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PII: S0958-6946(98)00116-2

Int. Dairy Journal **8** (1998) 813–818

Printed in Great Britain
0958-6946/99/$ – see front matter

Identification of the Principal Water-insoluble Peptides in Cheddar Cheese

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(Received 17 July 1998; accepted 11 September 1998)

ABSTRACT

The water-insoluble fraction (WISF) of a 20 week-old Cheddar cheese was fractionated by anion-exchange FPLC on Mono-Q® 5/5. Those fractions containing peptides were collected and analysed by urea-PAGE. Peptides of interest were isolated by electroblotting from the urea-PAGE gels and identified from their N-terminal amino acid sequence and mass. This study confirmed and completed identification of some of the WIS peptides partially identified by McSweeney *et al.* (*International Dairy Journal* **4,** 111–122, 1994) and identified a further 13 peptides. Most of the peptides can be attributed to cleavage of α_{s1}- and β-caseins by chymosin, plasmin or proteinase from *Lactococcus lactis* spp. © 1999 Elsevier Science Ltd. All rights reserved

Keywords: Cheddar cheese; water-insoluble peptides

INTRODUCTION

The biochemistry of cheese ripening involves three fundamental processes: glycolysis, lipolysis and proteolysis, all of which contribute to the texture, aroma and taste of the ripened cheese. Proteolysis is regarded as the most important biochemical event in Cheddar cheese ripening (Fox *et al.*, 1994). Primary proteolysis leads to the formation of large water-insoluble peptides and smaller water-soluble peptides. Many of the peptides in the water-soluble fraction have been isolated and identified (Singh *et al.*, 1994, 1995, 1997; Fernandez *et al.*, 1998). McSweeney *et al.* (1994) partially identified the principal water-insoluble peptides in Cheddar cheese by N-terminal sequencing.

The objective of this study was to isolate the principal peptides from the water-insoluble fraction (WISF) of Cheddar cheese and to identify them by N-terminal sequence analysis and mass spectrometry.

MATERIALS AND METHODS

Cheddar cheese was manufactured according to a standard protocol (Kosikowski, 1982). The coagulant used was standard calf rennet (Chr. Hansen's (Irl) Ltd., Rohan Industrial Estate, Little Island, Cork); *Lactococcus lactis* ssp. *cremoris* SK11 was used as starter. The cheese was ripened at 8°C. The WISF was prepared from the 20-week-old cheese, according to the method of Kuchroo and Fox (1983).

Peptides in the WISF were fractionated by FPLC on a Mono-Q® HR 5/5 column (Pharmacia Biotech Ltd., Uppsala, Sweden), that had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0), containing 4.5 mol L^{-1} urea and 8 mmol L^{-1} dithiothreitol (DTT) (Buffer A). A 500 μL sample, containing 15 mg mL^{-1} WISF dissolved in 50 mM Tris-HCl buffer (pH 8.0, 4.5 mol L^{-1} urea and 12 mmol L^{-1} DTT), was applied to the column. Elution was performed using a linear gradient of 0–85% Buffer B (i.e. Buffer A containing 0.5 mol L^{-1} NaCl). Eluate was monitored at 280 nm. Fractions from numerous runs were collected using a Frac-100 fraction collector (Pharmacia Biotech Ltd.) and those containing peptides were pooled, dialysed against distilled H_2O at 4°C and lyophilised.

Urea-PAGE was performed as described by Andrews (1983) and stained with Coomassie® Brilliant Blue by the method of Blakesley and Boezi (1977).

Peptides were isolated by electroblotting from urea-PAGE gels using a mini-Transblot™ electrophoretic transfer cell (Bio-Rad, Hercules, CA 94547, USA). Blotting conditions were as follows:

Peptides were transferred onto polyvinylidene difluoride (PVDF) membranes (pore size, 0.22 μm) at 90 V for 12 min in 10 mM cyclohexylamine sulphonic acid (CAPS) buffer, pH 11, in 10% methanol. Peptides were visualised by staining with Coomassie® Brilliant Blue R-250 in 50% methanol/1% acetic acid for 1 min, followed by destaining in 50% methanol.

Peptide-containing bands were excised from the PVDF membrane and subjected to N-terminal sequencing by Edman degradation on an Applied Biosystems model 477A pulsed liquid protein sequencer (Applied Biosystems Inc., Foster City, CA, USA). Liberated amino acids were detected as their phenylthiohydantoin derivatives using a model 120A analyser.

