Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese

By TANOJ K. SINGH*†‡, PATRICK F. FOX†‡ and ÁINE HEALY‡

†*Department of Food Chemistry and* ‡*National Food Biotechnology Centre, University College, Cork, Irish Republic*

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Summary. Several peptides were isolated from the diafiltration retentate, prepared using 10 kDa membranes, of the water-soluble extract from a commercial mature Cheddar cheese and identified by amino acid sequencing and mass spectrometry. Most of the peptides were from the N-terminal half of β-casein, but peptides from α_{s1}- and α_{s2}-caseins were also identified; the extract also contained α-lactalbumin. Identified peptides showed the important role played by lactococcal cell envelope proteinases in the degradation of primary proteolytic products from α_{s1}- and β-caseins, produced by chymosin and plasmin respectively. Plasmin seemed to be involved in the hydrolysis of α_{s2}-casein. Several phosphopeptides were identified and the action of phosphatase on these peptides was evident.

During the ripening of Cheddar cheese, caseins are hydrolysed by coagulant, plasmin, starter and non-starter bacterial proteinases and peptidases, resulting in the formation of products ranging from water-insoluble polypeptides, comparable in size to the intact caseins, through water-soluble intermediate-sized and small peptides to free amino acids (Fox *et al.* 1994). Several peptides in the water-insoluble (McSweeney *et al.* 1994) and water-soluble (Fox *et al.* 1994; Singh *et al.* 1994, 1995) fractions of Cheddar cheese have been isolated and identified.

Normally, $\sim 6\%$ of the coagulant (chymosin) is retained in Cheddar cheese curd (see Fox, 1989) and this rapidly hydrolyses α_{s1}-casein (CN) at the bond Phe^{23}–Phe^{24}, and possibly also the bond Phe^{24}–Val^{25}, producing the peptides α_{s1}-CN(24–199) (also called α_{s1}-I-CN) and α_{s1}-CN(1–23). α_{s1}-CN(1–23) is hydrolysed rapidly in cheese by lactococcal cell envelope proteinases (CEP) to several small peptides (Kaminogawa *et al.* 1986; Fox *et al.* 1994; Singh *et al.* 1994, 1995). The concentration of α_{s1}-CN(24–199) increases initially but it is further hydrolysed by chymosin and CEP (see Fox *et al.* 1994; Exterkate & Alting, 1995; Singh *et al.* 1995).

Chymosin has limited action on β-CN in Cheddar cheese, although some activity is evident by the presence of a low level of the peptide β-CN(1–192) (β-I-CN) in the water-insoluble fraction (McSweeney *et al.* 1994). Several γ-CN (γ_1-CN (β-CN(29–209), γ_2-CN (β-CN(106–209) and γ_3-CN (β-CN(108–209)) formed by the action of plasmin on the bonds Lys^{28}–Lys^{29}, Lys^{105}–His^{106} and Lys^{107}–Glu^{108} of β-CN (Eigel *et al.* 1984), have been identified in Cheddar cheese (McSweeney *et al.* 1994). The concentrations of γ-CN increase during ripening (Farkye & Fox, 1990) and they are more pronounced in Gouda (Visser & de Groot-Mostert, 1977) and Emmental

* For correspondence. Present address: Department of Food Science and Nutrition, University of Minnesota, St Paul, MN 55108, USA.

cheeses (Ollikainen & Kivelä, 1989) than in Cheddar. Singh *et al.* (1995) resolved the diafiltration (DF) retentate of the water-soluble extract into eight fractions by ion-exchange chromatography on DEAE-cellulose. Most (45) of the 51 peptides identified in fractions I and II of the retentate originated in β-CN, especially from the sequence Asp^{47}–Pro^{110}. The N-terminals of most of the peptides corresponded to lactococcal CEP cleavage sites, but the C-terminals of only a few corresponded to a reported CEP cleavage site. From the peptides identified so far, it appears that β-CN was hydrolysed initially by plasmin and the resulting polypeptides, especially N-terminal proteose peptones, were degraded further by starter CEP.

In this study, work on the identification of peptides from DF retentate was extended by characterization of peptides in DEAE fractions III and IV.

EXPERIMENTAL

Cheese sample and fractionation of cheese nitrogen

Several water-soluble peptides have been isolated from a sample of commercial mature Cheddar cheese and subsequently characterized (Fox *et al.* 1994; Singh *et al.* 1994, 1995). The same cheese sample was used in the present study. Fractionation of the cheese nitrogen and chromatography of the DF retentate of the water-soluble extract on DEAE-cellulose were described by Singh *et al.* (1995).

Reversed-phase high-performance liquid chromatography

Peptides in the ion-exchange fractions III and IV were resolved, and collected, by reversed-phase HPLC on a Nucleosil C_8-wide pore column (5 μm, 300 Å, 250 × 4·6 mm; HPLC Technology Ltd, Macclesfield SK11 6PJ, UK) as described by Singh *et al.* (1995). Lyophilized peaks (Fig. 1*a*, *b*) were dissolved in acetonitrile (100 ml l^{-1}, HPLC far u.v. grade; Labscan Ltd, Dublin, Irish Republic) containing trifluoroacetic acid (1 ml l^{-1}, sequencing grade; Sigma Chemical Co., St Louis, MO 63178, USA) and filtered through a 0·45 μm Acrodisk LC PVDF syringe filter (Gelman Sciences, Ann Arbor, MI 48103-9019, USA). Samples were further resolved, and collected, by rechromatography on the same Nucleosil C_8 column using a linear gradient of 100–500 ml/l of solvent B (1 ml trifluoroacetic acid/l acetonitrile) in solvent A (1 ml trifluoroacetic acid/l deionized water) over 80 min (result not shown).

