)	4178
15	58	1238 (MH⁺), 619 (MH₂²⁺)	1237
16	59	1060 (MH₄⁴⁺), 849 (MH₅⁵⁺), 707 (MH₆⁶⁺), 606 (MH₇⁷⁺)	4238
17	60	1287 (MH₃³⁺), 966 (MH₄⁴⁺), 773 (MH₅⁵⁺), 644 (MH₆⁶⁺)	3860
18	60	1085 (MH₃³⁺), 814 (MH₄⁴⁺)	3252
19	62	1202 (MH₃³⁺), 901 (MH₄⁴⁺), 721 (MH₅⁵⁺), 601 (MH₆⁶⁺)	3602
20	62	874 (MH₃³⁺), 656 (MH₄⁴⁺)	2621
21	64	941 (MH₂²⁺), 628 (MH₃³⁺)	1881

5% for the peptide amounts from twin preparations of the same sample), it appears that the nominal molecular cut-off of the filters can admit some variations, which can probably be ascribed to the different conformations of the various peptides. It should be noted that all the peptides with high molecular masses found are very lipophilic and likely to adopt a very compact conformation in water solutions. In the literature, a similar but opposite phenomenon has been reported (i.e., that peptides with a molecular mass lower than 3000 Da can be retained by ultrafilters (*10*)). Alternatively, the effect may be due to variations of pore sizes in ultrafilters, even if the high reproducibility of different preparations of the same cheese sample seems to rule out this possibility.

Identification of Peptides. The peptides reported in **Table 1** were identified in a very fast way by applying a new method, developed by us, based both on the in-source fragmentation detectable with a single-quadrupole mass analyzer and by a software analysis of the casein sequences, from which the peptides originate. In fact, since all these peptides derive from the four milk caseins generated by proteolysis during the aging period, the knowledge of their molecular weight offers a limited range of possibilities for the punctual identification of every single peptide. By using a suitable software developed by one of us (S. Sforza, unpublished), for every peptide of a given molecular weight we obtained a list of all the possible sequences contained in the caseins that were compatible with that particular molecular weight. Then, by applying another software developed by one of us (S. Sforza, unpublished), we calculated the molecular weight of the fragments generated by all the possible peptides for the loss of the first N- or C-terminal amino acids. In fact, in low-energy induced fragmentations, peptides are known to break up mainly at the peptidic junction, giving rise to two series of fragments indicated as b fragments (charge retained on the N-terminal) and y fragments (charged retained on the C-terminal). Therefore, it was possible to associate to every possible peptide its own pattern of fragments generated by the loss of few amino acids from the C-terminus or from
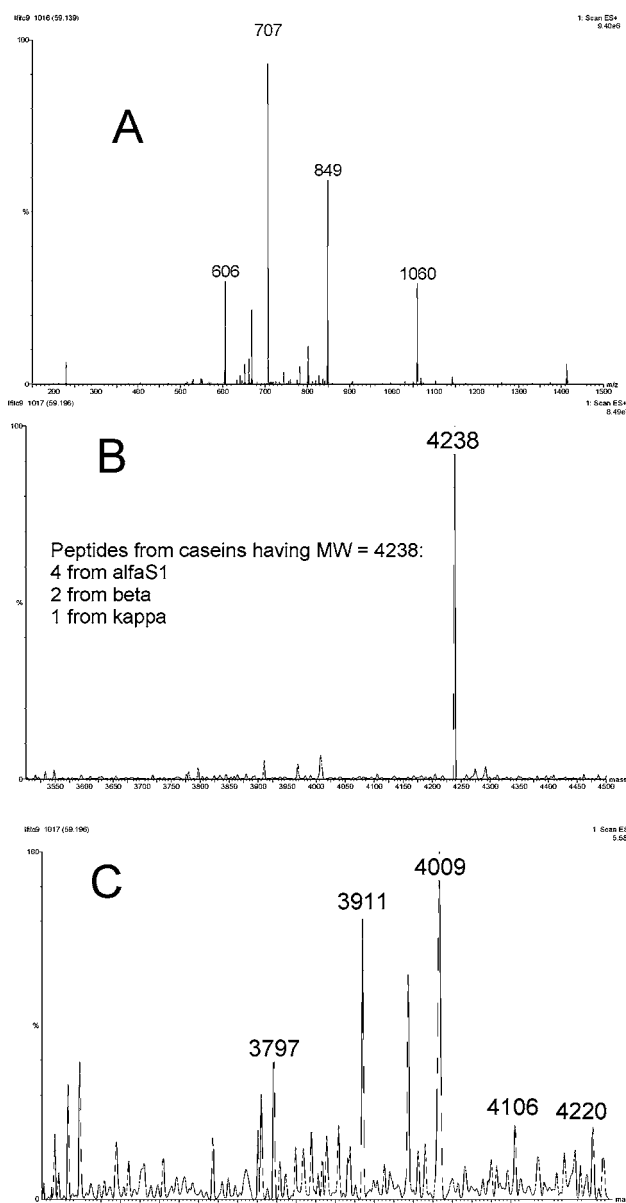
Figure 3. (A) Mass spectrum of the peak 16. (B) Reconstructed mass spectrum, which shows the actual MW (all the possible fragments coming from the caseins that have that MW are also shown). (C) Magnification of the reconstructed spectrum showing the fragmentation pattern.

