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Isolation and Identification of Peptides from the Diafiltration Permeate of the Water-Soluble Fraction of Cheddar Cheese

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The water-soluble extract of a mature Cheddar cheese was fractionated by diafiltration using 10 kDa nominal molecular weight cutoff membranes. The permeate had a savory, cheesy taste, whereas the retentate was bland. The permeate was resolved into nine fractions by gel permeation chromatography on Sephadex G-25. Fractions I−III contained only peptides, whereas fractions IV−IX comprised mainly free amino acids. Fraction IV contained a mixture of all amino acids except Phe (fraction V), Tyr (fraction VI), and Trp (fraction IX). Fraction III, which had the savory cheesy taste of the permeate, was dominated by one major peak with several minor ones. Fraction III was rechromatographed on a Sephadex G-25 column, and a number of peptides were isolated from subfractions thereof by reversed-phase high-performance liquid chromatography and characterized by N-terminal amino acid sequencing and mass spectrometry. The results showed that starter bacteria cell-envelope proteinase, endopeptidases, and aminopeptidases play an important role in the degradation of the primary proteolytic products produced by chymosin and plasmin from α_{s1}-, α_{s2}-, and β-caseins.

Keywords: *Cheddar cheese; proteolysis; peptides; cheese flavor*

INTRODUCTION

Although it is generally accepted that free amino acids and small peptides contribute to the background flavor of cheese, the proteolytic products responsible for the cheesy taste and the agents responsible for their formation have not yet been fully characterized. Therefore, isolation and identification of peptides from cheese and explanation of their formation will help to complete understanding of proteolysis during cheese ripening. The study of peptide profiles can also help to determine the degree of maturation based on the detection and evaluation of peptide indicators related to the age of the cheese (Addeo et al., 1995).

Various proteinases and peptidases in cheese hydrolyze caseins to peptides and free amino acids (Fox et al., 1994; McSweeney and Fox, 1997). Small peptides and free amino acids contribute directly to the background flavor of cheese (McGugan et al., 1979; Aston and Creamer, 1986; Cliffe et al., 1993; Singh et al., 1994). Most of the savory, cheesy taste of the water-soluble extract (WSE) of Cheddar cheese is in the diafiltration permeate (DFP) (10 kDa nominal molecular weight cutoff), which contains small peptides, free amino acids, salts, and other low molecular weight compounds, whereas the retentate, which contains intermediate size peptides, is bland (Singh et al., 1994). Engels and Visser (1994) concluded that, with the exception of Edam, the ultrafiltration permeate (UFP), with a molecular weight (MW) <500, of Cheddar, Gouda, Gruyère, Maasdam, Parmesan, and Proosdij cheeses contained the components responsible for flavor. The permeate of these cheeses contained low molecular weight peptides (probably not larger than tetrapeptides), amino acids, breakdown products of amino acids (e.g., γ-aminobutyric acid and ornithine), and short-chain fatty acids ($<C_9$).

The DFP of the WSE from Cheddar is dominated by two peptides, α_{s1}-CN f1−9 and α_{s1}-CN f1−13, which accumulate during ripening (Singh et al., 1994). Kaminogawa et al. (1986) reported that α_{s1}-CN f1−9, α_{s1}-CN f1−13, and α_{s1}-CN f1−14 also accumulated in Gouda cheese. These peptides are formed from α_{s1}-CN f1−23, produced on cleavage of the bond Phe^{23}−Phe^{24} of α_{s1}-casein by chymosin during the early stages of ripening, by the starter cell-envelope proteinase (CEP). Exterkate and Alting (1995) showed that incubation of α_{s1}-CN f1−23 with lactococcal proteinases and peptidases failed to generate cheeselike flavor. This observation indicates that flavor development, insofar as it is based on proteolysis, is apparently initiated relatively late in Cheddar or Gouda cheeses and is dependent on further proteolysis of other casein fragments.