Peptide identification

The isolated peptides were subjected to N-terminal amino acid sequencing by automated Edman degradation and mass analysis using a Plasma Desorption mass spectrometer (see Singh *et al.* 1995).

Peptides were identified from the sequence of their five to seven N-terminal residues (no seven residue sequence is duplicated in any of the caseins, and no more than two peptides were read from any sequencing report) and their molecular masses using GPMAW software (Lighthouse Data, DK-5250 Odense SV, Denmark). Average values for the molecular masses of amino acids were used to calculate the theoretical molecular masses of peptides.

RESULTS

Peptides from fraction III

Twenty-one peaks in fraction III of the DF retentate were collected, as shown in Fig. 1 (*a*). After lyophilization, peptides were subjected to N-terminal sequencing and

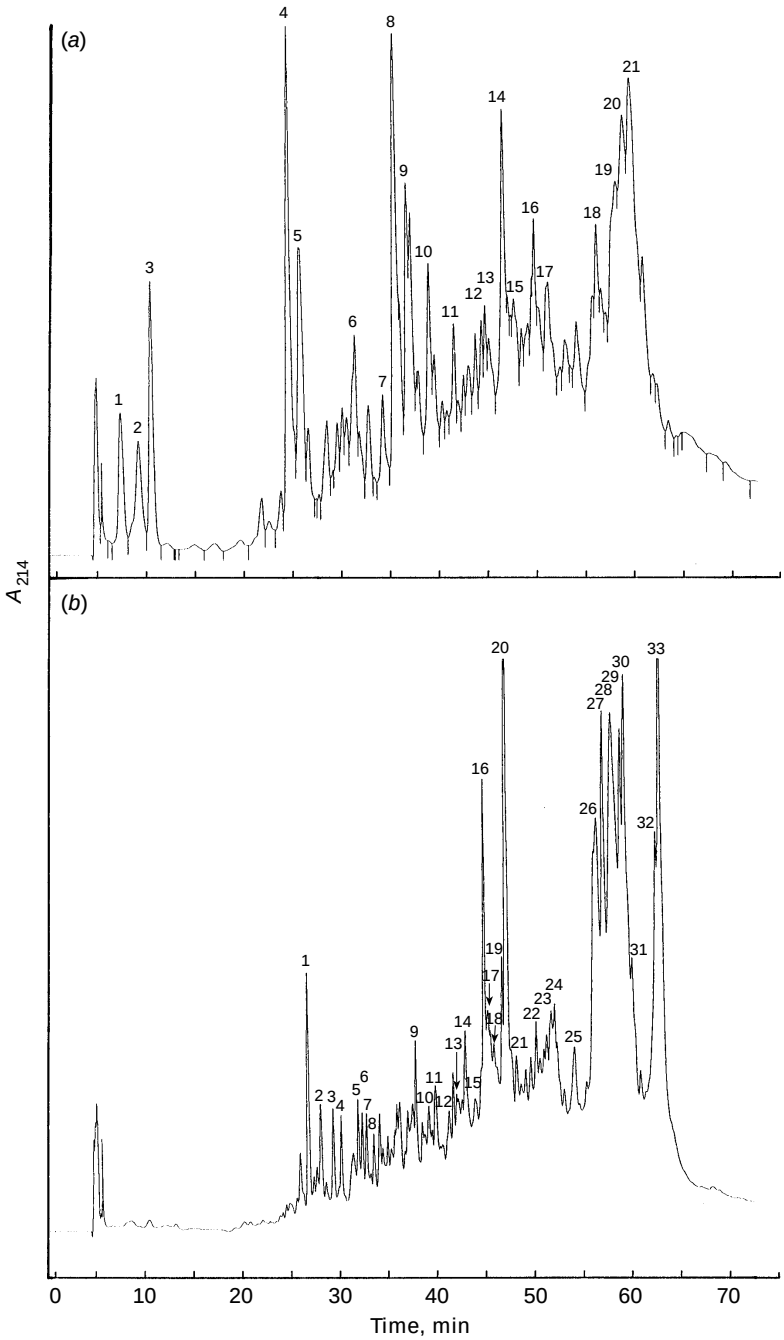

Fig. 1. Reversed-phase HPLC profile of (*a*) fraction III and (*b*) fraction IV of diafiltration retentate from a water-soluble extract of Cheddar cheese, carried out as described in the text, showing the peaks collected and identified. Peak numbers refer to (*a*) Table 1 and (*b*) Table 2.

analysed by mass spectrometry. Many of the peaks contained more than one peptide and a total of 27 peptides in fraction III were identified completely or partly (Table 1). Twelve of the peptides were derived from α_{s1}-CN. Five of these were present in peaks 1–5 and had Ser^{75} as their N-terminal. Unfortunately, no mass values were

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Table 1. *Peptides in fraction III from DEAE-cellulose chromatography of diafiltration retentate from a water-soluble extract of Cheddar cheese*