the N-terminus. Analysis of the reconstructed spectra showed that actually every molecular peak was always associated to small fragmentation peaks, which usually corresponded to only one of the possible calculated b or y patterns, thus allowing in most cases a clear-cut identification of the peptide sequence from its original casein portion. The complete procedure is exemplified for the peptide 16 (MW 4238). In **Figure 3**, the mass spectrum of the peptide (A) and the reconstructed mass spectrum showing the actual MW (B) are reported, together with all the possible fragments coming from the caseins that have the same MW. The magnified reconstructed spectrum showing the real fragmentation pattern is reported in panel C. For every possible sequence corresponding to the MW of 4238, the expected fragmentation patterns, arising either from the loss of few N-terminal or few C-terminal amino acids, are reported in **Table 2**. If we compare **Figure 3C** and **Table 2**, we see that the real fragmentation pattern is compatible only with the peptide

Oligopeptides in Grana Padano Cheese

J. Agric. Food Chem., Vol. 51, No. 8, 2003 **2133**

Table 2. High MW Fragments (*b* and *y*) Expected for All the Peptides Originating from Milk Caseins with MW = 4238 Da

peptide	b series	y series
α_{S1} f(80–114)	4107(*n*–1), 4010(*n*–2), 3911(*n*–3), 3798(*n*–4)	4102(*n*–1), 3989(*n*–3), 3861(*n*–3), 3732(*n*–4)
α_{S1} f(109–145)	4074(*n*–1), 3911(*n*–2), 3839(*n*–3), 3726(*n*–4)	4126(*n*-1), 3997(*n*-3), 3883(*n*-3), 3784(*n*–4)
α_{S1} f(125–159)	4058(*n*–1), 3987(*n*–2), 3871(*n*–3), 3758(*n*–4)	4110(*n*–1), 4053(*n*–3), 3939(*n*–3), 3802(*n*–4)
α_{S1} f(134–168)	4124(*n*–1), 4025(*n*–2), 3861(*n*–3), 3698(*n*–4)	4142(*n*–1), 4011(*n*–3), 3897(*n*–3), 3840(*n*–4)
β f(7–43)	4110(*n*–1), 3976(*n*–2), 3875(*n*–3), 3747(*n*–4)	4125(*n*–1), 4025(*n*–3), 3928(*n*–3), 3871(*n*–4)
β f(129–165)	4108(*n*–1), 4021(*n*–2), 3908(*n*–3), 3809(*n*–4)	4124(*n*–1), 4025(*n*–3), 3896(*n*–3), 3782(*n*–4)
κ f(103–140)	4108(*n*–1), 3979(*n*–2), 3878(*n*–3), 3749(*n*–4)	4138(*n*–1), 4039(*n*–3), 3942(*n*–3), 3871(*n*–4)