A scheme for the fractionation of WSE and identification of the principal peptides in Cheddar cheese was reported by Singh et al. (1994). So far, >150 peptides isolated from the DF retentate have been identified (Singh et al., 1995, 1997). The majority of the peptides identified were from β-casein, especially from the N-terminal half of the molecule. The DFP of the WSE from a commercial matured Cheddar cheese was resolved into nine fractions by chromatography on Sephadex G-25 (Figure 1). Fractions I−III contained peptides, whereas fractions IV−VI and IX contained amino acids only (Fox et al., 1994; Singh et al., 1994). Several peptides in fractions I and II were isolated and identified

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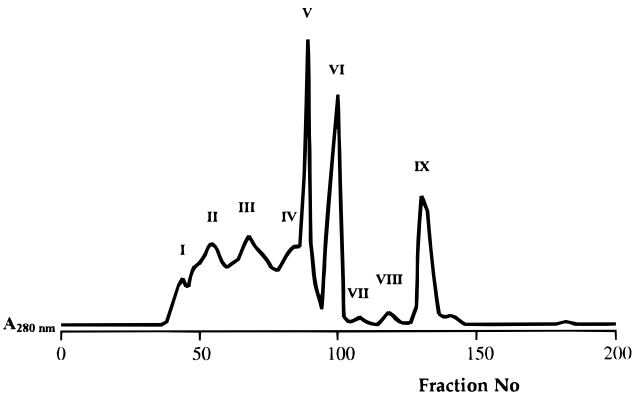

Figure 1. Gel permeation chromatogram of the DFP of a 9-month-old Cheddar cheese. Fraction size, 3.5 mL; flow rate, 0.7 mL min^{-1}; solvent, distilled water; temperature, 20 °C. Fractions were pooled as indicated.

(Fox et al., 1994; Singh et al., 1994). The aim of the present study was to purify and identify peptides in fraction III, which had the most savory cheesy taste among the nine fractions of the WSE-DFP obtained by chromatography on Sephadex G-25, and in fractions IV and V of the WSE-DFP of Cheddar cheese.

MATERIALS AND METHODS

Samples. Mature commercial Cheddar cheese (~9 months old), made using a pair of *Lactococcus lactis* subsp. *cremoris* strains (strains 227 and 303, Chr. Hansen's, Cork, Ireland), was used in this study. Several peptides from the same cheese were identified previously (Fox et al., 1994; Singh et al., 1994, 1995, 1997).

Fractionation of Cheese Nitrogen. A WSE of the cheese was prepared according to the method of Kuchroo and Fox (1982) and diafiltered using a Millipore Minitan ultrafiltration system (Millipore Corp., Bedford, MA) fitted with polysulfone membranes with a nominal molecular weight cutoff of 10 kDa, as described by O'Sullivan and Fox (1990). The DFP was fractionated by gel permeation chromatography on a column (80 cm × 3 cm) of Sephadex G-25 using distilled water as eluent at a flow rate 0.7 mL min^{-1}. Fractions (3.5 mL) were collected and pooled as shown in Figure 1. Fractions III−V were rechromatographed at 20 °C on an 80 × 2 cm column of Sephadex G-25 using distilled water as eluent at a flow rate of 1 mL min^{-1}. Fractions (5 mL) were collected, and the peptide content was monitored by measuring absorbance at 280 nm.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Lyophilized gel permeation fractions were dissolved in 0.1% trifluoroacetic acid (TFA) and filtered through 0.45 μm filters (Acrodisk, MI) for application to the column. HPLC was performed using an automated Waters HPLC system (consisting of a model 626 pump, a model 600S controller, and a model 717 autosampler; Waters Chromatography Division, CT) fitted with a Nucleosil C_8 wide-pore column (5 μm, 300 Å, 250 × 4.6 mm; HPLC Technology Ltd., U.K.) and guard column (10 × 4.6 mm). The solvents were 0.1% (v/v) TFA in deionized water (A) and 0.1% (v/v) TFA in acetonitrile (B). The samples were eluted initially with 100% A for 5 min, then with a gradient from 0 to 50% B over 55 min, maintained at 50% B for 6 min, followed by a linear gradient to 60% B over 4 min, and finally with 60% B for 3 min; the flow rate was maintained at 0.75 mL min^{-1}. The eluate was monitored at 214 nm using a model 486 programmable spectrophotometric detector (Millipore Corp.), interfaced with a personal computer using Millennium 2010 software (Millipore Corp.).