Peak	N-terminal residues	Molecular mass, Da: Experimental	Molecular mass, Da: Theoretical	Peptide identification
1	Ser–Val–Glu–Gln–Lys	—	—	α_{s1}-CN(75–?)†
2	Ser–Val–Glu–Gln–Lys	—	—	α_{s1}-CN(75–?)†
3	Ser–Val–Glu–Gln–Lys	—	—	α_{s1}-CN(75–?)†
4	Ser–Val–Glu–Gln–Lys	—	—	α_{s1}-CN(75–?)†
5	Ser–Val–Glu–Gln–Lys	968·8	968·1	α_{s1}-CN(75–82)‡
	Gly–Glu–Ile–Val–Glu	718·4	712·7	β-CN(10–15)§
6	Asp–Val–Pro–Ser–Glu	880·9	864·9	α_{s1}-CN(85–91)
	Asp–Val–Pro–Ser–Glu	1295·7	1311·5	α_{s1}-CN(85–95)
7	Val–Pro–Gly–Glu–Ile	742·8	741·9	β-CN(8–14)
	Val–Pro–Gly–Glu–Ile	1665·6	1661·8	β-CN(8–23)‖
8	Asn–Val–Pro–Gly–Glu	857·5	855·9	β-CN(7–14)
	Asn–Val–Pro–Gly–Glu	947·7	943·0	β-CN(7–15)¶
9	Gly–Tyr–Leu–Glu–Gln	—	—	α_{s1}-CN(93–?)†
10	Asp–Val–Pro–Ser–Glu	980·5	978·1	α_{s1}-CN(85–92)
11	Gly–Tyr–Leu–Glu–Gln	1752·5	1751·1	α_{s1}-CN(93–106)
12	Phe–Val–Ala–Pro–Phe–Pro	—	—	α_{s1}-CN(24–?)†
13	Lys–Ile–Glu–Lys–Phe	—	—	β-CN(29–?)†
14	Lys–Ile–Glu–Lys–Phe	3179·7	3174·4	β-CN(29–54)††
15	Ser–Leu–Val–Tyr–Pro	1399·3	1387·6	β-CN(57–69)‡‡
16	Ser–Asp–Ile–Pro–Asn	891·0	899·0	α_{s1}-CN(180–188)
17	Ser–Leu–Val–Tyr–Pro	1542·0	1540·8	β-CN(57–70)§§
18	Ser–Leu–Pro–Gln–Asn	2622·2	2611·1	β-CN(69–92)
19	Ala–Gln–Thr–Gln–Ser	—	—	β-CN(53–?)†
	Ser–Leu–Val–Tyr–Pro	—	—	β-CN(57–?)†
	Val–Tyr–Pro–Phe–Pro	—	—	β-CN(59–?)†
20	Leu–Val–Tyr–Pro–Phe	—	—	β-CN(58–?)†
21	Ser–Leu–Val–Tyr–Pro	4128·6	4121·9	β-CN(57–94)§§

† Samples failed to give results on mass spectrometer.
‡ Ser^{75} was dephosphorylated.
§ Ser^{15} was phosphorylated.
‖ Ser^{15}, Ser^{17}, Ser^{18} and Ser^{19} were dephosphorylated.
¶ Ser^{15} was dephosphorylated.
†† Ser^{35} was dephosphorylated.
‡‡ Pro^{67} (β-CN A^2 and A^3 variants).
§§ His^{67} (β-CN A^1, B and C variants).

obtained for peptides in peaks 1–4. In α_{s1}-CN, residue Ser^{75} is phosphorylated (Swaisgood, 1992). The residue Ser^{75} was dephosphorylated in α_{s1}-CN(75–82) (peak 5, based on molecular mass).

Fraction III contained 15 peptides which originated from three segments of the N-terminal half of β-CN. Five of the peptides in peaks 5, 7 and 8 (Table 1) originated from a small segment near the N-terminal of β-CN. The N-terminal region (especially residues 1–40) of β-CN is highly negatively charged, containing five phosphate groups esterified to serine residues 15, 17, 18, 19 and 35 (Swaisgood, 1992). Ser^{15} was present in phosphorylated form in peptide β-CN(10–15) but was dephosphorylated in β-CN(7–15). There are four phosphorylated serines in the sequence 8–23 of β-CN, but in the peptide β-CN(8–23) all of them were dephosphorylated (based on molecular mass).

Lys^{29} of β-casein was the N-terminal of two peptides (peaks 13 and 14). This region of β-CN contains one phosphorylated residue, Ser^{35}, and an amino acid substitution in genetic variants (Glu^{37} or Lys^{37}: β-CN A and B, or C variants). The peptide in peak 14 was found to contain a dephosphorylated seryl residue at position

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35 and glutamic acid as residue 37, i.e. it originated from genetic variants A or B of β-CN (based on molecular mass). Unfortunately, the molecular mass of peptide β-CN(29–?) in peak 13 could not be determined.

A number of peptides in peaks 15 and 17–21 had Ala^{53}, Ser^{57}, Leu^{58}, Val^{59} or Ser^{69} as the N-terminal residue. Residue 67 is another possible site for genetic substitution of amino acids in native β-casein, i.e. Pro^{67} in variants A^2 and A^3 and His^{67} in variants A^1, B and C. The amino acid residue at position 67 in β-CN(57–69) (peak 15) was proline (genetic variants A^2 and A^3) while in β-CN(57–70) (peak 17) and β-CN(57–94) (peak 21) it was histidine (genetic variants A^1, B and C) (based on molecular mass).

