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Phosphopeptides from Grana Padano cheese: nature, origin and changes during ripening

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Summary. Casein phosphopeptides (CPP) which develop in Grana Padano cheese at different ages were isolated by precipitation with Ba^{2+} and analysed by HPLC. Profiles were complex throughout the period between 4 and 38 months. CPP in a cheese sample 14 months old were identified by a combination of fast atom bombardment–mass spectrometry and Edman degradation. They were found to consist of a mixture of components derived from three parent peptides, β-CNf(7–28)4P, α_{s1}-CNf(61–79)4P and α_{s2}-CNf(7–21)4P. In total, 45 phosphopeptides were identified: 24 from β-CN, 16 from α_{s1}-CN and 5 from α_{s2}-CN. The presence of aminopeptidase activity during cheese ripening was deduced from the presence of a number of CPP of different lengths with the loss of one or more residues from the N-terminus. The longest had C-terminal lysine and seemed to be progressively hydrolysed by carboxypeptidases A and B to shorter peptides. CPP in cheese appeared to be shortened plasmin-mediated products. Moreover, those most resistant to further hydrolysis contained at least three closely located phosphoserine residues. The anticariogenic activity of CPP is also discussed.

When proteolysis affects the N-terminal regions of α_{s1}-, α_{s2}- and β-casein, multiple phosphoseryl-containing peptides are formed. Casein phosphopeptides (CPP), present in the N fraction soluble in trichloroacetic acid (TCA, 120 g/l) from young hard cheese samples, were not detected when ripening increased beyond 1 year (Addeo *et al*. 1992). High levels of phosphoserine (SerP) up to 2·3 g/kg have been reported in some varieties of hard cheese, e.g. Walliser Raclette, Tilsiter, Appenzeller, Gruyère and Sbrinz (Lavanchy & Bühlmann, 1983). Thus, CPP could be regarded as transient intermediate components susceptible to further hydrolysis by cheese enzymes to shorter peptides and free amino acids (FAA), including SerP. This degradation mechanism might explain the absence of CPP in Parmigiano-Reggiano (PR) cheese older than 12 months (Addeo *et al*. 1992), in contrast with the results of Dulley & Kitchen (1973), who reported an increase in CPP during cheese maturation without alteration of the P_i level. From our present knowledge, there are two possibilities for the fate of cheese CPP: they either accumulate or are degraded into

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FAA. SerP potentially represents either a terminal product or a substrate for indigenous milk or bacterial phosphatase, since it is degraded into Ser and P_i. However, it has been found recently (P. Resmini, unpublished results) that there are only trace amounts of free SerP in several types of cheese, including Grana Padano (GP) and PR.

In order to establish the ultimate fate of the soluble CPP formed in GP cheese, an Italian hard cooked variety, a detailed analytical study was undertaken. The aim of this work was to identify the cheese CPP components and to explore possible means of formation and degradation. The origin of CPP in cheese was also identified and the possible biological functions with special regard to anticariogenic activity are considered.

MATERIALS AND METHODS

Cheese samples

GP cheeses at different stages of ripening were obtained from the Consorzio per la Tutela del Formaggio Grana Padano, Milan, Italy. Single samples at 4, 8, 14, 18, 34 and 38 months ripening, with the 5 mm rind removed, were grated and the resulting powder thoroughly mixed to give a homogeneous sample.

Chemical

Dithiothreitol, glycerol, sequence grade trypsin (TPCK treated, product number T8642) and thioglycerol were purchased from Sigma Chemical Co. (St Louis, MO 63178, USA). Alkaline phosphatase was obtained from Boehringer (D-68298 Mannheim 31, Germany). HPLC grade solvents and reagents were obtained from Carlo Erba (I-21059 Milan, Italy). Reagents for automated sequencing were supplied by the sequencer manufacturer (Applied BioSystems, Warrington, WA3 7PB, UK).

Preparation of pH 4·6-soluble fraction of cheese

Grated cheese samples (500 mg) were freeze dried and the fat extracted with diethyl ether in a Soxhlet apparatus. The dry residue was suspended in 20 ml water, stirred 10 min at room temperature, and 10 ml ammonium acetate (4 g/l, pH 4·6) was added dropwise. The resulting suspension was centrifuged at 4000 ***g*** and 15 °C for 20 min and the soluble fraction carefully removed with a Pasteur pipette. The residue was washed twice with 5 ml ammonium acetate solution. The three extracts containing N soluble at pH 4·6 were pooled and the resulting solution filtered through a 0·45 μm filter (Millipore, Bedford, MA 01730, USA).

Selective precipitation of multiple phosphoseryl-containing casein peptides

Peptides containing multiple SerP residues were selectively prepared according to the modified procedure of Manson & Annan (1971). Barium nitrate (100 g/l, 1 ml) and aqueous ethanol (500 ml/l, 30 ml) were added to the pH 4·6-soluble N extract. Phosphopeptides were precipitated as insoluble barium salts and recovered by centrifugation at 4000 ***g*** for 10 min. The supernatant was discarded and the phosphopeptides redissolved in 10 ml water. Precipitation was repeated by adding 10 ml acetone and 0·1 M-HCl to pH 4·6. The precipitate was recovered by centrifugation at 4000 ***g*** for 10 min, freeze dried and weighed.

Tryptic phosphopeptides were obtained by the procedure of Adamson & Reynolds (1995).

HPLC analysis of phosphopeptides

The selectively precipitated casein phosphopeptides were fractionated by

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reversed-phase HPLC on a Vydac C_{18} column (214TP54, 5 μm, 250 × 4·6 mm; Vydac, Hesperia, CA 92345, USA). Solvent A was 1 ml trifluoroacetic acid/l water and solvent B was 0·7 ml trifluoroacetic acid/l acetonitrile. Dried sample (500 μg) was dissolved in 200 μl water and injected on to the HPLC column previously equilibrated with solvent A. A linear gradient from 0 to 370 ml B/l was applied at a flow rate of 1 ml/min over 60 min. Peptide detection was carried out at 220 nm. Peak integration was performed using the software program supplied by Kontron Instruments (I-20138 Milan, Italy). Peptide fractions were collected manually, dried by flushing under nitrogen and stored at −20 °C.