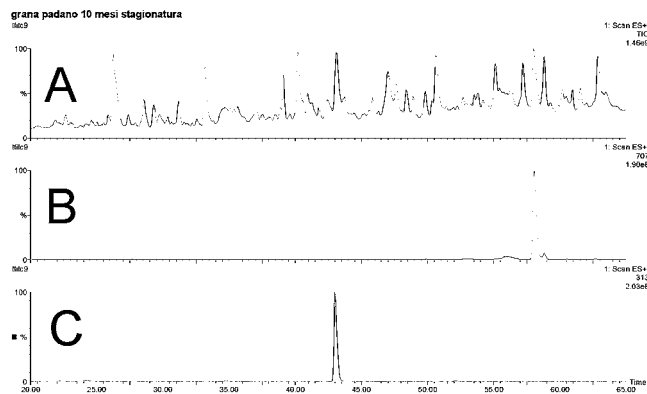**Table 3.** Identification of the Most Significant Peptides Detected in Grana Padano Cheese

peak number	t_r (min)	ext. MW (Da)	fragments assigned	identification
1	19	977	873 (b_{n-1}(3P)), 775 (b_{n-1}(2P)), 677 (b_{n-1}(P))	β-CNf(16–22)3P
2	23	310	182 (TyrH$^+$)	**Glu–Tyr**[a]
3	24	226	none	not identified
4	28	260	132 (LeuH$^+$ or IleH$^+$)	**Glu–Leu(Ile)**[a]
5	30	260	132 (LeuH$^+$ or IleH$^+$)	**Glu–Leu(Ile)**[a]
6	32	294	166 (PheH$^+$)	**Glu–Phe**[a]
7	34	1329	1231 (loss of H_3PO_4)	phosphopeptide
8	34	1319	1221 (loss of H_3PO_4)	phosphopeptide
9	34	1348	1073 (b_{n-2}(3P))	β-CNf(16–25)3P
10	40	1703	1136 (b_{n-3}(1P))	β-CNf(16–28)3P
11	41	2000	1872 (y_{n-1}(4P)), 1774 (y_{n-1}(3P)), 1675 (y_{n-1}(2P))	β-CNf(14–28)3P
12	51	2764	2599 (y_{n-1}), 2330 (b_{n-3}), 2103 (y_{n-4})	α_{S1}-CNf(1–23)
13	54	1708	1562 (y_{n-1}), 1462 (y_{n-2}), 1391 (y_{n-3})	α_{S1}-CNf(24–38)
14	56	4178	none	not identified
15	58	1237	992 (y_{n-2}), 921 (y_{n-3})	α_{S1}-CNf(24–34)
16	59	4238	4106 (b_{n-1}), 4009 (b_{n-2}), 3911 (b_{n-3}), 3797 (b_{n-4})	**α_{S1}-CNf(80–114)**[a]
17	60	3860	3631 (b_{n-2}), 3487 (y_{n-3})	**α_{S1}-CNf(83–114)**[a]
18	60	3252	3166 (y_{n-1}), 3121 (b_{n-1}), 3008 (b_{n-2}), 2860 (b_{n-3})	**β-CNf(164–192)**[a]
19	62	3602	3389 (b_{n-2}), 3374 (y_{n-2}), 3161 (b_{n-4})	**α_{S1}–CNf(85–114)**[a]
20	62	2621	2492 (b_{n-1}), 2410 (y_{n-2}), 2378 (b_{n-2}), 2230 (b_{n-3})	**β-CNf(170–192)**[a]
21	64	1881	1765 (b_{n-1}), 1652 (b_{n-2}), 1539 (b_{n-3}), 1441 (b_{n-4})	β-CNf(193–209)

[a] Not previously reported in the literature.

Figure 4. (A) TIC chromatogram of a cheese sample. (B) XIC obtained by extracting the mass peak at 707 *m/z* (the highest in the multicharged pattern generated by peptide **16**. (C) XIC obtained by extracting the 313 mass peak (protonated molecular ion for Phe–Phe).

