

Study of the oligopeptide fraction in Grana Padano and Parmigiano-Reggiano cheeses by liquid chromatography-electrospray ionisation mass spectrometry

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The oligopeptide fractions of Grana Padano and Parmigiano Reggiano cheese samples, at various stages of ageing (6, 12, 18 and 24 months), were analysed by means of high-performance liquid chromatography coupled to electrospray ionisation mass spectrometry (ESI-MS). The main oligopeptides (< 5000 Da) present in the samples were extracted by using an originally developed method which allowed good enrichment of the oligopeptide fractions and identified according to their molecular weights. The casein sequences compatible with the found molecular weights were determined and the exact oligopeptide sequences were identified by using the fragmentation peaks originated by in-source collisionally-induced dissociation (CID). The ESI-MS method reported here allowed the sequence identification of oligopeptides very quickly with a single liquid chromatography-mass spectrometry run. Oligopeptides were also semi-quantified by comparison with a suitable internal standard (the dipeptide phenylalanyl-phenylalanine, Phe-Phe). This methodology demonstrated that, as far as the main oligopeptides are concerned, Grana Padano and Parmigiano Reggiano presented very similar composition. Anyway, the evolution of the fractions during ageing time was characteristically distinct among the two cheeses: in Grana Padano cheeses most of the oligopeptides reached a maximum at 12 months of ageing and then decreased, whereas in Parmigiano Reggiano cheeses the oligopeptide amounts were usually lower and had a less regular trend. The reason for this different behaviour may be ascribed to the different production techniques.

Keywords: ESI-MS, Grana Padano, in-source CID, oligopeptides, Parmigiano Reggiano

Introduction

Grana Padano and Parmigiano Reggiano are Italian PDO (protected designation of origin) cheeses, produced in the northern part of Italy. They are extra-hard, cooked cheeses made from raw, partly-skimmed cow's milk supplemented with a natural whey starter and ripened for at least 9 (Grana Padano) or 12 (Parmigiano Reggiano) months. There

are some differences in production techniques of the two types of cheeses such as cow feed, manufacturing, ripening time and the use of lysozyme as an additive in Grana Padano.

Thermophilic lactic acid bacteria (LAB) selected by the process of curd cooking are the dominant microflora of the natural whey starter. Lysis of LAB and the release of intracellular enzymes are the determining factors in the ripening of Grana Padano and Parmigiano Reggiano cheeses. As far as the nitrogen fraction is concerned, during the ageing process milk curd proteins (namely α_{s1} , α_{s2} , β and κ caseins) undergo an extensive degradation. A variety of proteolytic enzymes released by lysed LAB cells,¹ are active in these cheeses^{2,3} and together with the indigenous milk proteases and residual milk clotting enzymes contribute to an extensive degradation of cheese caseins.^{4,5}

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The nitrogen components of aged cheeses, therefore, consist of a mixture of native caseins, high, medium and low molecular mass peptides and free amino acids. Several studies have been reported on the molecular characterisation of the oligopeptide fraction of "Grana-type" cheeses, mainly based on the high identificative power of mass spectrometry techniques. Addeo and co-workers reported the isolation and the identification of low molecular mass peptides formed during the ripening of Parmigiano Reggiano cheese and soluble in 12% trichloroacetic acid (TCA) by fast atom bombardment mass spectrometry (FAB MS).^{6,7} Ferranti *et al.* isolated and identified phosphopeptides using MS techniques by precipitation with calcium chloride at pH = 4.6.⁸ In another paper from the same group, the peptide fraction of Grana Padano was more extensively analysed 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 a molecular mass ranging from 500 to 2700 Da were identified by Edman degradation and subsequent analysis by FAB or electrospray ionisation (ESI) mass spectrometry.⁹ In a very recent paper,¹⁰ we reported an innovative high-performance liquid chromatography (HPLC)-MS method that allowed the peptides of the oligopeptide fraction to be identified easily on-line in a single HPLC-MS analysis. Moreover, we provided a semi-quantitative evaluation of the major oligopeptides present in Grana Padano cheese during the ageing time (from 2 to 33 months) in comparison with a suitable internal standard (the dipeptide phenylalanyl-phenylalanine, Phe-Phe).