Enzyme digestion

Trypsin and alkaline phosphatase action was carried out at 37 °C in ammonium bicarbonate (4 g/l, pH 8·5) for 6 h at a substrate:enzyme ratio of 50:1 (w/w) and stopped by freeze drying.

Free amino acid determination

Extraction of FAA from cheese with citrate buffer at pH 2·2, deproteination with sulphosalicylic acid (75 g/l) and the subsequent determination by ion-exchange chromatography and post-column ninhydrin derivatization with Biochrom 20 (Pharmacia, S-751 28 Uppsala, Sweden) were performed as described by Resmini *et al.* (1993).

Fast atom bombardment–mass spectrometry

Fast atom bombardment–mass spectrometry (FAB–MS) spectra were recorded on a VG Analytical ZAB 2SE double focusing mass spectrometer fitted with a caesium gun operating at 25 keV (2 mA). Samples were dissolved in HCl (1 ml/l) and loaded on to a glycerol-coated probe tip; thioglycerol was added to the matrix just before the introduction of the probe into the ion source. Amplification of the electric signal was decreased during the magnet scan according to the intensity of the mass signals observed on the oscilloscope. Mass spectra were recorded on u.v.-sensitive paper and counted manually.

Peptide identification

Signals recorded in the FAB spectra were associated with the corresponding tryptic peptides on the basis of the expected molecular mass deduced from the sequence of the casein fractions (Swaisgood, 1993). In order to confirm these assignments, manual Edman degradation steps and/or alkaline phosphatase action were performed on either mixtures of peptides or isolated peptides, followed by FAB–MS analysis of the truncated peptides (Addeo *et al.* 1992, 1994).

Sequence analysis

Automated Edman degradation was performed using an Applied BioSystems model 477A protein sequencer with on-line phenylthiohydantoinyl(PTH)-amino acid HPLC. The yield for each PTH-amino acid was calculated by analysis of a standard PTH solution.

RESULTS

Grana Padano cheese phosphopeptides soluble at pH 4·6

HPLC analysis of the pH 4·6-soluble N fraction of a 14 month old GP cheese sample gave a pattern typical of hard cheese varieties with a very high concentration of hydrophilic compounds within 30 min elution (Fox & McSweeney, 1994). In order

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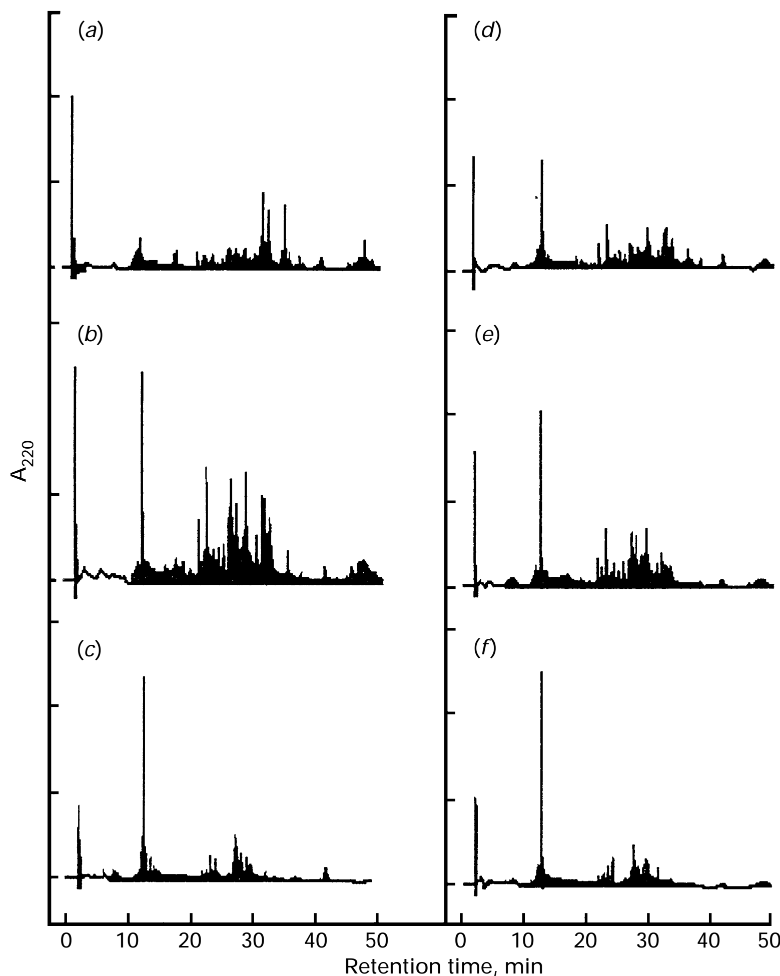

Fig. 1. HPLC patterns of phosphopeptides recovered as barium salts from the pH 4·6-soluble fraction of Grana Padano cheese ripened for (*a*) 4, (*b*) 8, (*c*) 14, (*d*) 18, (*e*), 34 and (*f*) 38 months.

to identify CPP components in this fraction, single HPLC peaks were collected and analysed by FAB–MS. No CPP were detected in the HPLC fractions except for one with a retention time (RT) of 26·2 min which contained, among others, α_{s1}-CNf(64–74)4P.

This was partly consistent with our previous results on PR cheese where no CPP were found in the N fraction soluble in 120 g TCA/l of cheese samples older than 6 months (Addeo *et al.* 1994).