α_{S1}CNf(80–114) (i.e., with the fragment included between the positions 80 and 114 of the α_{S1} casein).

By applying the method here described, 19 peptides were identified. Their identification is reported in **Table 3**. It is interesting to notice that, while some peptides were already reported in the literature as being present in hard cheeses (*10, 11*), others (in **Table 3** marked in bold characters) are here identified for the first time in Grana Padano cheese.

Method for the Semiquantification of Peptides. All the peptides identified were semiquantified by comparison with an internal standard (Phe−Phe). For the correct integration of the peaks, being the total ion chromatograms (TIC) were very crowded, we applied the extract ion chromatogram (XIC) technique. Following a procedure previously described by our group (*11*), for every peptide the XICs were derived from the TICs, by extracting, the chromatogram was defined by its characteristic mass peak. The oligopeptides were semiquantitated by measuring the ratio between the peptide area in the XIC and the area of the Phe−Phe in its relative XIC. The overall

procedure is exemplified for the peptide 16 (MW 4238). In **Figure 4A** the TIC is reported; in **Figure 4B** the XIC obtained by extracting the mass peak at 707 *m/z* (the highest in the multicharged pattern generated by peptide 16) is reported; in **Figure 4C** the XIC obtained by extracting the 313 mass peak (the protonated molecular ion for Phe−Phe) is shown. The average relative standard deviation (RSD) of the same sample in two preparations was 5%.

DISCUSSION

Oligopeptide Extraction. TCA precipitation of high molecular mass protein and peptides is a common method to extract the oligopeptide fraction from cheese samples (*9*). However, discrepancies are present in the literature. Some authors reported that all the oligopeptides with a MW lower than 10 kDa are kept in solution (*12*), whereas others indicate that the maximum number of amino acid residues present in the oligopeptides extracted is seven amino acids and very hydrophilic (*13*). Our results are consistent with the latter findings: in our hands, the use of TCA as an extracting agent led to an oligopeptide fraction constituted by very short peptides (2−3 residues) or very polar ones (phosphopeptides). In contrast, by using 0.1 N HCl it was possible also to maintain in solution longer peptides (up to 34 residues), thus obtaining a richer and much more informative oligopeptide fraction.

Main Oligopeptides Found in the Grana Padano Cheese. The new method reported above (extraction with 0.1 N HCl, HPLC−MS analysis with in-source fragmentation) allowed us to identify the most abundant peptides with low molecular weight (less than 5000 Da). This method is much faster and reproducible than those previously reported, which were based on HPLC purifications, Edman degradation of the different fractions, and redetermination of the MW. We studied only the peptides giving rise to strong signals in HPLC both by PDA and by MS detection, which were present in the oligopeptide
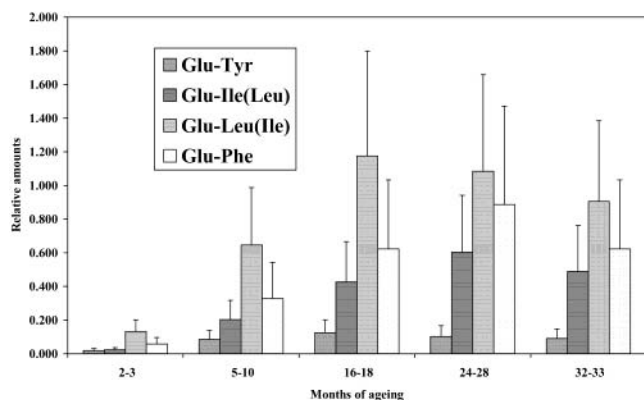
Figure 5. Average relative amounts of glutamyl-dipeptides (peptide peak area/Phe–Phe peak area) in samples of similar age.

Figure 6. Average relative amounts of phosphopeptides (peptide peak area/Phe–Phe peak area) in samples of similar age.

fractions of Grana Padano cheese. Nine new oligopeptides (of 21 taken in consideration) never previously reported in the literature were identified.