In the present paper, we apply the method reported above, which is based on the in-source collisionally-induced dissociation (CID) of the analytes, and used it for an analysis of the main patterns of the oligopeptide fractions of the Grana Padano and Parmigiano Reggiano cheeses at different stages of ageing.

Material and methods

Materials

Eight samples of Grana Padano cheese (aged 6, 12, 18 and 24 months, two for every age) were obtained from the Istituto Lattiero Caseario (Lodi, Italy). Eight samples of Parmigiano Reggiano cheese (aged 6, 12, 18 and 24 months, two for every age) were obtained from Parmalat SpA (Parma, Italy). The samples, obtained from 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

To 5 g of finely-grated cheese, suspended in 45 mL of 0.1 N HCl, (L,L)-phenylalanyl-phenylalanine (Phe-Phe) was added as an internal standard (2.25 mL of a 0.1N solution). The suspension was homogenised for 1 min by Ultra Turrax T50 (Janke and Hunkel Italabortechnik), 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), 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. 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), then through Amicon Ultrafilters YM3 (nominal molecular cut-off 3000 Da), by using an Amicon Micropartition system MPS-1 in both cases. The filtrate was dried under nitrogen, redissolved in H₂O (0.2% CH₃CN and 0.1% HCOOH) and analysed by HPLC.

HPLC-MS analysis

The HPLC equipment consisted of a Waters Alliance 2695 separation module connected with a Micromass ZMD mass spectrometer. HPLC conditions: Jupiter (Phenomenex) C18 column, 5 µm, 300 Å, 250 × 4.6 mm. Eluent A: H₂O (0.2% CH₃CN and 0.1% HCOOH), eluent B: H₂O : CH₃CN 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⁻¹. MS conditions: ESI interface with 90% splitting of the column flow, positive ions, single quadrupole analyser. Capillary voltage 3500 V, cone voltage 30 V, source temperature 100°C, desolvation temperature 150°C, cone gas (N₂) 60 L h⁻¹, desolvation gas (N₂) 450 L h⁻¹. Acquisition in total ion mode (150–1500 Da), scan time 2.9 s, inter-scan delay 0.1 s. Every cheese samples was prepared and analysed in duplicate. Reconstructed spectra were obtained by means of MassLynx 1.0 software.

Results and discussion

The extraction of the oligopeptide fraction from cheese samples was performed according to the method developed in our group (details in the experimental section),¹⁰ which avoids the use of strong deproteinising agents such as TCA in order to get a richer and more informative oligopeptide fraction.

Chromatographic analysis was performed on a reversed-phase HPLC system (C18 column), by using an ESI-MS mass spectrometer equipped with a single quadrupole analyser as a detector. The chromatograms were acquired in the full scan mode (positive ions). The dipeptide phenylalanyl-phenylalanine (Phe-Phe) was used as the

internal standard. The duplicate analyses of the same samples confirmed that the method was extremely reproducible, the average relative standard deviation (RSD) of the amounts of peptides in the duplicate analyses being about 5%.

Under the present chromatographic conditions, oligopeptides eluted later, in the part of the chromatogram between 20 and 70 min. Before 20 min, small and polar nitrogen components (i.e. mainly amino acids) were dominant.

The analysis of the chromatographic peaks with their associated mass spectra revealed that the oligopeptide fractions extracted from the two cheese types mainly contained the same oligopeptides, independently of the type of cheese (Grana Padano or Parmigiano Reggiano) and of the months of ageing. Every single peptide identified in a given sample was always found in all the other samples, although often in very different amounts. About 30 peptides were considered during this first preliminary analysis. These findings indicated a homogeneity of the two cheeses as far as the oligopeptide fraction is concerned, probably related to the similarity in the proteolytic enzyme composition.

Twelve peaks were chosen as characteristic for the oligopeptide fractions, which were usually present in relatively high amounts in all the samples of both types of cheese. A typical chromatogram of the oligopeptide fraction is reported in Figure 1, both for Grana Padano (12 months of ageing) and for Parmigiano Reggiano (12 months of ageing) cheese samples, together with an indication of the 12 peaks considered.