Peptides in fraction IV

Ion-exchange fraction IV of the DF retentate was resolved into 33 peaks by reversed-phase HPLC (Fig. 1*b*). A total of 53 peptides were completely or partly identified in the peaks collected (Table 2). This fraction contained seven peptides originating from α_{s1}-CN, which contains eight phosphorylated serine residues (Swaisgood, 1992), although only Ser^{75} and Ser^{115} are relevant here. Ser^{115} was phosphorylated in peptides α_{s1}-CN(115–121) (peak 1) and α_{s1}-CN(115–124) (peak 3) but dephosphorylated in α_{s1}-CN(115–121) in peak 2 (based on molecular mass). Ser^{75} was dephosphorylated in α_{s1}-CN(70–76) (peak 11). No mass was obtained for the α_{s1}-CN peptides in peaks 13, 14 and 22, which were identified by their N-terminal amino acid sequences.

Fraction IV contained four peptides originating from α_{s2}-CN, two each in peaks 1 and 24. Native α_{s2}-CN has 11 phosphoseryl residues (Swaisgood, 1992) but only Ser^{61} is relevant here. Ser^{61} was phosphorylated in the peptide α_{s2}-CN(61–70) but dephosphorylated in α_{s2}-CN(61–71) (based on molecular mass); both of these peptides were present in peak 1.

Fraction IV contained numerous β-CN peptides (42 of 53). Two peptides containing the six or seven N-terminal amino acid residues of β-CN, i.e. β-CN(1–6) and β-CN(1–7), were present in peak 10. Peptide β-CN(7–15), phosphorylated at Ser^{15}, was identified in peak 15. The same peptide, with Ser^{15} dephosphorylated, was identified in peak 8 of fraction III (Table 1). Peptide β-CN(7–18), in peak 13, contained three possible phosphorylation sites (serine residues 15, 17 and 18) but only two phosphate residues were confirmed by molecular mass measurement, while in peptide β-CN(7–19) (peak 12) three of the four seryl residues (at 15, 17, 18 and 19) were phosphorylated. Peptide β-CN(28–?) (peak 19) failed to yield a result on mass spectrometry. Nine peptides had Lys^{29} as their N-terminal amino acid. Peptides β-CN(29–56), β-CN(29–37) and β-CN(29–39), present in peaks 5, 16 and 18 respectively, contained Glu^{37} (genetic variants A and B of β-CN) but the peptides β-CN(29–56) and β-CN(29–39) in addition had Ser^{35} in phosphorylated form. No mass was obtained for five peptides, β-CN(29–?), in peaks 7, 8, 17, 20 and 21. Three peptides were found to start at Ile^{30}, present in peaks 4, 6 and 20. Peptide β-CN(30–36) (peak 4) contained an esterified phosphate group at Ser^{35} but in a peptide with same sequence in peak 6 it was dephosphorylated (based on molecular mass). Numerous β-CN peptides identified in fraction IV had Ala^{53}, Ser^{57}, Tyr^{60}, Ser^{69} or Asn^{73} as their N-terminal amino acid, especially Ser^{57} and Asn^{73}. Most of the peptides that included residue 67 contained His^{67} (β-CN genetic variants A^1 and B) and only three peptides, β-CN(60–68), β-CN(57–72) and β-CN(60–97) in peaks 18, 23 and 27 respectively, contained Pro^{67}, based on molecular mass (β-CN genetic variants A^2 and A^3). Only two peptides, β-CN(171–?) (peak 13) and β-CN(177–191) (peak 22) were found to

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Table 2. *Peptides in fraction IV from DEAE-cellulose chromatography of diafiltration retentate from a water-soluble extract of Cheddar cheese*