Peptide Identification. The sequence of the five N-terminal amino acid residues of isolated peptides was determined by automated Edman degradation using a pulsed liquid-phase protein/peptide sequencer (model-477A, Applied Biosystem Inc., CA), interfaced with a 120A HPLC (Applied Biosystem Inc.) for the on-line analysis of the phenylthiohydantoin derivatives of liberated amino acids.

The mass of isolated peptides was determined at the Department of Biochemistry, Faculty of Medicine, Nottingham University, Nottingham, U.K., using a BioIon 20 plasma desorption mass spectrometer (BioIon AB, Uppsala, Sweden).

Amino Acid Analysis. The total amino acid composition (after hydrolysis with 6 M HCl, 110 °C for 24 h) and the free amino acids in fraction IV were determined on a Beckman 6300 analyzer (Beckman Instruments Ltd., U.K.) using a cation exchange column (Na form, 12 cm × 4 mm i.d.). A standard amino acid mixture was used to calibrate the column, and norleucine was added to samples as an internal standard. Samples and standards were diluted in 0.2 M sodium citrate buffer, pH 2.2, for injection, and 50 μL was applied to the column. Samples were eluted with different sodium citrate buffers, first with 0.2 M sodium citrate, pH 3.28, for 17.8 min, then with 0.2 M, pH 4.25, for 14.2 min, then with 1.0 M, pH 6.4, for 30 min, and finally with 0.2 M, pH 3.28, for 13 min. Amino acids were postcolumn derivatized with ninhydrin and detected by absorbance at 570 and 440 nm. Results were analyzed using a PC Minichrom system.

RESULTS

The gel filtration profile of the WSE-DFP obtained from the sample used in this study (Figure 1) was similar to that of numerous other cheese samples analyzed in the Department of Food Chemistry (Singh et al., 1994; Fernández and Fox, 1998), although there are quantitative differences between fractions depending on the degree of ripening (Fernández and Fox, 1998). The profile of the DFP obtained in this work also showed remarkable similarity with the gel permeation chromatograms obtained by Mulholland (1991) in methanol-clarified WSE from different Cheddar cheese samples.

Fractions III−V of the DFP were rechromatographed on Sephadex G-25 to obtain a better separation of peptides (Figure 2). Subfractions were pooled, as indicated; 12 subfractions were collected from fraction III, 8 from IV, and 5 for V.

The RP-HPLC profiles of fraction III and the 12 subfractions thereof are shown in Figure 3. Although fraction III was very heterogeneous, it was dominated by two peaks; one, with a retention time of ~6 min (peak 1), eluted mainly in subfractions III-9−12, and the second largest peak (peak 10), with a retention time ~37 min, eluted mainly in subfractions III-4−9. These two peaks, as well as some minor ones, were collected from the fractions in which they were best resolved by RP-HPLC for identification by N-terminal amino acid sequence. No peptides were identified in peaks 1 and 2, which contained mixtures of amino acids, as reported in Table 1. Peak 1 contained mainly Glu and Lys, whereas peak 2 contained mainly Val, but also Ile, Leu, Pro, and Cys in high proportions. Several peptides were identified in peaks 3−15 of fraction III (Table 2). A number of peaks contained more than one peptide. In total, 19 peptides were identified in fraction III, 14 of which originated from α_{s1}-, 4 from α_{s2}-, and 1 from β-casein.