Mass spectrometry was performed on peptides which were extracted from the electroblotting membranes by

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the method of Sutton *et al.* (1995). Coomassie® Brilliant Blue-stained bands were excised and destained using 70% acetonitrile. The bands were then placed in a 1:1 mixture of formic acid and ethanol (300 μL) and extracted at 27–28°C for 2 h. The extract was removed, diluted with deionised H_2O, to facilitate freezing, and lyophilised. Mass spectrometry was performed on a LASERMAT 2000 MALDI time-of-flight mass spectrometer (Finnigan MAT Ltd., Paradise, Hemel Hempstead, UK). The accuracy of peptide mass determination was improved by using internal standard calibrants, as described by Gouldsworthy *et al.* (1996). Peptide mass searches of the different caseins were performed using the Protein Abacus software program, version 2.0.2 (Lighthouse Data, Finnigan MAT Ltd). The primary structure of the caseins and their variants are described by Swaisgood (1992).

RESULTS AND DISCUSSION

The cheese was ripened for 20 weeks and samples taken weekly up to week 6 and then fortnightly up to week 20. Electrophoretograms of the water-insoluble fraction of the cheeses are shown in Fig. 1. The 20 week sample was used for fractionation and isolation of peptides because (1) all the main peptides were present at reasonable concentrations (all were apparent after 1 week of ripening but increased or decreased in concentration to some extent during further ripening) and (2) 20 weeks is the approximate age of commercial Cheddar cheese. The breakdown of α_{s1}-casein and accumulation and subsequent breakdown of α_{s1}-CN (f24-199) (α_{s1}-I-casein) are apparent. The breakdown of β-casein and build-up of the γ-caseins are also apparent, although the breakdown of β-casein was far less extensive than that of α_{s1}-casein. A number of other large peptides also accumulated and the objective of this study was to isolate and identify these peptides.

Various methods were assessed for the fractionation of the WISF of the cheese, including open-column anion exchange chromatography on DEAE-cellulose, RP-HPLC (on a C_8 nucleosil column), hydrophobic interaction chromatography and cation and anion exchange FPLC on Mono-S® and Mono-Q® columns, respectively. Anion exchange FPLC on a Mono-Q® column gave the most effective and reproducible resolution (Fig. 2). However, the 13 fractions collected were found to be

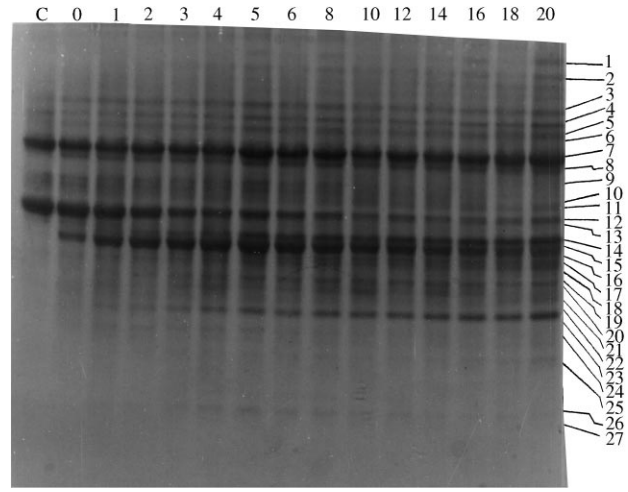

Fig. 1. Urea–polyacrylamide gel electrophoretogram of sodium caseinate (C) and the water-insoluble nitrogen fraction of Cheddar cheese after manufacture (slot 0) and ripening for 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20 weeks (slots 1–20, respectively). Peptides isolated from the 20 week sample or in earlier studies are as follows: **1.** β-CN f106-149, **2.** β-CN f106-128, **3.** β-CN f106-209 (γ2), **4.** β-CN f29-209 (γ1), **5.** β-CN f108-209 (γ3), **6.** β-CN f30-*, **7.** β-CN, **8.** β-CN f1-192 (β-I-CN), **9.** β-CN f29-*, **10.** β-CN f1-*, **11.** α_{s1}-CN f99-199, **12.** α_{s1}-CN f80-*, **13.** α_{s1}-CN, **14.** α_{s1}-CN f102-199, **15.** α_{s1}-CN f102-191, **16.** α_{s1}-CN f24-199 (α_{s1}-I-CN), **17.** α_{s1}-CN f121-199, **18.** α_{s1}-CN f33-*, **19.** α_{s1}-CN f104-199, **20.** α_{s1}-CN f129-*, **21.** α_{s1}-CN f106-199, **22.** α_{s1}-CN f60-*, **23.** α_{s1}-CN f110-199, **24.** α_{s1}-CN f70-156, **25.** α_{s1}-CN f24-186/7, **26.** α_{s1}-CN f24-*, **27.** α_{s1}-CN f24-*. * = peptide not identifed completely.