Peak	N-terminal residues	Molecular mass, Da: Experimental	Molecular mass, Da: Theoretical	Peptide identification
1	Ser–Ala–Glu–Glu–Arg	922·7	920·9	α_{s1}-CN(115–121)†
	Ser–Ala–Glu–Val–Ala	1143·6	1142·1	α_{s2}-CN(61–70)‡
	Ser–Ala–Glu–Val–Ala	1172·6	1175·1	α_{s2}-CN(61–71)§
2	Ser–Ala–Glu–Glu–Arg	842·5	840·9	α_{s1}-CN(115–121)‖
3	Ser–Ala–Glu–Glu–Arg	1264·6	1267·3	α_{s1}-CN(115–124)†
4	Ile–Glu–Lys–Phe–Gln	962·1	959·9	β-CN(30–36)¶
5	Lys–Ile–Glu–Lys–Phe	3483·3	3483·6	β-CN(29–56)¶
6	Ile–Glu–Lys–Phe–Gln	881·6	880·0	β-CN(30–36)††
7	Lys–Ile–Glu–Lys–Phe	—	—	β-CN(29–?)‡‡
8	Lys–Ile–Glu–Lys–Phe	—	—	β-CN(29–?)‡‡
9	Lys–Ile–Glu–Lys–Phe	670·1	663·8	β-CN(29–33)
10	Arg–Glu–Leu–Glu–Glu	790·0	787·9	β-CN(1–6)
	Arg–Glu–Leu–Glu–Glu	903·5	902·0	β-CN(1–7)
11	Glu–Ile–Val–Pro–Asn	757·4	756·8	α_{s1}-CN(70–76)§§
12	Asn–Val–Pro–Gly–Glu	1559·4	1557·4	β-CN(7–19)‖ ‖
13	Asn–Val–Pro–Gly–Glu	1385·0	1390·3	β-CN(7–18)¶¶
	Glu–Ile–Val–Pro–Asn	—	—	α_{s1}-CN(70/110–?)‡‡
	Leu–Pro–Val–Pro–Glu	—	—	β-CN(171–?)‡‡
14	Asn–Val–Pro–Gly–Glu	—	—	β-CN(7–?)‡‡
	Glu–Ile–Val–Pro–Asn	—	—	α_{s1}-CN(70/110–?)‡‡
15	Asn–Val–Pro–Gly–Glu	1044·0	1023·0	β-CN(7–15)††† ‡‡‡
16	Asp–Glu–Leu–Gln–Asp	—	—	β-CN(43–?)‡‡
	Lys–Ile–Glu–Lys–Phe	1142·4	1137·3	β-CN(29–37)††
17	Lys–Ile–Glu–Lys–Phe	—	—	β-CN(29–?)‡‡
18	Lys–Ile–Glu–Lys–Phe	1468·5	1473·5	β-CN(29–39)¶
	Tyr–Pro–Phe–Pro–Gly	1002·2	1001·2	β-CN(60–68)§§§
19	Lys–Lys–Ile–Glu–Lys	—	—	β-CN(28–?)‡‡
20	Lys–Ile–Glu–Lys–Phe	—	—	β-CN(29–?)‡‡
	Ile–Glu–Lys–Phe–Gln	—	—	β-CN(30–?)‡‡
21	Lys–Ile–Glu–Lys–Phe	—	—	β-CN(29–?)‡‡
22	Ser–Leu–Val–Tyr–Pro	1085·2	1089·3	β-CN(57–66)
	Ala–Val–Pro–Tyr–Pro	1767·7	1746·1	β-CN(177–191)‡‡‡
	Tyr–Leu–Gly–Tyr–Leu	—	—	α_{s1}-CN(91–?)‡‡
23	Ser–Leu–Val–Tyr–Pro	1725·9	1726·0	β-CN(57–72)§§§
	Ser–Leu–Val–Tyr–Pro	1340·1	1340·5	β-CN(57–68)‖ ‖ ‖
	Ser–Leu–Val–Tyr–Pro	1542·4	1540·8	β-CN(57–70)‖ ‖ ‖
	Ser–Leu–Val–Tyr–Pro	1765·4	1766·0	β-CN(57–72)‖ ‖ ‖
24	Asn–Met–Ala–Ile–Asn	1352·0	1333·5	α_{s2}-CN(25–36)
	Ala–Ile–Asn–Pro–Ser	1637·1	1654·9	α_{s2}-CN(27–41)
25	Ser–Leu–Pro–Gln–Asn	2574·2	2512·0	β-CN(69–91)‡‡‡
	Asn–Ile–Pro–Pro–Leu	2371·6	2373·9	β-CN(73–94)
	Asn–Ile–Pro–Pro–Leu	2087·4	2086·5	β-CN(73–91)
26	Asn–Ile–Pro–Pro–Leu	3538·5	3543·3	β-CN(73–105)
27	Ser–Leu–Val–Tyr–Pro	3837·7	3834·5	β-CN(57–91)‖ ‖ ‖
	Tyr–Pro–Phe–Pro–Gly	4080·6	4096·9	β-CN(60–97)§§§
28	Ala–Gln–Thr–Gln–Ser	4494·0	4493·3	β-CN(53–93)‖ ‖ ‖
	Ser–Leu–Val–Tyr–Pro	3937·5	3933·6	β-CN(57–92)‖ ‖ ‖
	Tyr–Pro–Phe–Pro–Gly	4010·0	4008·7	β-CN(60–96)‖ ‖ ‖
	Tyr–Pro–Phe–Pro–Gly	4494·0	4493·3	β-CN(60–100)‖ ‖ ‖
29	Ser–Leu–Val–Tyr–Pro	4311·7	4308·1	β-CN(57–96)‖ ‖ ‖
30	Ser–Leu–Val–Tyr–Pro	4065·7	4064·8	β-CN(57–93)‖ ‖ ‖
31	Ser–Leu–Val–Tyr–Pro	4062·5	4064·8	β-CN(57–93)‖ ‖ ‖
	Ser–Leu–Val–Tyr–Pro	4221·6	4221·0	β-CN(57–95)‖ ‖ ‖
32, 33	Glu–Gln–Leu–Thr–Lys	14174·0	14174·0	α-Lactalbumin

† Ser^{115} was phosphorylated.
‡ Ser^{61} was phosphorylated.
§ Ser^{61} was dephosphorylated.

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have originated from the C-terminal region of β-CN. The whey protein, α-lactalbumin, was identified in peaks 32–33.

The peptides identified in ion-exchange fractions III and IV of the DF retentate ranged from 664 to 4494 Da. Mass measurements correlated well with theoretical calculated mass. Most of the β-CN peptides identified in the two fractions originated from the N-terminal half, residues 1–107, of the molecule, especially from the sequence 53–107. A similar observation was made in the case of β-CN peptides in fractions I and II of the DF retentate (Singh *et al*. 1995).

DISCUSSION

Peptides isolated from fractions III (Table 1) and IV (Table 2) originated from α_{s1}-CN, α_{s2}-CN and especially from β-CN. Theoretically, all these peptides were small enough (664–4494 Da) to pass through the ultrafiltration membranes (nominal molecular mass cut-off 10 kDa) used in this study. Most of the hydrophobic peptides in the cheese water-soluble extract were retained and concentrated in the DF retentate (Singh *et al*. 1995).