Isolation of phosphopeptides from Grana Padano cheese

An additional selective purification step was performed using the modified Manson & Annan (1971) procedure to isolate the peptides containing multiple SerP residues from the mixture of peptides in the pH 4·6-soluble N cheese fraction. The HPLC pattern of the isolated peptides was complex and dependent on the age of the cheese samples, as shown in Fig. 1. The relative proportion of components with RT > 40 min decreased with age. In addition, there was an increase of the number of HPLC peaks between 4 and 8 months, indicating the formation of additional soluble

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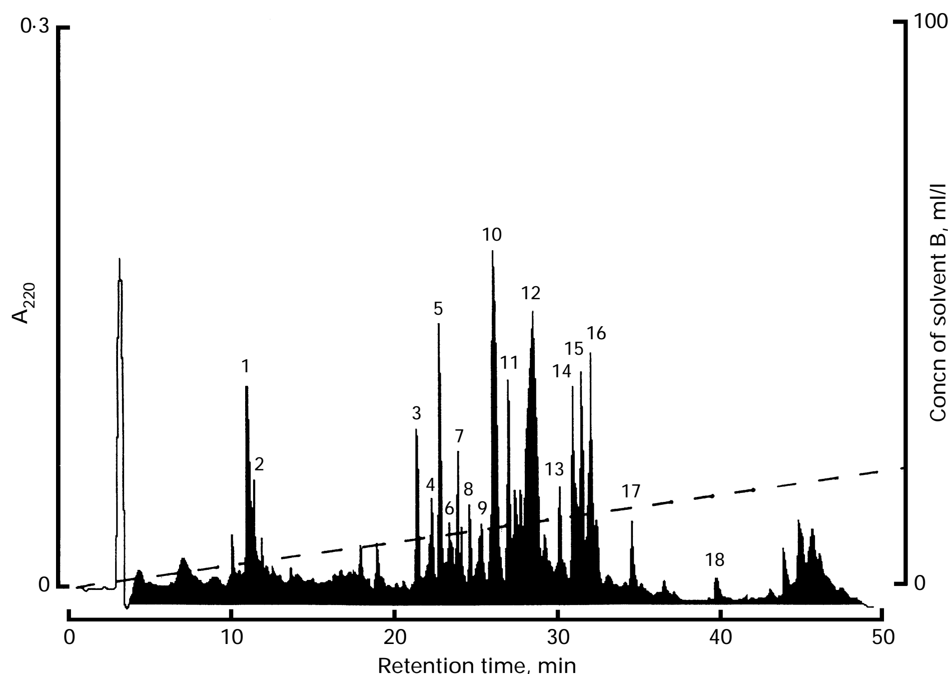

Fig. 2. HPLC pattern of phosphopeptides obtained as barium salt precipitate from the pH 4·6-soluble fraction of a Grana Padano cheese sample ripened for 14 months. ––––, Concentration (ml/l) of solvent B (see text for HPLC conditions).

peptides. Thereafter, both the RT and the relative intensity of these components decreased, being lowest in the oldest cheese. In contrast, the intensity of the peak at RT 12·7 min increased with ripening, being highest in the most ripened samples. Although this observation was limited to a few samples, we inferred a progressive reduction in the size of peptides from the decrease of RT and an accumulation of one or more components under the peak at low RT. We focused on the 14 month old GP sample since it was the most numerous of the commercially available products (Fig. 2). A limited number of peaks were also examined from cheese samples with other ripening times.

No FAB–MS signal was obtained from the HPLC peaks at RT between 44 and 47 min (Fig. 2). Since the elution time of the cheese peptides was found to increase with molecular mass (Addeo *et al.* 1992), it was estimated that the molecular masses of these peptides exceeded 3000 atomic mass units, at the upper limit of detection of our FAB–MS apparatus.

In order to reduce the size of these peptides, the whole pH 4·6-soluble N fraction was submitted to trypsin digestion. Afterwards the insoluble barium salt fraction was recovered, and the resolubilized peptides were again analysed by HPLC (results not shown). Comparing the HPLC patterns of the native fraction and after trypsin digestion, new components appeared between RT 32 and 35 min, while those between RT 44 and 47 min disappeared. The identity of the newly formed tryptic peptides from the pH 4·6-soluble N fraction of 14 month old GP cheese is indicated in Table 1. The parent peptides were most likely medium sized peptides. None of the newly formed tryptic peptides originated from β-casein, confirming that β-casein was extensively hydrolysed, forming oligopeptides, within the first year of ripening (Addeo *et al.* 1988).

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Table 1. *Newly formed tryptic phosphopeptides from the pH 4·6-soluble N fraction of a 14 month old ripened Grana Padano cheese sample*

Retention time, min	MH^+	N-terminal sequence	Peptide
31·4	2720	Gln–*Met–Glu*‡	α_{s1}-CNf(59–79)5P
32·6	3007	Asn–*Ala–Asn*	α_{s2}-CNf(46–70)4P
32·7	2640	Gln–*Met–Glu*	α_{s1}-CNf(59–79)4P
35·7	1927	Asp–*Ile–Gly*	α_{s1}-CNf(43–58)2P

MH^+, quasi-molecular ion value observed in the fast atom bombardment–mass spectrometry spectrum.
‡ Amino acid residues in italics indicate N-terminal sequence determined by fast atom bombardment mapping.

Table 2. *Phosphopeptides derived from β-casein in a Grana Padano cheese sample ripened for 14 months*