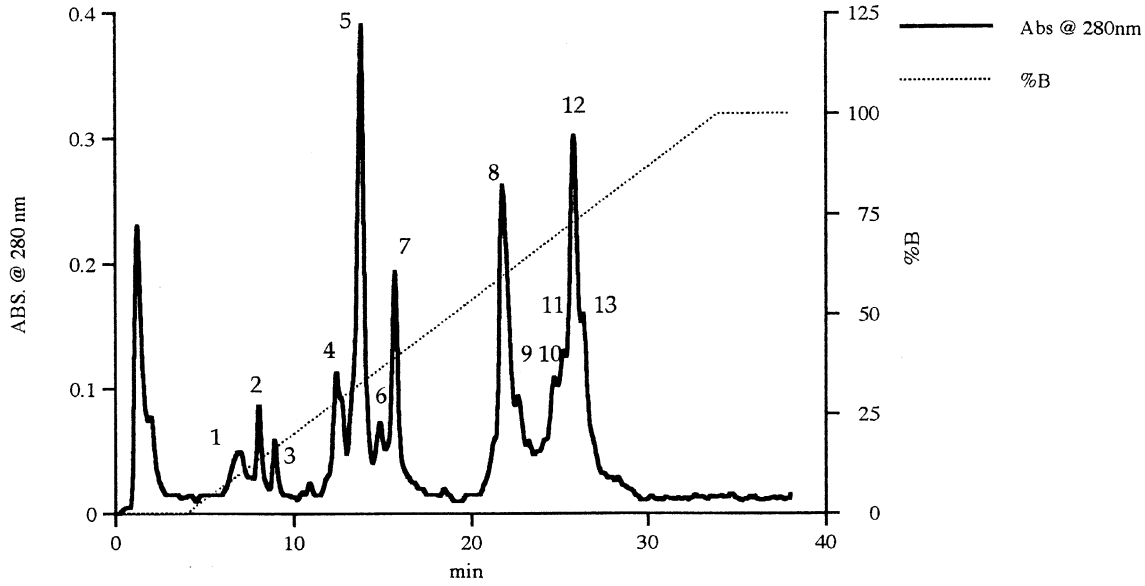

Fig. 2. Fast protein liquid chromatogram of the water-insoluble fraction of a 20 week-old Cheddar cheese by anion exchange on a Mono-Q® 5/5 HR column.

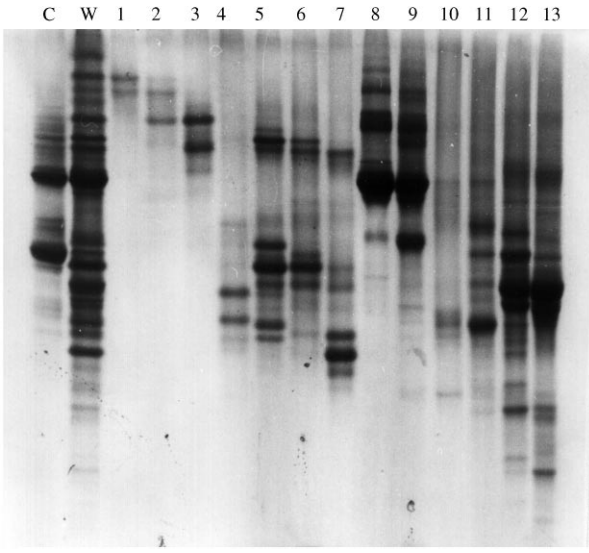

Fig. 3. Urea–polyacrylamide gel electrophoretogram of sodium caseinate (C), the water-insoluble fraction of a 20-week-old Cheddar cheese (W) and fractions 1–13 of the water-insoluble nitrogen fraction obtained by anion exchange Fast Protein Liquid Chromatography on Mono-Q® (Fig. 2).

heterogeneous by urea-PAGE analysis (Fig. 3). Therefore, the isolation of individual peptides suitable for N-terminal sequencing and mass spectrometry required a further step. Attempts were made to isolate individual peptides from FPLC fractions by RP-HPLC, but electroblotting of the peptides from urea-PAGE gel electrophoretograms of the fractions from Mono Q was found to be the most satisfactory method.