The α_{s1}-CN peptides identified are summarized on a line diagram of α_{s1}-CN in Fig. 2, together with the known cleavage sites of chymosin and lactococcal CEP (mixture of CEP_I and CEP_{III}). The peptides previously identified in fractions I and II of the DF retentate (Singh *et al*. 1995) and permeate of the water-soluble extract (Fox *et al*. 1994; Singh *et al*. 1994) and the water-insoluble fraction (McSweeney *et al*. 1994) are also included. The α_{s1}-CN peptides so far isolated from the retentate originated from the larger peptide α_{s1}-CN(24–199) produced very early in ripening by chymosin action on the Phe^{23}–Phe^{24} bond of α_{s1}-CN. All the α_{s1}-CN peptides in fractions I and II (Singh *et al*. 1995) were from the N-terminal region of α_{s1}-CN(24–199), but with two exceptions, α_{s1}-CN(24–?) and α_{s1}-CN(180–188), the α_{s1}-CN-derived peptides identified in this study were from the sequence Glu^{70}–Lys^{124}.

In solution, chymosin cleaves the bond Trp^{164}–Tyr^{165} of α_{s1}-CN rapidly following cleavage of the primary site Phe^{23}–Phe^{24} (McSweeney *et al*. 1993; Exterkate & Alting, 1995); however, there is no evidence so far that this bond is cleaved in cheese. Overall, lactococcal CEP appears to play a very important role in the breakdown of α_{s1}-CN(24–199) during cheese ripening. Activity of some lactococcal endopeptidases, e.g. PepO or PepF, intracellular proteinases and/or proteinases and peptidases of non-starter lactic acid bacteria may have been involved in the production of those α_{s1}-CN peptides the release of which cannot be explained on the basis of known specificities of chymosin or lactococcal CEP. α_{s1}-CN peptides so far identified in fractions I–IV of the DF retentate originated mainly from the sequences residues 24–39 and 70–124, but four peptides from the gap (residues 40–69) were identified in the DF permeate of the water-soluble extract (Fox *et al*. 1994). Peptides from the N-terminal region of the chymosin-produced peptide α_{s1}-CN(1–23) were identified in

‖ Ser^{115} was dephosphorylated.
¶ Ser^{35} was phosphorylated.
†† Ser^{35} was dephosphorylated.
‡‡ Samples failed to give results on mass spectrometer.
§§ Ser^{75} was dephosphorylated.
‖‖ Three phosphate groups among Ser^{15}, Ser^{17}, Ser^{18} and Ser^{19}.
¶¶ Two phosphate groups among Ser^{15}, Ser^{17} and Ser^{18}.
††† Ser^{15} was phosphorylated.
‡‡‡ Differences between theoretical and experimental masses were probably due to Na^+, K^+ or HCO_3^-.
§§§ Pro^{67} (β-CN A^2 and A^3 variants).
‖‖‖ His^{67} (β-CN A^1, B and C variants).

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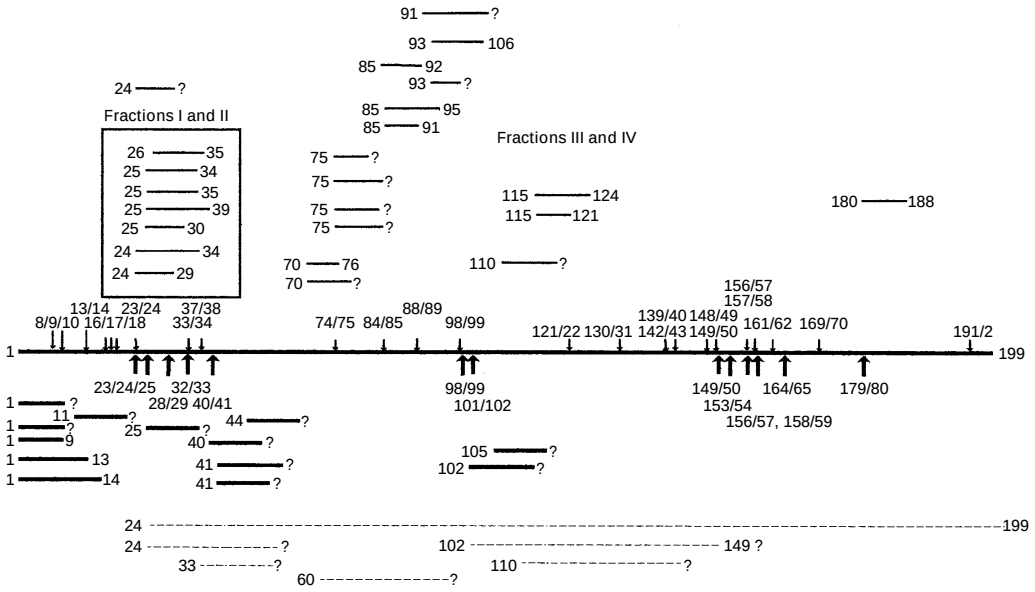

Fig. 2. α_{s1}-Casein-derived peptides isolated from ——, the water-insoluble fraction (McSweeney *et al.* 1994), and from ——, the diafiltration permeate (Fox *et al.* 1994; Singh *et al.* 1994) and ——, the diafiltration retentate (fractions I–II, Singh *et al.* 1995, and III–IV, present study) of the water-soluble fraction of Cheddar cheese. ↑, Chymosin cleavage sites; ↓, cleavage sites of *Lactococcus* sp. cell envelope proteinase (mixture of CEP_I and CEP_{III}); ?, incomplete sequence.

the DF permeate, e.g. α_{s1}-CN(1–9), α_{s1}-CN(1–13), α_{s1}-CN(1–14) and α_{s1}-CN(11–?) (Fox *et al.* 1994; Singh *et al.* 1994), but the peptides from the C-terminal region of α_{s1}-CN(1–23) have not yet been isolated.