HPLC peak†	MH^+	N-terminal sequence	C-terminal residue	Peptide
18	2707	*Asn–Val–Pro*–‡	Lys^{28}	β-CNf(7–28)4P
17	2211	*Ile–Val–Glu*–SerP–Leu–SerP–SerP–…	Lys^{28}	β-CNf(12–28)4P
16	2098	*Val–Glu–SerP*	Lys^{28}	β-CNf(13–28)4P
14	1999	*Glu–SerP–Leu*	Lys^{28}	β-CNf(14–28)4P
13	1870	*SerP–Leu–SerP*	Lys^{28}	β-CNf(15–28)4P
16	1790	*Ser –Leu–SerP*	Lys^{28}	β-CNf(15–28)3P
13	1703	*Leu–SerP–SerP*	Lys^{28}	β-CNf(16–28)3P
16	1970	*Val–Glu–SerP*–Leu–SerP–SerP–	Asn^{27}	β-CNf(13–27)4P
15	1871	*Glu–SerP–Leu*	Asn^{27}	β-CNf(14–27)4P
13	1742	*SerP–Leu–SerP*	Asn^{27}	β-CNf(15–27)4P
16	1662	*Ser –Leu–SerP*	Asn^{27}	β-CNf(15–27)3P
13	1575	*Leu–SerP–SerP*	Asn^{27}	β-CNf(16–27)3P
13	1856	*Ile–Val–Glu*–SerP–Leu–SerP–SerP–…	Arg^{25}	β-CNf(12–25)4P
10	1743	*Val–Glu–SerP*	Arg^{25}	β-CNf(13–25)4P
8	1644	*Glu–SerP–Leu*	Arg^{25}	β-CNf(14–25)4P
9	1435	*Ser –Leu–SerP*	Arg^{25}	β-CNf(15–25)3P
6	1348	*Leu–SerP–SerP*	Arg^{25}	β-CNf(16–25)3P
9	1587	*Val–Glu–SerP*–Leu–SerP–SerP–…	Thr^{24}	β-CNf(13–24)4P
6	1488	*Glu–SerP–Leu*	Thr^{24}	β-CNf(14–24)4P
4	1359	*SerP–Leu–SerP*	Thr^{24}	β-CNf(15–24)4P
7	1279	*Ser –Leu–SerP*	Thr^{24}	β-CNf(15–24)3P
3	1192	*Leu–SerP–SerP*	Thr^{24}	β-CNf(16–24)3P
2	1065	*Ser–Leu–SerP*–SerP–SerP–Glu–Glu–Ser	Ser^{22}	β-CNf(15–22)3P
1	978	*Leu–SerP–SerP*–SerP–Glu–Glu–Ser	Ser^{22}	β-CNf(16–22)3P

MH^+, quasi-molecular ion value observed in the fast atom bombardment–mass spectrometry spectrum.
† HPLC peak numbers refer to Fig. 2.
‡ Amino acid residues in italics indicate N-terminal sequence determined by fast atom bombardment mapping.

The identity of the peptide components under the other HPLC peaks is shown in Tables 2, 3 and 4. Examination of these results revealed that the peptides all derived from the CPP family belonging to the three calcium-sensitive casein fractions: α_{s1}-, α_{s2}- and β-casein. No components derived from κ-casein were found in the CPP fraction since para-κ-casein, the only fragment entrapped in cheese, contains no phosphorylated residue. The three parent peptides were β-CNf(7–28)4P, α_{s1}-CNf(61–79)5P and α_{s2}-CNf(7–21)4P, and the components in the mixture were phosphopeptides, belonging to the three families, each of different lengths without one or more residues at the N- and/or C-terminus. This indicated the presence of aminopeptidase and carboxypeptidase enzymes during cheese ripening, producing a

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Table 3. *Phosphopeptides derived from α_{s1}-casein in a Grana Padano cheese sample ripened for 14 months*

HPLC peak†	*MH*⁺	N-terminal sequence	C-terminal residue	Peptide
12	2461	*Glu–Ala–Glu*–SerP–Ile–SerP–SerP–SerP–Glu–…	Lys^{79}	α_{s1}-CNf(61–79)5P
12	2332	*Ala–Glu–SerP*‡	Lys^{79}	α_{s1}-CNf(62–79)5P
12	2261	*Glu–SerP–Ile*	Lys^{79}	α_{s1}-CNf(63–79)5P
11	2133	*SerP–Ile–SerP*	Lys^{79}	α_{s1}-CNf(64–79)5P, (Glu^{78})
12	2132	*SerP–Ile–SerP*	Lys^{79}	α_{s1}-CNf(64–79)5P
11	1966	*Ile–SerP–SerP*	Lys^{79}	α_{s1}-CNf(65–79)4P, (Glu^{78})
11	1965	*Ile–SerP–SerP*	Lys^{79}	α_{s1}-CNf(65–79)4P
11	1810	*Glu–Ala–Glu*–SerP–Ile–SerP–SerP–SerP–Glu–…	Asn^{74}	α_{s1}-CNf(61–74)4P
11	1681	*Ala–Glu–SerP*	Asn^{74}	α_{s1}-CNf(62–74)4P
11	1610	*Glu–SerP–Ile*	Asn^{74}	α_{s1}-CNf(63–74)4P
10	1481	*SerP–Ile–SerP*	Asn^{74}	α_{s1}-CNf(64–74)4P
11	1401	*Ser–Ile–SerP*	Asn^{74}	α_{s1}-CNf(64–74)3P
10	1314	*Ile–SerP–SerP*	Asn^{74}	α_{s1}-CNf(65–74)3P
11	1234	*Ile–Ser –SerP*	Asn^{74}	α_{s1}-CNf(65–74)2P
6	1034	*SerP–SerP–Glu*	Asn^{74}	α_{s1}-CNf(67–74)2P
9	954	*Ser –SerP–Glu*	Asn^{74}	α_{s1}-CNf(67–74)1P

MH⁺, quasi-molecular ion value observed in the fast atom bombardment–mass spectrometry spectrum.
† HPLC peak numbers refer to Fig. 2.
‡ Amino acid residues in italics indicate N-terminal sequence determined by fast atom bombardment mapping.

Table 4. *Phosphopeptides derived from α_{s2}-casein in a Grana Padano cheese sample ripened for 14 months*

HPLC peak†	*MH*⁺	N-terminal sequence	C-terminal residue	Peptide
15	2006	*Val–SerP–SerP*–SerP–Glu–Glu–Ser–Ile–Ile–SerP–…	Lys^{21}	α_{s2}-CNf(7–21)4P
12	1715	*Val–SerP–SerP*‡	Thr^{19}	α_{s2}-CNf(7–19)4P
10	1614	*Val–SerP–SerP*	Glu^{18}	α_{s2}-CNf(7–18)4P
10	1534	*Val–Ser –SerP*	Glu^{18}	α_{s2}-CNf(7–18)3P
7	1515	*SerP–SerP–SerP*	Glu^{18}	α_{s2}-CNf(8–18)3P

MH⁺, quasi-molecular ion value observed in the fast atom bombardment–mass spectrometry spectrum.
† HPLC peak numbers refer to Fig. 2.
‡ Amino acid residues in italics indicate N-terminal sequence determined by fast atom bombardment mapping.

progressive reduction in the size of the primary peptides and the concomitant formation of shorter peptides and FAA.