Peptides isolated by electroblotting from fractions 1, 3, 4, 5, 6, 7, 9 and 12 and from the WISF were identified. The peptides isolated are indicated on the urea-PAGE gel in Fig. 1 and their N-terminal sequences and experimental and theoretical masses listed in Table 1. Some of the peptide masses fell between two theoretical values—an example is the peptide α_{s1}-CN 24-186/187. As no proteinase involved in cheese ripening has been reported to cleave either Ile_{186}–Gly_{187} or Gly_{187}–Ser_{188}, it is difficult to identify the correct C-terminus. The experimental mass of two other peptides was also between two theoretical values, i.e. α_{s1}-CN f104-198/199 and α_{s1}-casein f121-198/199. However, since *Lactococcus* spp. do not possess carboxypeptidases and the bond Leu_{198}–Trp_{199} of α_{s1}-casein has not been reported as a cleavage site for chymosin or plasmin, it is likely that the peptides are α_{s1}-casein f104-199 and α_{s1}-casein f121-199.

Some of the peptides identified in this study arise from hydrolysis of bonds which have not been reported to be cleaved by chymosin, plasmin or the CEP of *Lc. lactis* subsp. *cremoris* SK11 (although some of these bonds have been reported to be cleaved by other lactococcal CEPs). Fraction 1 (Fig. 2) contained two such peptides, derived from β-casein, which had not been identified previously, i.e. β-CN f106-128 and β-CN f106-149, both

Table 1. Identity of Peptides Isolated from the Water-insoluble Fraction of Cheddar Cheese[a]

Fraction no.[b]	N-terminal sequence	Experimental mass (Da)	Position in casein sequence	Theoretical mass (Da)	Proteinase responsible	
					N-terminal	C-terminal
1	H.K.E.M.P	5234	β-CN f106-149	5222	Plasmin	Unknown
	H.K.E.M.P	2701	β-CN f106-128	2767	Plasmin	Unknown
3	H.K.E.M.P	11,811	β-CN f106-209	11,823.9	Plasmin	C-term. of β-CN (γ_2-CN)
	E.M.P.F.P	11,620	β-CN f108-209	11,558.6	Plasmin	C-term. of β-CN (γ_3-CN)
4	H.S.M.K.E	9122.2	α_{s1}-CN f121-199	9142.6	Unknown	C-term. of α_{s1}-CN
	A.Q.Q.K.E	[c]	α_{s1}-CN f129-[d]		Chymosin	Unidentified C-term.
5	K.I.E.K.F	20,418	β-CN f29-209	20,447	Plasmin	C-term. of β-CN (γ_1-CN)
	L.R.L.K.K	11,771	α_{s1}-CN f99-199	11,807	Chymosin/ CEP (SK11)	C-term. of α_{s1}-CN
	K.K.Y.K.V	11,453	α_{s1}-CN f102-199	11,424	Chymosin	C-term. of α_{s1}-CN
	K.K.Y.K.V	10,459	α_{s1}-CN f102-191	10,437	Chymosin	Unknown
6	I.E.K.F.Q.	[c]	β-CN f30-[d]		Plasmin	Unidentified C-term.
	K.I.E.K.F.	[c]	β-CN f29-[d]		Plasmin	Unidentifed C-term.
	H.I.Q.K.E	[c]	α_{s1}-CN f80-[d]		Plasmin	Unidentified C-term.
	Y.K.V.P.Q	11,239	α_{s1}-CN f104-199	11,168	Plasmin	C-term. of α_{s1}-CN
	V.P.Q.L.E	10,915	α_{s1}-CN f106-199	10,877	Plasmin	C-term. of α_{s1}-CN
7	E.I.V.P.N	10,435	α_{s1}-CN f110-199	10,439.5	Pepsin[e]	C-term. of α_{s1}-CN
	E. I.V.P.N	10,601	α_{s1}-CN f70-156	10,560	Unknown	Chymosin/CEP (SK11[f])
9	R.E.L.E.E	[c]	β-CN f1-[d]		N-term. of β-casein	Unidentified C-term.
12	F.V.A.P.F	19,428	α_{s1}-CN f24-186/187	19,398/19,455	Chymosin	Unknown
	F.V.A.P.F	[c]	α_{s1}-CN f24-[d]		Chymosin	Unidentified C-term.
	F.V.A.P.F	[c]	α_{s1}-CN f24-[d]		Chymosin	Unidentified C-term.