β-CN peptides are summarized on a line diagram of β-CN in Fig. 3, together with known plasmin and selected CEP cleavage sites (mixture of CEP_I and CEP_{III}). Plasmin plays an important role in the primary hydrolysis of β-CN; bonds Lys^{28}–Lys^{29}, Lys^{105}–His^{106} and Lys^{107}–Glu^{108} are the main cleavage sites in solution (Eigel *et al.* 1984). These bonds are also cleaved in Cheddar cheese, as indicated by the presence of the C-terminal fragments, i.e. γ-CN, in the water-insoluble fraction (McSweeney *et al.* 1994) and two N-terminal fragments, proteose peptone (PP)-5 (β-CN(1–105) and β-CN(1–107)) in the DF retentate of the water-soluble fraction (Singh *et al.* 1995). Several peptides originated from β-CN(1–28), e.g. PP-8 fast, which is produced on hydrolysis of the Lys^{28}–Lys^{29} bond of β-CN by plasmin. In the present study, most of the β-CN peptides originated from the polypeptide β-CN(29–105/107, i.e. PP-8 slow, produced by plasmin. The sum of peptides β-CN(29–56), β-CN(57–72) and β-CN(73–105) represents the complete sequence of PP-8 slow (β-CN(29–105)). Peptides with an N-terminus corresponding to Ala^{53}, Ser^{57}, Leu^{58}, Val^{59} or Ser^{69} fall directly on CEP cleavage sites. The peptide β-CN(58–72) was isolated from the water-soluble extract of Cheddar and Jarlsberg cheeses by Stepaniak *et al.* (1995) and found to inhibit intracellular lactococcal endopeptidase (PepO). From the above results it appears that the lactococcal CEP actively hydrolysed peptide bonds in the N-terminal half of β-CN, especially in the region His^{50}–Lys^{107}. Considering the large number of peptides found, bonds Gln^{56}–Ser^{57} and Asn^{68}–Ser^{69} seemed to be the preferred cleavage sites for CEP under the conditions prevailing in Cheddar cheese. In solution, the specificity of CEP_I on β-CN is rather broad but glutamine and serine residues are most frequently found in the cleavage sites while all cleavage sites lie in

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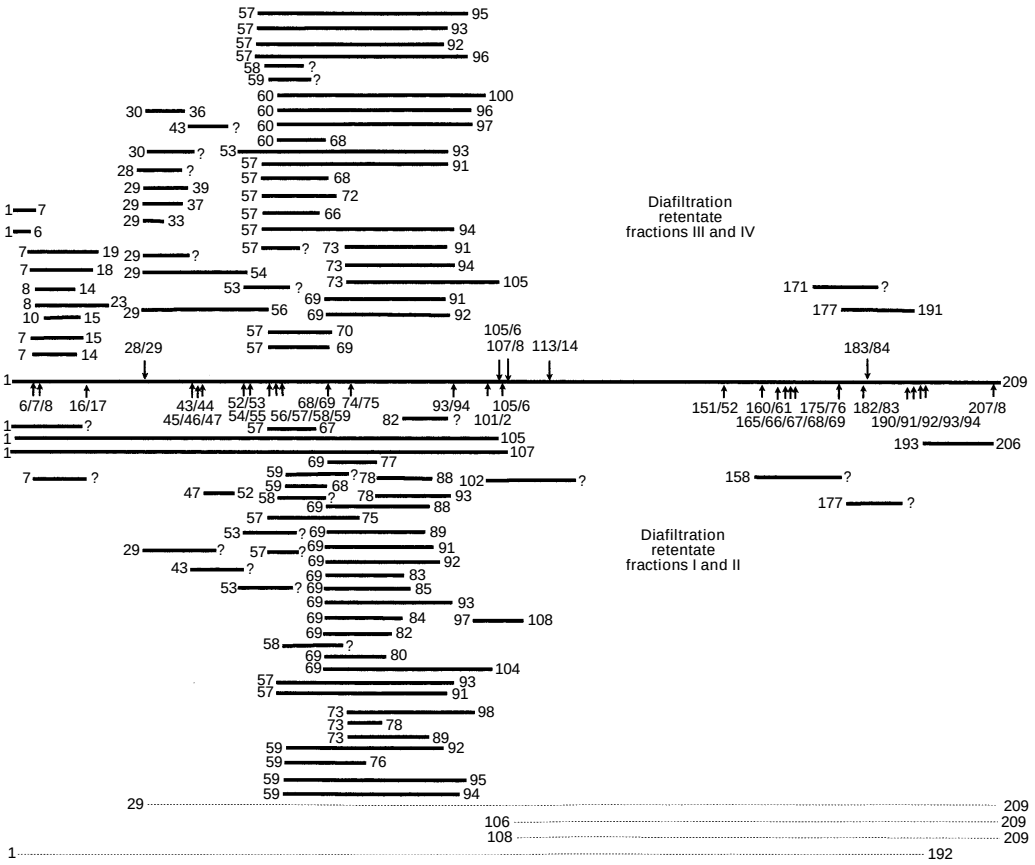