In total, 45 phosphopeptides were identified in the sample of 14 month old GP cheese: 24 derived from β-casein, 16 from α_{s1}-casein and 5 from α_{s2}-casein. The largest peptides each had lysine as the C-terminal residue, probably formed from the parent protein by action of a trypsin-like enzyme. Since plasmin cleaves Lys–X and Arg–X bonds with a slight preference for Lys–X (Weinstein & Doolittle, 1972), this could be the enzyme actually responsible for the formation of these peptides.

Phosphopeptides from β-, α_{s1}- and α_{s2}-caseins

Four sets of CPP from the N-terminal part of β-CN were identified, two pairs of peptides having either Ile^{12} or Val^{13} as N-terminal residues (Table 2). It is likely that these peptides arose from Glu^{11}–Ile^{12} bond cleavage by an acid proteinase active in the cheese. The substrate may have been the N-terminal fragment β-CNf(1–28), previously identified as one of the plasmin-mediated products of β-casein (Andrews,

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1978), which contains four of the five SerP residues of β-casein. Peptide β-CNf(1–28) was found to be one of the five peptides soluble in 120 g TCA/l identified in GP cheese whey (L. Chianese, unpublished results). Although peptide β-CNf(1–28) does not contain peptide bonds sensitive to plasmin-like enzymes other than $Arg^{1}–Glu^{2}$, it was absent from the 14 month old GP cheese sample. Unfortunately, our analytical procedure allowed us to detect only phosphorylated peptides, so that only β-CNf(7–28)4P and β-CNf(12–28)4P were isolated together with four derived products having one to four amino acid residues truncated at the N-terminus (Table 2).

From finding closely related β-casein CPP (Table 2), we could deduce the activities of general and specific aminopeptidases (which release the N-terminal amino acid from oligopeptides), of phosphatase, and of carboxypeptidases A and B. One set of six CPP was detected with C-terminal Lys^{28}. Two other sets, each including five components, had Arg^{25} and Thr^{24} as their C-terminal residues. The presence of peptides with Lys as the C-terminal residue can be explained by an active plasmin in cheese. The three sets of phosphopeptides with C-terminal Asn^{27}, Arg^{25} and Thr^{24} can be related to each other, assuming in cheese both active carboxypeptidases B and A, which remove basic and neutral amino acid residues respectively from the C-terminal end. However, it must be taken into account that the optimum pH of carboxypeptidase B activity is close to 8·5 whereas that of carboxypeptidase A is 4·5, close to that in GP cheese ($\sim$ 5).

Two sets of peptides derived from α_{s1}-casein had Lys^{79} and Asn^{74} as C-terminal residues (Table 3). The peptide α_{s1}-CNf(61–79)5P could arise from the parent peptide α_{s1}-CNf(1–79)7P through cleavage at $Met^{60}–Glu^{61}$ by an unknown endopeptidase. The resulting two peptides may be further hydrolysed by cheese aminopeptidases and carboxypeptidases giving rise to FAA and shorter peptides. Two sets of phosphopeptides were identified (Table 3) differing either in the C-terminal extension, e.g. f(61–79)5P and f(61–74)5P, or at the N-terminal end, e.g. f(61–79)5P to f(65–79)5P, and f(61–74)4P to f(67–74)1P and f(67–74)2P. Here also, in order to explain the occurrence of the peptide set having C-terminal Asn^{74}, a concerted action of both carboxypeptidases B and A on the parent peptide f(61–79)5P could be assumed, with the release first of the C-terminal Lys^{79} and subsequently of neutral and acid amino acid residues.

Five phosphopeptides were derived from the N-terminal part of α_{s2}-casein (Table 4). The precursor of these peptides may be the α_{s2}-CNf(1–21) fragment probably formed as a consequence of plasmin attack on $Lys^{21}–Gln^{22}$, identified as one of the most plasmin-sensitive bonds (Le Bars & Gripon, 1989). This could open the peptide to further endopeptidase attack, forming two shorter peptides, α_{s2}-CNf(1–6) and α_{s2}-CNf(7–21)4P. Exposure of α_{s2}-CNf(7–21)4P to cheese carboxypeptidases B and A probably caused the release of three amino acid residues from the C-terminal part. The formation of two partly digested phosphopeptides f(7–19)4P and f(7–18)4P and the partly dephosphorylated peptide f(7–18)3P possibly indicates that digestion proceeded at both C- and N-terminal portions of the peptide. The shortest phosphopeptide formed from α_{s2}-CNf(7–21)4P was the undecapeptide α_{s2}-CNf(8–18)3P (Table 4).

Formation of casein phosphopeptides during ripening

The CPP level increased from $\sim$ 10 to 16–19 g/kg cheese within 4–8 and 14–38 months of ripening. This indicated that CPP were formed mainly in the first period of ripening, and that later CPP formation was balanced by their enzymic degradation.

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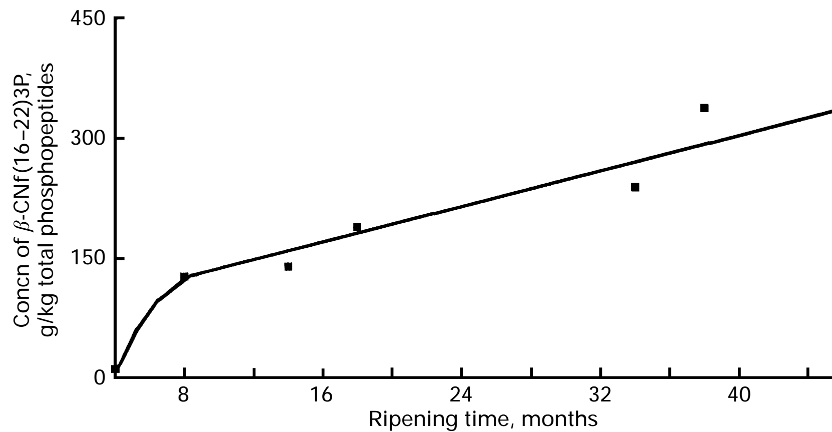

Fig. 3. Increase in the β-CNf(16–22)3P peptide level during Grana Padano cheese ripening.