[a] These peptides are indicated in Fig. 4.
[b] The fractions are indicated in Figs 2 and 3.
[c] No mass found.
[d] C-terminal not determined.
[e] Unconfirmed.
[f] Cell wall-associated proteinase of *Lactococcus lactis* subsp. *cremoris* SK11.

of which are breakdown products of β-CN f106-209 (γ_2-casein), which is formed by the action of plasmin.

Fraction 3 contained β-CN f106-209 and β-CN f108-209, i.e. γ_2-casein and γ_3-casein, respectively. Fraction 4 contained two peptides derived from α_{s1}-casein, α_{s1}-CN f121-199 and α_{s1}-CN f129-*. Chymosin cleaves α_{s1}-casein at Leu_{120}–His_{121} (Exterkate *et al*., 1997) and His_{128}-Ala_{129} (Exterkate *et al*., 1995) and probably hydrolyses α_{s1}-CN f24-199 to form these peptides.

A number of peptides were identified from fraction 5. The largest of the γ-caseins, γ_1-casein (β-CN f29-209) was isolated from fraction 5. The other peptides in this fraction were products of α_{s1}-casein. One of these peptides, α_{s1}-CN f99-199, migrated slightly slower than α_{s1}-casein itself; the bond Leu_{98}–Leu_{99} is cleaved by chymosin (Exterkate *et al*., 1997) and by the CEP of *Lc. lactis* subsp. *cremoris* SK11. All the other α_{s1}-casein peptides isolated in this study migrated faster than α_{s1}-casein. α_{s1}-CN f102-199 was also isolated from fraction 5; this peptide, which is formed by chymosin, was evident in the 1 day-old cheese (Fig. 1) and is a major product of α_{s1}-casein in Cheddar and similar cheeses. A minor peptide isolated from fraction 5 was α_{s1}-CN f102-191; this was probably formed by cleavage of α_{s1}-CN f102-199 at Ser_{191}–Glu_{192} but this bond has not been reported to be cleaved by chymosin, plasmin or the CEP of *Lc. lactis* ssp. *cremoris* SK11, although it was identified as a cleavage site for the CEP of *Lc. lactis* ssp. *lactis* NCDO 763 (Monnet *et al*., 1992).

Fraction 6 contained two β-casein-derived peptides with *N*-terminal sequences of Lys–Ile–Glu–Lys–Phe and Ile–Glu–Lys–Phe–Gln. These were identified as β-CN f29-* and β-CN f30-*, respectively, but the masses of either peptide could not be determined. O'Malley (1995) identified the peptide β-CN f30-*, which is probably formed by plasmin, in cheese analogues. Three other peptides isolated from fraction 6 were probably produced by plasmin from α_{s1}-casein: α_{s1}-CN f80-*, α_{s1}-CN f104-199 and α_{s1}-CN f106-199; the bonds Lys_{79}–His_{80}, Lys_{103}–Tyr_{104} and Lys_{105}–Val_{106} have been identified as plasmin cleavage sites in α_{s1}-casein in solution (McSweeney *et al*., 1993b; Le Bars and Gripon, 1993). The peptide α_{s1}-CN f80-* had the same mobility as α_{s1}-casein. Inspection of the literature shows that electrophoretograms of mature Cheddar cheese appear to contain α_{s1}-casein; however, α_{s1}-casein was not isolated from this position during this study, suggesting that it is completely hydrolysed by 20 weeks and that which appears to be residual α_{s1}-casein is actually α_{s1}-Cn f80-*.

The peptide α_{s1}-CN f110-199 was isolated from fraction 7. The bond Leu_{109}–Gly_{110} is cleaved by chymosin in phosphate buffer at pH 6.5, but not at pH 5.2, in the presence of 4–5% NaCl (McSweeney *et al*., 1993a; Exterkate *et al*., 1995). The peptide α_{s1}-CN f110-199 has been identified in cheese made with porcine pepsin, but not in cheese made with recombinant chymosin (Lane, 1997). The other peptide isolated from fraction 7 was α_{s1}-CN f70-156. Both chymosin and the CEP of *Lc. lactis* ssp. *cremoris* SK11 are reported to cleave the bond Leu_{156}–Asp_{157} of α_{s1}-CN in solution (Reid *et al*., 1991; Exterkate *et al*., 1995). The enzyme which cleaves the bond at the N-terminal (Glu_{69}–Glu_{70}) is unknown.