The identification of the peptide nature by mass spectrometry is usually performed by MSⁿ approaches, in which the fragmentation of the parent peptide ions gives information on their amino acid sequence.¹¹ In a single quadrupole

instrument, such a procedure is hampered by the limited fragmentation occurring in the ion source of the instrument. However, the procedure developed by us,¹⁰ which combines the information coming from in-source CID and an accurate mass analysis based on the knowledge of the casein sequences, allowed the direct on-line identification in the mass chromatogram of the peptide sequences with a high degree of reliability. Since the in-source CID was performed under quite low-energy conditions, peptides were mainly expected to fragment at the most labile linkage, i.e. the amidic bond, thus generating b or y fragments. Having calculated the possible casein sequences compatible with the found molecular masses for all the considered peptides, it was quite easy to calculate the fragments generated by the loss of the first N-terminal or C-terminal amino acids. Among all the calculated fragmentation patterns, usually only one corresponded to the actual fragments found in the reconstructed spectra, thus allowing a clear-cut identification of the peptide sequence corresponding to the peak of interest.

As a striking example, in the reconstructed spectrum of peptide F, reported in Figure 2, among the peaks with molecular weight lower than the main one (2764 Da), a clear fragmentation pattern composed of b-type fragments can be detected, demonstrating how reconstructed spectra can be used to exploit in-source fragmentation.

In that case, the main fragments found in the reconstructed spectra corresponded to the expected b fragments coming from the sequence α S1CN(1–23). This procedure worked perfectly well for peptides from F to L.

As far as the short peptides are concerned (peptides A and B, MW 260 and 294, respectively), a strong fragment was observed corresponding to a loss of a glutamyl residue. The residual MW corresponded to the amino acid leucine

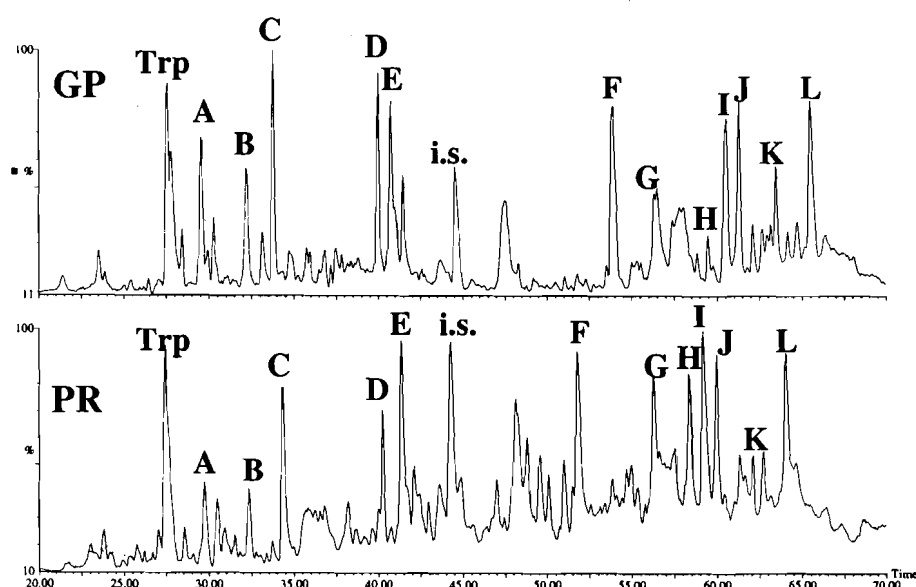


Figure 1. TIC chromatogram of a Grana Padano (GP, top) and Parmigiano-Reggiano (PR, bottom) cheese samples both aged 12 months. Characteristic peaks are marked with letters from A to L.