The structural features of the peak at RT 12·7 min (Fig. 2, peak 1), MH^+ 978 and Leu–SerP–SerP– as N-terminal sequence, indicated the peptide β-CNf(16–22)3P. The identity of this component was also confirmed through automated amino acid sequencing of the cheese samples at different ages and corresponded to the β-casein fragment with the amino acid sequence H_2N.Leu^{16}–SerP–SerP–SerP–Glu–-Glu–Ser.OH^{22}. This allowed us to conclude that no components other than β-CNf(16–22)3P were eluted within the peak at RT 12·7 min in GP cheese samples between 4 and 38 months ripening. The changes in the β-CNf(16–22)3P level during GP ripening are shown in Fig. 3. After 38 months, β-CNf(16–22)3P became the most abundant CPP, making up $\sim$ 25% of the total and indicating that the peptide was resistant to cheese enzymes. Owing to the high content of negatively charged residues, two glutamic and three SerP residues, this phosphopeptide probably exhibits a potent ability to form soluble complexes with calcium phosphate at various Ca:P ratios (Berrocal *et al.* 1989) and trace elements such as Fe, Mn, Cu, and Se, functioning as a carrier for a variety of minerals (West, 1986).

Dephosphorylation of casein phosphopeptides

The same peptides with different phosphate levels were identified within the cheese samples examined (Tables 2–4): β-CNf(15–28), β-CNf(15–27), β-CNf(15–25) and β-CNf(15–24) contained SerP^{15} partly dephosphorylated: α_{s1}-CNf(64–74) and α_{s1}-CNf(65–74) contained SerP^{64} and SerP^{66} respectively; α_{s1}-CNf(67–74) contained SerP^{67} and α_{s2}-CNf(7–18) SerP^{8}, partly dephosphorylated. The fact that only partly phosphorylated β-casein peptides containing N-terminal SerP^{15} were found means that dephosphorylation occurred on the N-terminal exposed SerP by active phosphatase action, followed by release of the N-terminal Ser through an aminopeptidase.

In order to check if both the fully phosphorylated and partly phosphorylated peptide forms either coexist naturally in casein or are artificially produced during ripening, a search was carried out in a laboratory preparation of casein tryptic phosphopeptides. Two peptides were found, β-CNf(1–25) and β-CNf(2–25), each with 4P and 3P in an approximate ratio of 90:10. Therefore, bovine β-casein, at variance with previous reports, appeared to be a 4P and 5P phosphorylated chain producing two parent phosphorylated peptides, β-CNf(1–28)4P and β-CNf(1–28)3P, each susceptible to enzyme action. No direct determination of the partly phosphorylated sites in peptides β-CNf(1–25)3P and β-CNf(2–25)3P was carried out.

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Formation of free amino acids during ripening

The experimental values for both FAA and free Ser in our samples were consistent with those expected from the calculated model for formation of total FAA and free Ser (Resmini *et al.* 1985, 1993), indicating that the cheese samples analysed in the present work were representative of both cheese variety and age. Only trace amounts of SerP were detected in cheese of different ages, which means that free Ser was the terminal product of CPP hydrolysis, at variance with the high levels of free SerP found in some hard Swiss cheeses (Lavanchy & Bühlmann, 1983).

DISCUSSION

Origin of casein phosphopeptides

Plasmin, more than rennet, has long been recognized as being responsible for casein degradation during ripening of hard cheeses (Collin *et al.* 1988; Boudjellab *et al.* 1994). The relatively high heating intensity attained during cheesemaking probably partly inactivates or destroys thermolabile enzymes such as chymosin, leaving plasmin as the main enzyme producing hydrolysis. The effects of heating on plasmin and chymosin activity in GP cheese have not been directly evaluated. However, action of these enzymes in the first phases of cheesemaking has been deduced from the identification of the peptides β-CNf(1–28)4P and α_{s1}-CNf(1–23) in GP whey (L. Chianese, unpublished results). Further, in GP cheese, β-casein was extensively degraded to complete hydrolysis with concomitant formation of some plasmin-derived peptides, among which γ-caseins are the most important (Addeo *et al.* 1995). This explains the predominance in some hard cheeses of plasmin-mediated over chymosin-mediated peptides. Therefore, composition of the pH 4·6-soluble N cheese peptides depends on the specificities of the enzymes degrading caseins. In GP cheese, since it is hydrolysed by plasmin more than other casein fractions, β-casein is the main source of the oligopeptides (Addeo *et al.* 1995) hydrolysed by proteinases and peptidases to give shorter peptides and free amino acids. Although CPP were mainly located in the N-terminal regions of β-, α_{s1}- and α_{s2}-casein, recent evidence has shown that the C-terminal region was also affected by hydrolysis (P. Ferranti, unpublished results). Among the pH 4·6-soluble oligopeptides with molecular masses < 3 kDa identified in the same sample of 14 month old GP cheese, five β-casein components originated from the β-CNf(1–28)4P plasmin-mediated peptide, 23 from the peptide region between residues 44 and 100, and two from the C-terminal region of β-casein, as a consequence of peptide bond cleavage between the residue pairs 192–193 and 193–194. These two latter are cleavage sites of the *Lactococcus* cell envelope proteinase (Singh *et al.* 1995). Of the 44 peptides identified from α_{s1}-casein, 30 were from the 1–79 region, and the others were from peptide bond cleavage between residues 89 and 166. Of the 13 peptides derived from α_{s2}-casein and identified, 10 originated from the C-terminal region. This demonstrated that CPP only partly describe the complexity of cheese proteolysis.