Only one peptide was isolated from fraction 9; this peptide had the N-terminal sequence of β-casein, but no mass was obtained and the peptide was not fully identified. Fraction 9 also contained β-CN f1-192 (β-I-CN) but this was not isolated in the present study.

Three peptides were isolated from fraction 12, all having Phe_{24} of α_{s1}-casein as the N-terminal. One of these was fully identified as α_{s1}-CN f24-186 or α_{s1}-CN f24-187. As previously mentioned, the actual C-terminal was uncertain. Masses were not obtained for the other two peptides, but one of these may be α_{s1}-CN f24-98 which was identified in solution by Exterkate et al. (1995); the complementary C-terminal fragment, i.e. α_{s1}-CN f99-199 was identified in fraction 5.

Two bands, the slower of which was a doublet, were electroblotted from urea-PAGE of the 20 week WISF. The N-terminal sequence of both peptides in the doublet was found to be Gly–Lys–Glu–Lys–Val, which corresponds to α_{s1}-CN f33-*. No mass was obtained for the peptides. The peptide bond Phe_{32}-Gly_{33} of α_{s1}-casein is hydrolysed by chymosin. The N-terminal sequence of the third isolated peptide was Met–Glu–Ala–Glu–Ser, which corresponds to α_{s1}-CN f60-*; no mass was obtained. The bond Gln_{59}–Met_{60} of α_{s1}-casein has not been reported to be cleaved by chymosin, plasmin or *Lc. lactis* ssp. *cremoris* SK11 CEP; this bond may be cleaved by an intracellular proteinase or endopeptidase of the starter or non-starter bacteria.

A total of 23 peptides, including α_{s1}-CN f24-199 and β-CN f1-192, have been isolated from the WISF of a mature Cheddar cheese and fully or partially identified; these are summarized in Fig. 4. In some cases, full identification was not achieved as results from mass spectrometry were not conclusive, because the concentration of peptide was too low or the peptide failed to ionize properly. Thus, a number of peptides in the WISF remain to be identified. Some peptides in fractions 8, 9, 12 and 13 from Mono Q chromatography (Fig. 2) were not sufficiently well separated on urea-PAGE gels to allow them to be removed cleanly from the electroblotting membrane. Additional techniques will be required for isolation of the above.

The results of this study clearly show the major role played by chymosin in primary proteolysis in Cheddar cheese, being mainly responsible for the hydrolysis of α_{s1}-casein which is completely hydrolysed in 20 week-old cheese. An α_{s1}-casein-derived peptide, i.e. α_{s1}-CN f80-*, co-migrates with α_{s1}-casein on urea-PAGE gels, causing the extent of α_{s1}-casein hydrolysis to be underestimated. Plasmin was mainly responsible for the hydrolysis of β-casein and it also produced three large peptides from α_{s1}-casein, i.e. α_{s1}-CN f80-*, α_{s1}-CN f104-199 and α_{s1}-CN f106-199. Lactococcal CEP was involved in the formation of some water-insoluble peptides, e.g. α_{s1}-CN f99-199. The formation of a few peptides cannot be explained based on the known specificities of chymosin, plasmin or *Lc. lactis* ssp. *cremoris* SK11 CEP e.g. α_{s1}-CN f70-156. Lactococcal endopeptidases or non-starter enzymes may, therefore, contribute the formation of a few large water-insoluble peptides in Cheddar cheese.

Although the bond Trp_{164}–Tyr_{165} is the second most susceptible bond in α_{s1}-casein in solution to chymosin (after Phe_{23}–Phe_{24}) (McSweeney *et al*., 1993a), no peptide arising from cleavage of this bond has been found either in water-soluble or water-insoluble fraction of Cheddar (Singh *et al*., 1994, 1995, 1997; Fernandez *et al*., 1998; this study) or in a model Gouda cheese (Exterkate *et al*., 1995). Presumably, the bond Trp_{164}–Tyr_{165} is rendered inaccessible in cheese, perhaps owing to