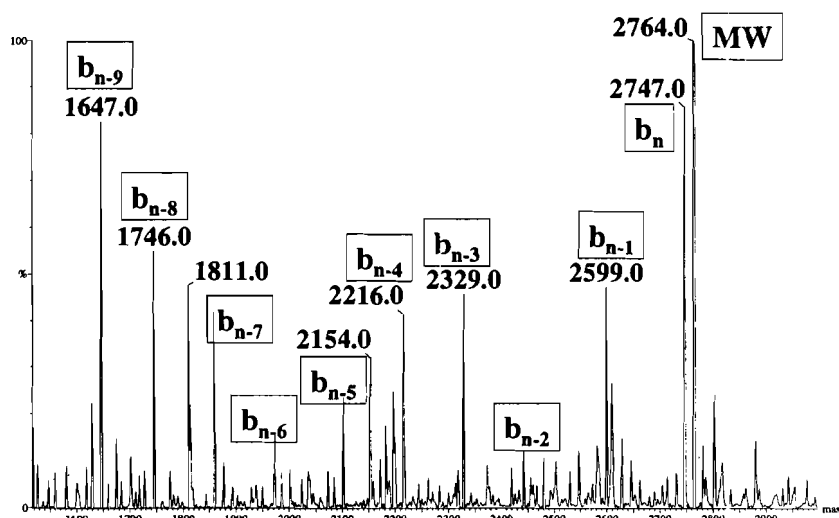


Figure 2. Reconstructed spectra for peptide F (MW 2764). The series of the b-fragments is clearly evident, allowing the identification of the peptide as the fragment(1-23) of α_{s1} -casein.

(or isoleucine) and phenylalanine, respectively, allowing the identification of their sequence as Glu-Leu(Ile) and Glu-(Phe). No unequivocal results were obtained for peptides C, D and E. Given the relatively high MW and the relatively low chromatographic retention time, these peptides were supposed to be phosphopeptides and actually

phosphorylated peptides with the same MW have already been reported in the literature.⁴ A more accurate analysis of the spectra revealed fragmentation patterns compatible with sequences of phosphopeptides already reported in the literature, allowing the identification of the three peaks as phosphopeptides derived from hydrolysis of β -casein.

Table 1. The 12 most characteristic peptides found in Grana Padano and Parmigiano Reggiano cheese samples, their diagnostic fragmentation peaks and the proposed identification.

Peptide ID	MW peptide	Diagnostic peaks identified	Identification
A	260	132 ^a (y_{n-1})	Glu-Leu (or Ile)
B	294	166 ^a (y_{n-1})	Glu-Phe
C	1348	1174 ^a (b_{n-1}), 1073 ^a (b_{n-2}), 961 ^a (b_{n-3})	β CN(16-25)3P ^c
D	1703	1419 ^b (y_{n-3}), 1333 ^b (y_{n-4})	β CN(16-28)3P ^c
E	1870	1643 ^b (b_{n-1} -H ₃ PO ₄), 1590 ^b (y_{n-2} -H ₃ PO ₄)	β CN(15-28)4P ^c
F	2764	2599 ^b (b_{n-1}), 2329 ^b (b_{n-3}), 2216 ^b (b_{n-4})	α_{s1} CN(1-23)
G	1707	1560 ^b (b_{n-1}), 1462 ^b (b_{n-2}), 1390 ^b (b_{n-3})	α_{s1} CN(24-38)
H	1237	992 ^a (b_{n-1}), 920 ^a (b_{n-2})	α_{s1} CN(24-34)
I	4238	4005 ^b (b_{n-2}), 3909 ^b (b_{n-3}), 3795 ^b (b_{n-4})	α_{s1} CN(80-114)
J	3860	3631 ^b (b_{n-2}), 3531 ^b (b_{n-3}), 3487 ^b (y_{n-3})	α_{s1} CN(83-114)
K	3602	3388 ^b (y_{n-2}), 3372 ^b (b_{n-2}), 3159 ^b (b_{n-4})	α_{s1} CN(85-114)
L	1881	1764 ^b (b_{n-1}), 1651 ^b (b_{n-2}), 1538 ^b (b_{n-3})	β CN(193-209)

^apeaks identified in the original spectra

^bpeaks identified in the reconstructed spectra

^closs of 98 (H₃PO₄) in the reconstructed spectra has been observed for these peptides

The peptide identifications, together with the diagnostic fragmentation peaks, are reported in Table 1.