Enzyme activity and amino acid release

The identification of some closely related phosphopeptides in GP cheese helps to highlight the degradative mechanisms active during ripening. The presence of peptides shorter than parent peptides may result from the concerted action of cell-wall-associated proteinases (Ezzat *et al.* 1985, 1993; Laloi *et al.* 1991) and

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exopeptidases and cytoplasmic enzymes, released by the flora of the cheese. *Lactobacillus helveticus* is considered the predominant species of GP cheese (Torriani *et al.* 1994) but *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* have also been found. *Lb. helveticus* is generally recognized as having one of the strongest proteolytic activities among the lactic acid bacteria (Torriani *et al.* 1994). Knowledge of the proteolytic systems of thermophilic lactic acid bacteria is less advanced than for lactococci. Identification of the low molecular mass peptides released from α_s- and β-casein by the cell envelope proteinase from *Lb. helveticus* demonstrated that some of the cleavage sites in β-casein (Zevaco & Gripon, 1988) were the same as those hydrolysed by the lactococcal P_1-type proteinase (Juillard *et al.* 1995). Others were different, although there appear to be several different specificity types among the proteinases from lactobacilli (Khalid *et al.* 1991). There are significant differences between bacterial genera (Atlan, 1996). Proteinases cover the cell surface of lactobacilli, whereas this is not the case for lactococci (Atlan, 1996). In addition, the proteolytic system of thermophilic lactic acid bacteria is characterized by the presence of several specific cell peptidase activities, mainly aminopeptidase and iminopeptidase (for review, see Atlan, 1996).

Essentially there may be two classes of cheese exopeptidases, aminopeptidases and carboxypeptidases of different origin, reducing the size of water-soluble peptides. The aminopeptidase system of thermophilic lactobacilli may be formed by at least two distinct types of aminopeptidase, a metalloenzyme and an X-prolyl dipeptidyl aminopeptidase (a serine-type enzyme), having some homology of general properties with those of lactococci (Pritchard & Coolbear, 1993). In addition, carboxypeptidase A and B activity, deriving from starter lactobacilli, bacterial lysis or another source, was involved in the release of FAA from peptides. Except for the intracellular enzyme of *Lb. casei* (El Soda *et al.* 1978), supported by a further report of Abo-Elnaga & Plapp (1987), no carboxypeptidase activity has previously been reported. However, a carboxypeptidase B-like enzyme discovered in human plasma (Plummer & Hurwitz, 1978) might explain this enzymic activity in cheese. Another source of a carboxypeptidase B-like activity is the flora contaminating milk, mainly moulds and yeasts, transferred to cheese.

In any case, the occurrence of several closely related soluble peptides in GP cheese suggests the presence of endopeptidase and exopeptidase activities, explaining mechanisms of peptide degradation and the increase in FAA during cheese ripening. More FAA were detected in the outer parts of GP cheese (Resmini *et al.* 1993), probably because of the higher enzyme activities there than in the cheese core. This could indicate a thermal gradient and a consequent gradient of alkaline phosphatase inactivation between the outer part and the core of GP cheese during moulding (Pellegrino *et al.* 1995).

Mechanism of phosphorylated peptide formation from caseins

The lists of CPP peptides suggest the hypothesis that primary soluble peptides are continuously hydrolysed to give a mixture of components from seven amino acid residues upwards. Roudot-Algaron *et al.* (1994) also isolated six β-casein phosphopeptides from Comté cheese: f(13–28), f(14–28), f(15–28) and f(14–27), f(15–27) and f(16–27), probably derived from the parent plasmin-mediated product β-CNf(1–28) (Le Bars & Gripon 1989). Addeo *et al.* (1994) identified several peptides from β-CNf(1–28) in the fraction soluble in 120 g TCA/l of 6 month old PR cheese, e.g. β-CNf(1/2/3/4/5–20) and β-CNf(6/7/8/9–28). Roudot-Algaron *et al.* (1994) suggested that, before being digested by aminopeptidases, β-CNf(1–28) was first

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cleaved by a proteinase and then digested by aminopeptidases, but no β-casein CPP larger than β-CNf(7–28) was found in 14 month GP cheese. However, the degradation mechanism of β-casein CPP seems similar in the hard cheese varieties involving release of amino acids and/or oligopeptides from the parent peptide β-CNf(1–28).

The phosphopeptides derived from α_{s1}-casein reported in Table 3 may come from a common parent plasmin-mediated product consisting of the peptide α_{s1}-CNf(1–79)7P, complementary to the α_{s1}-CNf(80–199) fragment. This latter was identified as one of the main components of the pH 4·6-insoluble fraction of PR cheese (Addeo *et al.* 1995). Cleavage of the Lys^{79}–Glu^{80} bond and the formation of the α_{s1}-CNf(80–199) fragment were also recorded both in the *in vitro* hydrolysate of α_{s1}-casein by plasmin (Addeo *et al.* 1995) and in PR cheese, indicating that Lys^{79}–Glu^{80} was one of the bonds intrinsically sensitive to plasmin. Two peptides arising from the cleavage of the Lys^{79}–Glu^{80} bond were also found in the mixture derived from an *in vitro* hydrolysate of α_{s1}-casein by plasmin (Le Bars & Gripon, 1989). The formation of α_{s1}-CNf(61–79) may be explained by the further attack on the Met^{60}–Glu^{61} bond by an endopeptidase and the consequent formation of the three peptides α_{s1}-CNf(1–60)2P, α_{s1}-CNf(23–60)2P and α_{s1}-CNf(61–79)5P. No phosphopeptide component from the 1–60 region was detected in the 14 month old GP cheese sample because the molecular mass exceeded the upper limit of detection of our FAB–MS apparatus. However, two of the above peptides could give rise to α_{s1}-CNf(43–58)2P after trypsin treatment of the pH 4·6-soluble N fraction of the cheese (Table 1). The direct determination of these CPP peptides would require a mass spectrometric technique suitable for high molecular mass measurement.

From a Comté cheese sample Roudot-Algaron *et al.* (1994) isolated three phosphopeptides derived from α_{s2}-casein, f(5–21), f(6–21) and f(7–21). They deduced that these peptides were derived from an N-terminal plasmin-mediated product consisting of α_{s2}-CNf(1–21). In both GP and Comté cheese, the formation of α_{s2}-casein CPP and peptide digestion seemed to proceed in similar manner, with an identical substrate, α_{s2}-CNf(1–21), involving endopeptidases and aminopeptidases progressively reducing the size of parent peptides. While the degradation of the α_{s2}-casein CPP proceeded in a similar manner at the N-terminal end in the GP and Comté cheese, only the GP CPP peptides were C-terminally processed, which may indicate a lower activity of carboxypeptidases B and A in Comté cheese.