All peptides can be roughly divided in three groups: small peptides (glutamyl dipeptides 2, 4, 5, and 6), phosphopeptides (1, 7, 8, 9, 10, and 11), and bigger apolar peptides (12, 13, 15, 16, 17, 18, 19, 20, and 21). They seem to cluster according to the casein region from which they arise: phosphopeptides (except one) come from the region 14−28 of β-casein, while the others come from the 164−209 region of β-casein and from the 1−38 and 80−114 regions of α_{S1}-casein.

As a tentative hypotesis, a high amount of these peptides in Grana Padano cheese may be due to the fact that the degradation process is slower than their relative formation process. Thus, the presence of these peptides gives us two different types of information. First, since many peptides show the same N- or C-terminal amino acid, their presence confirms the preferential cleavage sites of the endopeptidases found in Grana Padano cheese (79−80, 23−24, and 38−39 of α_{S1}-casein and 28−29, 163−164, and 192−193 of β-casein). Moreover, since the amounts of these peptides increase with aging, it seems that they are resistant to further degradation. Many of these cleavage sites are known: positions 28−29 of β-casein and 79−80 of α_{S1}-casein are known cleavage sites for plasmin (*14*), position 23−24 of α_{S1}-casein is known for chimosin (*15*), and position 192−193 of β-casein is known for bacterial proteases (*16*). Consistently with the model above proposed, it has been reported that Grana Padano cheese contains many exopeptidases able to act on the products of the endopeptidasic action, thus generating new oligopeptides (*17*). New and very interesting is the presence of glutamyl dipeptides and oligopeptides arising from the cleavage betweeen position 79−80 and 114−115 of α_{S1}-casein.

All peptides were present in all samples from 2 to 33 months, although in different amounts. This means that the same proteolytic enzymes are active, independently from the production site or the starting ingredients (milk, rennet, starter), but are modulated according to the production procedures.

Semiquantitative Determination of Oligopeptides During the Aging Process. All the 21 peptides reported above were semiquantified following the method described: the data are reported by grouping the samples of similar age in **Figures 5** (glutamyl-dipeptides), **6** (phosphopeptides), **7** (peptides deriving from the N-terminal part of α_{S1}-casein), **8** (peptides deriving from the middle part of α_{S1}-casein), and **9** (peptides deriving from β-casein). General trends emerge during aging. The oligopeptide contents are generally quite low after the first months of aging, increase rapidly between the 10th and the 16th month, and then decrease, particularly in the case of phospho-
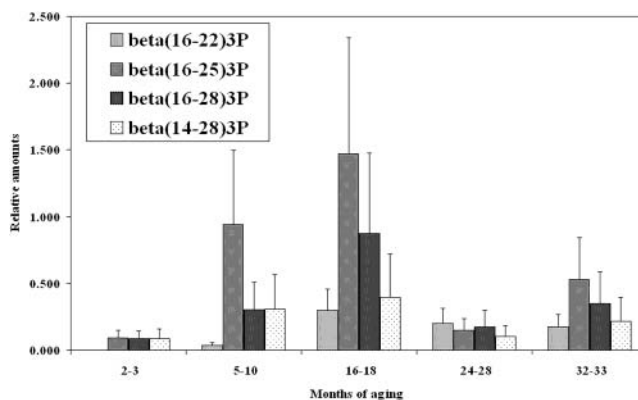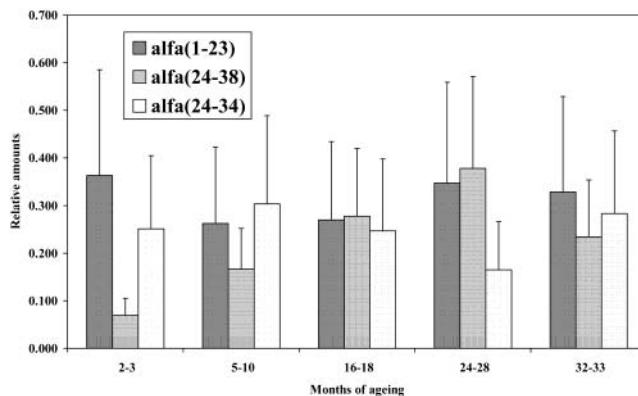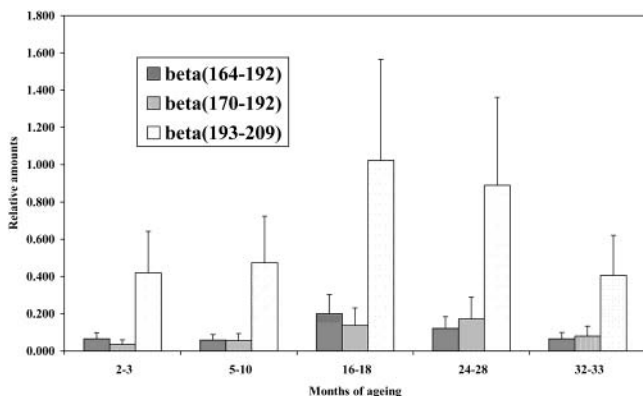