Although all the sequences found have already been reported in the literature as being found in Grana Padano or Parmigiano Reggiano cheeses,^{4–9} the method reported here enabled them to be confirmed very quickly within a single chromatographic run, without any off-line degradation reaction. Moreover, these peptides are of importance because they are present in relatively high amounts in both types of cheese and, therefore, can be considered as characterising for hard “Grana-type” cheeses.

Finally, as we have already demonstrated in the case of Grana Padano,¹⁰ the extraction method which makes use of HCl in place of TCA allowed a richer oligopeptide fraction. Actually, few peptides (in this paper peptides **I**, **J**, and **K**) were identified by us for the first time in Grana Padano cheese samples.¹⁰ In the present paper, they were identified for the first time in Parmigiano Reggiano cheese as well. The presence of these peptides implies a proteolytic event occurring at position 79–80 of α_{s1} -casein (Lys–His residues) and subsequent degradation by aminopeptidases.³ Although these particular peptides have never been reported before in “Grana-type” cheeses, the α_{s1} -casein 79–80 site has already been demonstrated to be cleaved by plasmin.¹² Moreover, the hydrolysis of other Lys–His residues was observed in previous works concerning the oligopeptide fraction of Grana Padano. In particular α_{s1} -CN oligopeptides 8–16, derived from the cleavage between the positions 7–8 (Lys–His) was evidenced both in cheese whey¹³ and in 14-month old Grana Padano cheese.⁷

Therefore, the oligopeptides identified in Grana Padano and Parmigiano Reggiano cheeses, essentially, appeared to be the same. However, although the peptide pattern was very similar in the two types of cheeses, the amount of peptides present at the different ageing periods was quite different.

All the peptides identified were semi-quantified by comparison with an internal standard (Phe–Phe). For the correct integration of the peaks, we applied the extract ion chromatogram (XIC) technique. Following a procedure previously described by our group for the analysis of the oligopeptide fraction in hams,¹⁴ for every peptide the XICs were derived from the TICs, by extracting the chromatogram defined by its characteristic mass peak. The oligopeptides were semi-quantitated 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 **F** (MW 2764). In Figure 3, the TIC is reported (panel a), together with the XIC obtained by extracting the mass peak at 692 m/z (the highest in the multi-charged pattern generated by peptide **F**) (panel b) and the XIC obtained by extracting the 313 mass peak (the protonated molecular ion for Phe–Phe) (panel c).

The average RSD of the same samples in two different preparations was 5%. The semi-quantitative results for the peptides are depicted in Figure 4.

As far as the peptides coming from the N-terminal part of α_{s1} -casein are concerned (Figure 4, upper left corner), characteristically the α_{s1} -casein(1–23) peptide is related to the chimosin activity,¹³ whereas the α_{s1} -casein(24–34) peptide has been associated with the plasmin proteolytic action.¹² In Grana Padano cheese, they both reached a maximum in the 12-month samples, suggesting that the endoproteolytic action follows the same trend. The observed decreasing trends in the following months may be explained by assuming that the degradation of the peptides by exoproteases was faster than their production. In Parmigiano Reggiano cheese, the trend was quite different, being all peptides present in relatively constant amounts at all ageing times. This indicates that the proteolytic process in Grana Padano cheese by chimosin and plasmin is accelerated in the