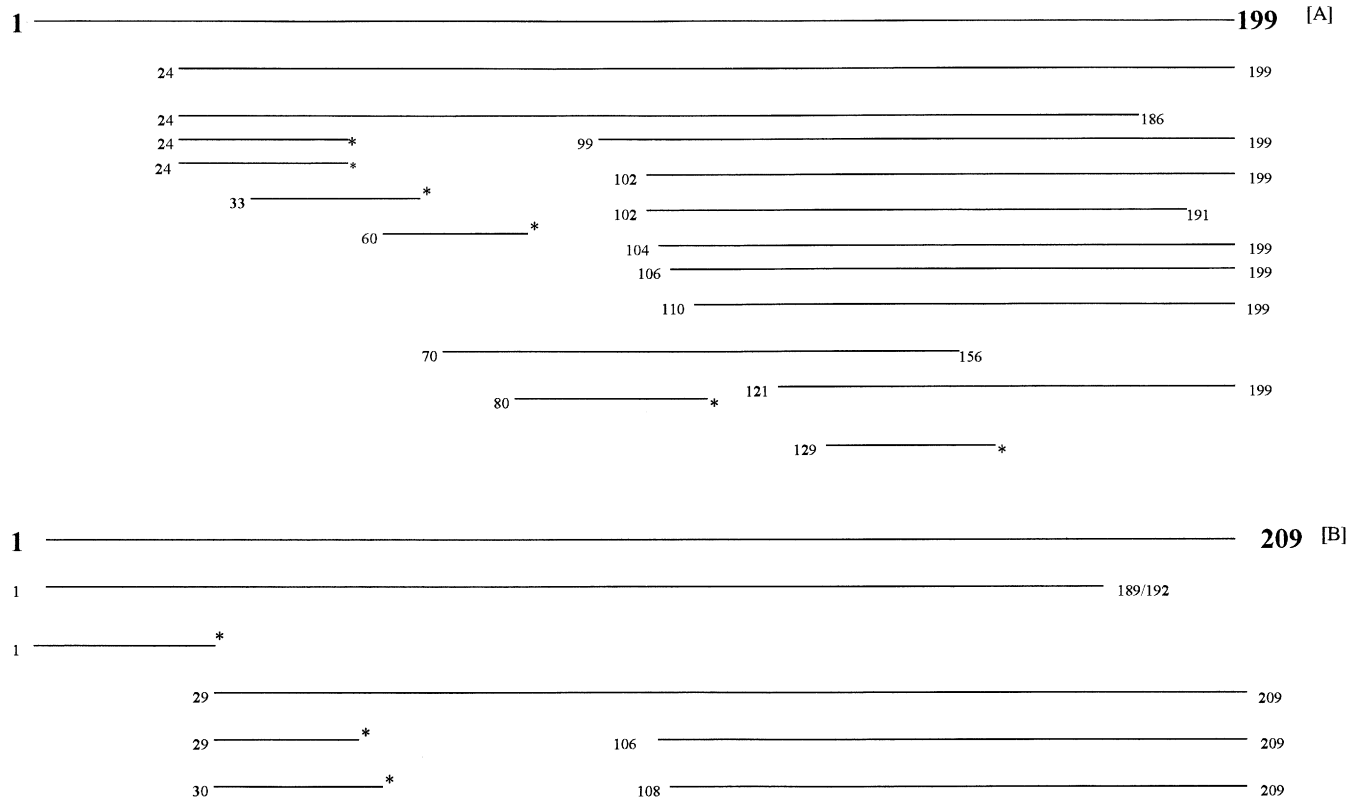

Fig. 4. Water-insoluble peptides derived from α_{s1}-casein [A] and β-casein [B], isolated from Cheddar cheese in this and earlier studies.

intermolecular hydrophobic interactions. It is noteworthy that with the exception of low concentration of β-CN f1-192 and β-CN f1-* (both in fraction 9), all the water-insoluble peptides in Cheddar cheese are C-terminal fragments of α_{s1}- and β-caseins (and para-κ-casein). The complementary N-terminal fragments are present in the water-soluble fraction, frequently after hydrolysis by lactococcal CEP and/or exopeptidases (see Singh *et al.*, 1994, 1995, 1997; Fernandez *et al.*, 1998). No α_{s2}-casein-derived peptide was found in the WISF although a few peptides derived from this protein have been found in the WSF (Singh *et al.*, 1994, 1995, 1997).

ACKNOWLEDGEMENTS

This research has been part-funded by grant aid under the Food Sub-Programme of the Operational Programme for Industrial Development which is administered by the Department of Agriculture, Food and Forestry and supported by national and EU funds.

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