Fig. 3. β-Casein-derived peptides isolated from ·····, the water-insoluble (McSweeney *et al.* 1994) and from ——, the diafiltration retentate (fractions I–II, Singh *et al.* 1995, and III–IV, present study) of the water-soluble fraction of Cheddar cheese. ↓, Plasmin cleavage sites; ↑, cleavage sites of *Lactococcus* sp. cell envelope proteinase (mixture of CEP_I and CEP_{III}); ?, incomplete sequence.

the C-terminal region of β-CN, which has a high hydrophobicity, a high proline content and a low charge (Monnet *et al.* 1989). For CEP_{III}, most of the primary cleavage sites are Glx–X or X–Glx bonds and in most cases a large hydrophobic residue (methionine, phenylalanine, leucine or tyrosine) is present at either P1 or P1′ (Reid *et al.* 1991; Visser *et al.* 1991). Peptides with sequences X–Pro–Y–Pro–Z–, e.g. β-CN(60–68) and β-CN(171–?), should be degraded by PepX into several X–Pro dipeptides. However, Singh *et al.* (1994) and Kaminogawa *et al.* (1986) demonstrated the accumulation of α_{s1}-CN(1–9), α_{s1}-CN(1–13) and α_{s1}-CN(1–14), which contain the N-terminal sequence Arg–Pro–, in Cheddar and Gouda type cheeses. The persistence of these peptides, i.e. α_{s1}-CN(1–9), α_{s1}-CN(1–13), α_{s1}-CN(1–14), β-CN(60–68) and β-CN(171–?), in cheese indicates that PepX has no or little activity in cheese, perhaps because it is unstable. Wilkinson *et al.* (1994) reported low levels of PepX activity in Cheddar cheese extract, and in the present study no PepX activity on Gly–Pro– and Ala–Pro-*p*-nitroanilide substrates was detected in a cheese slurry or extract.

The C-termini of some of the β-CN peptides stretched as far as the nearest known lactococcal CEP cleavage site, e.g. β-CN(29–54), β-CN(29–56), β-CN(53–93), β-CN(57–68), β-CN(57–93), β-CN(60–68) and β-CN(177–191), or a plasmin site, e.g. β-CN(73–105). However, the C-termini of most peptides fell short of the nearest

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reported cleavage site. Peptides β-CN(7–15), β-CN(57–72), β-CN(57–92), β-CN(69–92) and β-CN(60–100) were just one residue short of the nearest known lactococcal CEP cleavage site. Based on the known specificities of lactococcal CEP, the formation of these peptides would require carboxypeptidase activity. However, carboxypeptidase activity has not been reported in *Lactococcus* spp., although it has been reported in several lactobacilli (Atlan *et al*. 1993).

Addeo *et al*. (1992, 1994), who characterized water-soluble, low molecular mass peptides from Parmigiano-Reggiano cheese, found that the majority of β-CN peptides soluble in trichloroacetic acid (120 g/l) originated from PP-8 fast (β-CN(1–28)), while the trichloroacetic acid-insoluble peptides were mainly from the region β-CN(57–110).

Four peptides in fraction IV originated from α_{s2}-CN; these are the first reported peptides derived from α_{s2}-CN in Cheddar cheese. Plasmin is known to hydrolyse the bond Lys^{24}–Asn^{25} of α_{s2}-CN (Le Bars & Gripon, 1989; Visser *et al*. 1989), which may explain the origin of α_{s2}-CN(25–36), while the peptide α_{s2}-CN(27–41) may be formed by the removal of two residues by an aminopeptidase. The origin of peptides α_{s2}-CN(61–70) and α_{s2}-CN(61–71) cannot be explained on the basis of known specificities of chymosin, plasmin and CEP on α_{s2}-CN.

In this study, several phosphopeptides were identified that originated from α_{s1}-CN, α_{s2}-CN and β-CN and extensive dephosphorylation was observed (Tables 1 and 2). Weller (1979) reported that for a peptide derived from casein, which contained four closely located SerP residues, the first phosphate group was lost more easily than the second and so on. The peptides β-CN(7–18) and β-CN(7–19) identified in the present study contained two and three phosphate groups respectively, showing removal of one phosphate group which may have occurred at Ser^{15} (these sequences in β-CN contain three and four phosphate residues respectively). Complete dephosphorylation of the peptide β-CN(8–23), which in intact β-CN contains four phosphate groups, was observed. Addeo *et al*. (1992, 1994) identified numerous phosphopeptides in the pH 4·6-soluble extract of Parmigiano-Reggiano cheese. Thirteen low molecular mass phosphopeptides were isolated from the water-soluble extract of Comté cheese (Roudot-Algaron *et al*. 1994). Partial dephosphorylation of peptides was observed in the above studies.

The peptides in fractions I and IV characterized so far clearly showed that lactococcal CEP played a very important role in the further breakdown of primary proteolysis products, i.e. polypeptides produced by chymosin or plasmin from α_{s1}-CN and β-CN respectively. Plasmin seems to be involved in the breakdown of α_{s2}-CN but the role of CEP is not yet clear. Aminopeptidase and carboxypeptidase activities may have been involved. PepX seemed to have a limited or no role during cheese ripening. Work on the isolation and identification of peptides in the ion-exchange fractions V–VIII of the DF retentate is continuing.

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