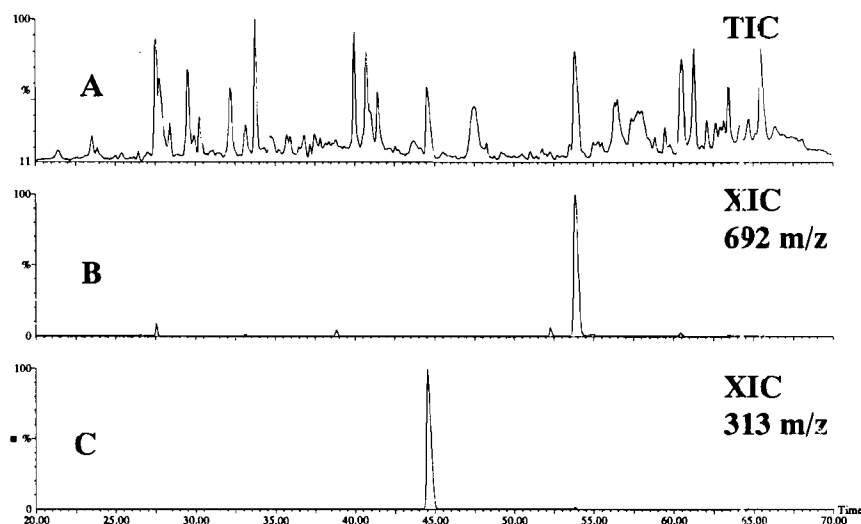


Figure 3. A) TIC chromatogram of a cheese sample; B) XIC obtained by extracting the mass peak at 692 m/z (the highest in the multi-charged pattern of peptide **F**); C) XIC obtained by extracting the 313 mass peak (protonated molecular ion for Phe–Phe).

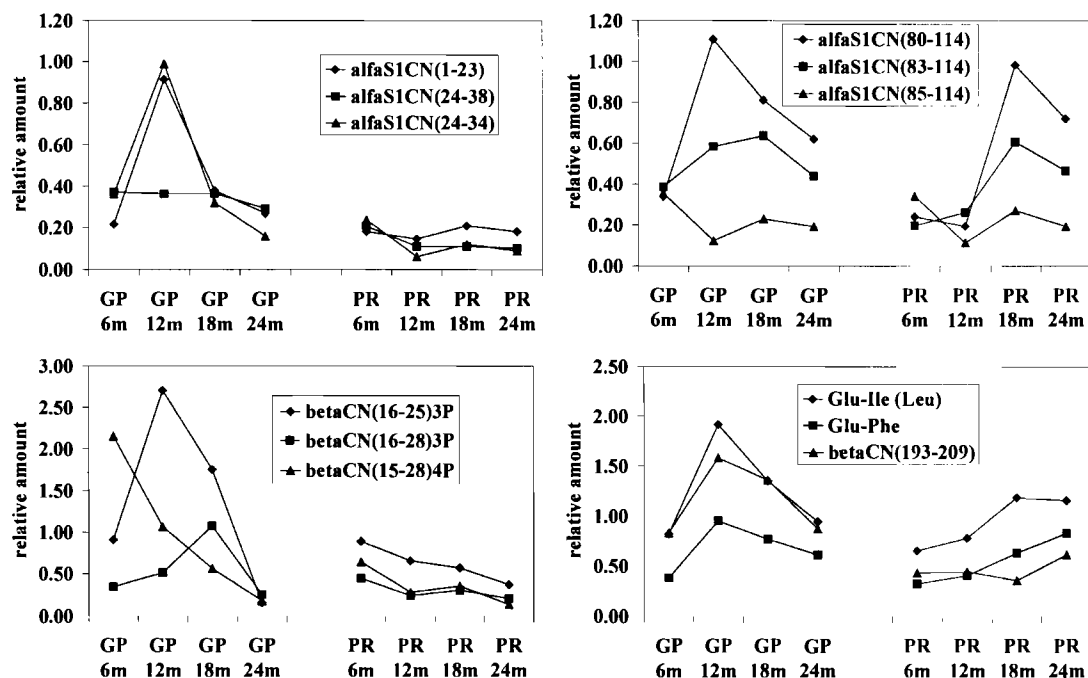


Figure 4. Semi-quantitative evaluation of the identified peptides. Peptides generated from N-terminal region of α_{s1} -casein (upper left corner); peptides generated from the internal region of α_{s1} -casein (upper right corner); phosphopeptides generated from β -casein (lower left corner); glutamyl dipeptides and the peptide associated to the C-terminal part of β -casein (lower right corner). In all panels the points on the left side are associated to the Grana Padano (GP) samples and the points on the right side are associated to the Parmigiano Reggiano (PR) samples. Months of ageing are also indicated. The relative amounts were calculated as peptide area / internal standard area.

first months of ageing as compared with Parmigiano Reggiano cheese.