Figure 7. Average relative amounts of peptides coming from the N-terminal part of α_{S1}-casein (peptide peak area/Phe–Phe peak area) in samples of similar age.
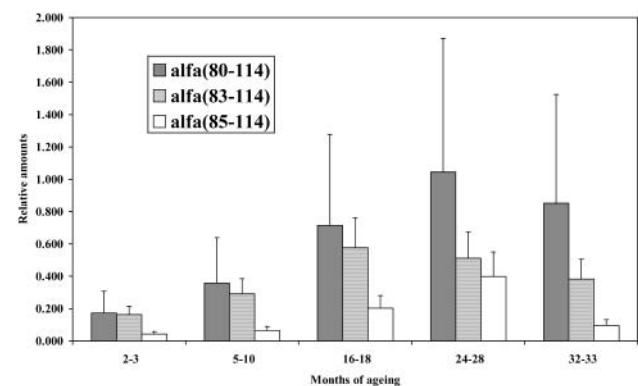
Figure 8. Average relative amounts of peptides coming from the middle part of α_{S1}-casein (peptide peak area/Phe–Phe peak area) in samples of similar age.

peptides. The trend is generally the same for all peptides, except for those derived from the N-terminal part of the casein, which do not vary significantly during aging. This behavior is likely to be related to the activity of the endoproteases. According to this hypotesis, plasmin (which cleaves between the positions 28−29 of β-casein and between the positions 79−80 of α_{S1}-casein, thus generating phosphopeptides and oligopeptides deriving from the middle part of α_{S1}-casein) should be quite active until the 16−24th month of aging. After that, the oligopeptides generated by its action are degraded faster than they are produced, decreasing regularly. Quite interestingly, the cleavage between positions 28−29 of β-casein, which has been described as a process mainly happening in milk, seems to occur

Oligopeptides in Grana Padano Cheese

J. Agric. Food Chem., Vol. 51, No. 8, 2003 **2135**

Figure 9. Average relative amounts of peptides coming from β-casein (peptide peak area/Phe–Phe peak area) in samples of similar age.

Table 4. Bioactive Sequences Contained in the Identified Oligopeptides

sequence	bioactivity	contained in peptide:
α_{S1}-CN(90–96)	opioid-like	16, 17, 19
α_{S1}-CN(25–27)	ACE-inhibitor	13, 15
β-CN(177–183)	ACE-inhibitor	18, 20
β-CN(193–202)	ACE-inhibitor	21
phosphate group-containing	ion binding	1, 9, 10, 11

also during the aging time, given the strong increase of phosphopeptides clearly arising from the proteolysis of the 1−28 β-casein peptide. Bacterial proteases (which cleave between the positions 192−193 of β-casein generating oligopeptides coming from the C-terminal part) show a very similar pattern of activity: they are active up to the 16−18th month, then they slow their activity, resulting in a corresponding decrease of the oligopeptide amounts. In contrast, the amount of the 1−23 α_{S1}-casein peptide seems to remain constant, consistently with the finding that the cleavage between positions 23−24 of α_{S1}-casein takes place in the first steps of cheesemaking (*3*). However, there are high variations among samples of the same age, coming from different factories, which may be due to different technological conditions (different aging conditions, areas of origin, natural whey starter, bacterial composition, etc.).