Dephosphorylation of peptides

As is well known, phosphatase also plays an important role during cheese ripening, by removing phosphate groups from phosphorylated residues of phosphopeptides. In GP cheese, phosphatase may affect SerP residues differently, depending on their different affinities for Ca^{2+}. The pK_1 values of SerP residues in β-CNf(1–25)4P ranged from 6·57 for $SerP^{15}$ to 7·10 for $SerP^{17}$, $SerP^{18}$ and $SerP^{19}$. Thus Ca^{2+} binding to β-CNf(1–25)4P took place first on SerP residues 17, 18, and 19 at the higher pK_1; then, when these sites were saturated, on residue 15 at the lower pK_1 (Baumy *et al.* 1989). Since GP cheese contains high levels of calcium ($\sim$ 15 g/kg), one may suppose that β-CNf(15–28)4P may exist with $SerP^{17}$, $SerP^{18}$ and $SerP^{19}$ saturated by Ca^{2+}, and $SerP^{15}$ only partly saturated. Even taking into account that Ca^{2+} bound to SerP residues is completely exchangeable (Pierre *et al.* 1983), bound calcium may hinder the action of phosphatase. This mechanism seems to be confirmed by preliminary results of the kinetics of phosphatase action on phosphopeptides in solution. In the absence of Ca^{2+}, alkaline phosphatase was able to dephosphorylate phosphopeptides completely, while an excess of Ca^{2+} caused the reaction to proceed

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at a slower rate (F. Barone, unpublished results). Accordingly, the susceptibility of SerP to dephosphorylation may increase when [Ca^{2+}] decreases.

Our results confirm the dephosphorylation mechanism proposed by Weller (1979) consisting, for a phosphopeptide containing four closely located residues, of the removal of one phosphate group more easily than the second one and so on for successive residues. In GP cheese, phosphatase removed phosphate groups only from the N-terminal SerP, exposing the dephosphorylated residue to aminopeptidase action. The dephosphorylated internal Ser sites occur naturally in cheese, since they derive from the parent discretely phosphorylated proteins. This mechanism apparently does not fully agree with the results on Comté cheese of Roudot-Algaron *et al.* (1994) who, in addition to the dephosphorylated N-terminal $SerP^{15}$, found other dephosphorylated residues at interior sites of the peptide chain.

Alkaline milk phosphatase(s) and other indigenous enzymes may assist in degrading peptides to FAA during GP ripening. It has been reported recently (Resmini *et al.* 1985) that only trace amounts of free SerP occur in cheese during ripening and only free Ser accumulated, regardless of the age. Moreover, in the same study it was demonstrated that during *in vitro* hydrolysis by a sequence of enzymes, SerP is not split from casein, and only in the presence of phosphatase activity is the theoretical amount of constituent amino acids of casein recovered, all SerP being recovered as free Ser. Together with the findings of the present study, these results suggest that CPP degradation during the ripening of GP cheese occurs first by removal of P_i from the N-terminal SerP and then by degradation of partly dephosphorylated peptides by aminopeptidases to give peptides depleted of the N-terminal Ser (or other residue).

Phosphopeptides resistant to hydrolysis

Both α_{s1}- and β-casein contain peptides of similar sequence containing the phosphorylated stretch -SerP-X-SerP-SerP-SerP-Glu-Glu-Y, where X is Ile or Leu and Y is Ile or Ser for α_{s1}- and β-casein respectively. Only two components containing these sequences, β-CNf(16–22)3P and β-CNf(15–22)3P, were detected in 14 month old GP cheese, their counterparts derived from α_{s1}-casein not being detected. In addition, the level of β-CNf(16–22)3P increased during GP ripening while that of α_{s1}-CNf(64–74)5P decreased, as it was partly digested to give several closely related peptides among which α_{s1}-CNf(67–74)1P was found (see Table 3). The formation of α_{s1}-CNf(67–74)1P, and of intermediate phosphorylated forms of α_{s1}-CNf(64–74)5P, indicated that the phosphopeptides derived from α_{s1}-casein are more sensitive to phosphatase action than those from their β-casein counterpart. The difference in susceptibility to phosphatase and exopeptidase of phosphopeptides derived from α_{s1}- and β-casein is surprising, since their sequences are similar. Similarly, the peptide α_{s2}-CNf(7–18)4P partly digested to α_{s2}-CNf(8–18)3P was affected by dephosphorylation less than the counterpart from α_{s1}-casein. In conclusion, ripening in GP cheese consisted of a series of successive events starting with the solubilization of peptides from calcium para-caseinate followed by a further degradation to oligopeptides of decreasing size and FAA.

Cheese and anticariogenic activity

Soluble CPP seem very important to the potential anticariogenic activity of hard cheese. Silva *et al.* (1986) showed that cheese eaten immediately after sucrose rinses results in a large reduction in demineralization of the enamel. An average reduction of 55·7 % in the cariogenicity of sucrose was reported, indicating that cheese contains

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water-soluble components that inhibit the development of tooth decay in humans (Silva *et al*. 1987). The pioneering work of Reynolds and co-workers (for review, see Reynolds, 1994) showed that the amorphous calcium phosphate complexes of CPP were responsible for *in vitro* anticariogenic activity. CPP produced by an *in vitro* trypsinolysis of casein stabilized calcium phosphate in solution and substantially increased the level of calcium phosphate in dental plaque. The current view is that CPP transport amorphous calcium phosphate to dental plaque, buffering the Ca^{2+} and phosphate ions and helping to maintain a state of supersaturation with respect to tooth enamel. This depresses demineralization and enhances mineralization. Since 14 month old GP cheese contains $\sim$ 15 g soluble CPP/kg cheese, a mechanism similar to that demonstrated by Reynolds (1994) probably occurs *in vivo*, helping to stabilize and localize amorphous calcium phosphate complexes at the tooth surface.

The characterization of phosphopeptide components in Grana Padano cheese first demonstrates a close relationship between cheese ripening and a possible bioavailability of cheese components, and furthermore seems to open new horizons on the possible role of the cheese phosphopeptides for the control of dental caries in humans.

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