As far as the peptides coming from the middle part of α_{s1} -casein are concerned (Figure 4, upper right corner), they may be generated by the endoproteolytic action of plasmin with subsequent degradation by aminopeptidases. Again, the semi-quantitative trend of these peptides in the two cheese types was quite different, although they reach a maximum and then decrease in both cases.

As far as the semi-quantitative results for the phosphopeptides coming from β -casein are concerned (Figure 4, lower left corner), in Grana Padano they reached a maximum and then decreased, except for one which decreased regularly. In Parmigiano Reggiano cheeses the trend was different, consisting in lower amounts and in a regular decrease from 6 to 24 months of ageing.

As far as the semi-quantitative results for the glutamyl dipeptides and for the peptide corresponding to the C-terminal part of β -casein are concerned (Figure 4, lower right corner), in Grana Padano cheeses the trend was the same as already seen: a maximum was reached in the 12-month samples, then a decrease occurred. Again, in Parmigiano Reggiano cheeses, these peptides were present in a different fashion, regularly increasing during the ageing period.

Although the number of samples considered was low, the general trends outlined a different evolution of the

proteolytic action in the two types of cheeses, which seemed to be faster in Grana Padano. In this case, the use of lysozyme probably induced an anticipated LAB lysis and, consequently, an anticipated release of cytoplasmic proteolytic enzymes. This phenomenon could be related to the higher amount of oligopeptides observed in Grana Padano cheeses aged 6 or 12 months, when compared with Parmigiano Reggiano samples.

A further consideration concerns the oligopeptides related to plasmin activity. The important presence of these peptides in Grana-type cheeses has been attributed to the inactivation of chimosin during cooking.¹⁵ Accordingly, in both types of cheeses we found several oligopeptides attributable to the action of plasmin. Nevertheless, the trend during the ageing of oligopeptides associated with chimosin action (particularly $\alpha_{s1}(1-23)$ peptide in Grana Padano samples) seems to indicate that a chimosin activity is still present in the cheeses at least until 12 months of ageing.

Conclusions

In the present paper, we have demonstrated the full feasibility of in-source fragmentation approaches in HPLC-ESI-MS for obtaining a fast on-line identification of the oligopeptides in hard "Grana-type" cheeses. It should be remarked that this method may have a very general applica-

bility for peptide identification, when the protein sequence from which the oligopeptides are generated is already known. In the present paper, the analysis of the oligopeptide fractions in Grana Padano and Parmigiano Reggiano cheeses showed that the two products present very similar oligopeptide composition, as far as the types of peptides are concerned but in quite different amounts. These data provided important information on the action of proteolytic enzymes in hard "Grana-type" cheeses, evidencing differences in the proteolytic events occurring in Grana Padano and Parmigiano Reggiano. These results may allow an evaluation of the ageing time of long-aged cheeses on the basis of their oligopeptide content, although observed amounts are usually different in cheese samples of same type and age but coming from different factories. This fact may be ascribed to the cheese-making techniques used in the "Grana Cheese-type" production area which, for Parmigiano Reggiano in particular, are very artisan. It could be hypothesised that the small size of dairies induce a strong variability in specific traits of the cheese-making process (percentage of fat, thermal parameters of processing, whey starter activity, NaCl penetration in curd) thus inducing modifications in the proteolytic events occurring during cheese ripening. Therefore, studies on other samples and an accurate statistical analysis of the generated results is necessary to outline a more general scheme. The LC-ESI-MS approach reported here appears to be fully suitable for this purpose.

Acknowledgements

We are grateful to Istituto Lattiero Caseario (Lodi, Italy) for providing Grana Padano cheese samples and to Parmalat S.p.A. (Collecchio, Parma, Italy) for providing Parmigiano Reggiano cheese samples.

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Received: 23 April 2003

Revised: 30 September 2003

Accepted: 2 October 2003

Web Publication: 4 December 2003