Potentially Bioactive Peptides. Another important point is the potential biological activity of the oligopeptides found in the Grana Padano cheese samples. Many studies have been focused on the structure−activity relationships of oligopeptides derived from milk proteins, outlining many different potential activities, such as angiotensin converting enzyme (ACE)-inhibitory, opioid-like, immunomodulatory, antimicrobial, and ion-binding (*18*). Some sequences of the oligopeptides reported in this paper are consistent with those reported in the literature (**Table 4**).

The sequences may be active also if included in longer peptides, or alternatively, the corresponding bioactive peptide can be formed in the gastrointestinal tract, after ingestion, by pepsin or trypsin digestion, thus potentially exhibiting functional properties on the human metabolism.

LITERATURE CITED

(1) Grappin, R.; Tank, T. C.; Olson, N. F. *J. Dairy Sci.* **1985**, *68*, 531−540.

(2) Le Bars, D.; Gripon, J. C. *J. Dairy Res.* **1989**, *56*, 817−821.

(3) McSweeney, P. L. H.; Olson, N. F.; Fox, P. F.; Healy, A.; Hoeirup, P. *J. Dairy Res.* **1993**, *60*, 401−412.

(4) Pritchard, G. G.; Colbear, T. *FEMS Microbiol. Rev.* **1993**, *12*, 179−206.

(5) Giraffa, G.; Neviani, E. *Food Microbiol.* **1999**, *16*, 205.

(6) Addeo, F.; Garro, G.; Intorcia, N.; Pellegrino, L.; Resmini, P.; Chianese, L. *J. Dairy Res.* **1995**, *62*, 297−309.

(7) Ferranti, P.; Barone, F.; Chianese, L.; Addeo, F.; Scaloni, A.; Pellegrino, L.; Resmini, P. *J. Dairy Res.* **1997**, *64*, 601−615.

(8) Ferranti, P.; Itolli, E.; Barone, F.; Malorni, A.; Garro, G.; Laezza, P.; Chianese, L.; Migliaccio, F.; Stingo, V.; Addeo, F. *Lait* **1997**, *77*, 683−697.

(9) Fox, P. F.; McSweeney, P. L. H.; Singh, T. K. In *Chemistry of Structure−Function Relationship in Cheese*; Malin, E. L., Tunick, M. H., Eds.; Plenum Press: New York and London, 1995.

(10) Singh, T. J.; Fox, P. F.; Healy, A. *J. Dairy Res.* **1995**, *62*, 629−640.

(11) Sforza, S.; Pigazzani, A.; Motti, M.; Porta, C.; Virgili, R.; Galaverna, G.; Dossena, A.; Marchelli, R. *Food Chem.* **2001**, *75*, 267−273.

(12) O'Sullivan, M.; Fox, P. F. *J. Dairy Res.* **1990**, *57*, 135−139.

(13) Yvon, M.; Chabanet, C.; Pelisser, J. P. *Intern. J. Peptide Protein Res.* **1989**, *34*, 166−172.

(14) Addeo, F.; Chianese, L.; Salzano, A.; Sacchi, R.; Cappuccio, U.; Ferranti, P.; Malorni, A. *J. Dairy Res.* **1992**, *49*, 401−411.

(15) Chianese, L.; Caira, S.; Ferranti, P.; Laezza, P.; Malorni, A.; Mucchetti, G.; Garro, G.; Addeo, F. *Lait* **1997**, *77*, 693−709.

(16) Larsen, L. B., Benfeldt, C., Rasmussen, L. K., Petersen, T. E. *J. Dairy Res.* **1996**, *63*, 119−130.

(17) Gatti, M.; Fornasari, M. E.; Mucchetti, G.; Addeo, F.; Neviani, E. *Lett. Appl. Microbiol.* **1999**, 368.

(18) For a recent review, see: Meisel, H.; Bockelmann, W. *Antonie van Leeuwenhoek* **1999**, *76*, 207−215.