RP-HPLC of fraction IV showed that this fraction contained three main peaks, eluting at the beginning of the chromatogram with retention times of 5−8 min, which were present in all gel permeation subfractions (Figure 4), especially IV-3−8, and were found to contain amino acids (Table 1). Peaks 1 and 2 contained almost the full range of amino acids, especially Glu and Ser in

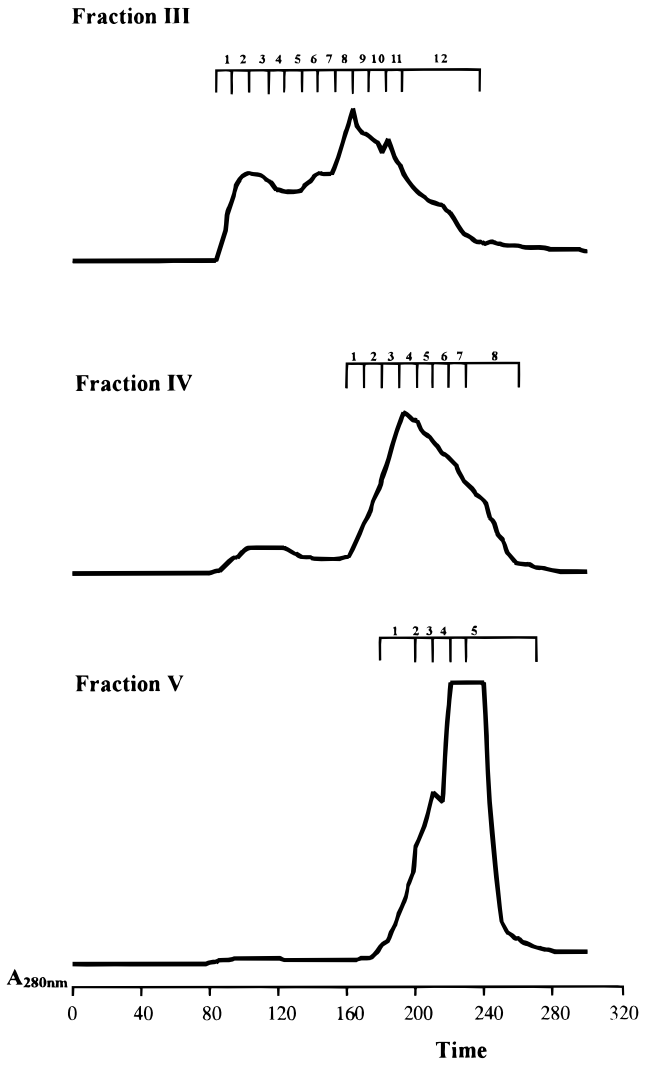

Figure 2. Rechromatography on Sephadex G-25 of fractions III−V obtained from the gel permeation chromatography of the 10 kDa WSE-DFP of a 9-month-old Cheddar cheese (Figure 1). Fraction size, 5 mL; flow rate, 1 mL min^{-1}; solvent, distilled water; temperature, 20 °C. Subfractions were pooled as indicated.

peak 1 and Pro, Val, and Glu in peak 2; peak 3 contained mainly Leu and Met. Peaks 4−6 were collected for amino acid sequencing; no result was obtained for fractions 4 and 5, and one peptide, α_{s2}-CN f204−207, was identified in peak 6.

Although preliminary work showed that fraction V contained mainly Phe, it was also rechromatographed on Sephadex G-25 to check its profile (Figure 2). Five subfractions were collected and analyzed by RP-HPLC. The chromatogram (Figure 5) was dominated by a large peak (retention time ~12 min) that was identified as Phe. It has been reported (Singh et al., 1994; Silvestre et al., 1994) that the aromatic amino acids are retarded in gel permeation chromatography, due mainly to hydrophobic interactions. Some minor peaks were observed in the RP-HPLC profiles, probably containing some very short peptides that were retarded due to interaction with the column packing, but they were not collected.

DISCUSSION

The majority of the peptides characterized in fraction III of the WSE-DFP originated from α_{s1}-casein. The

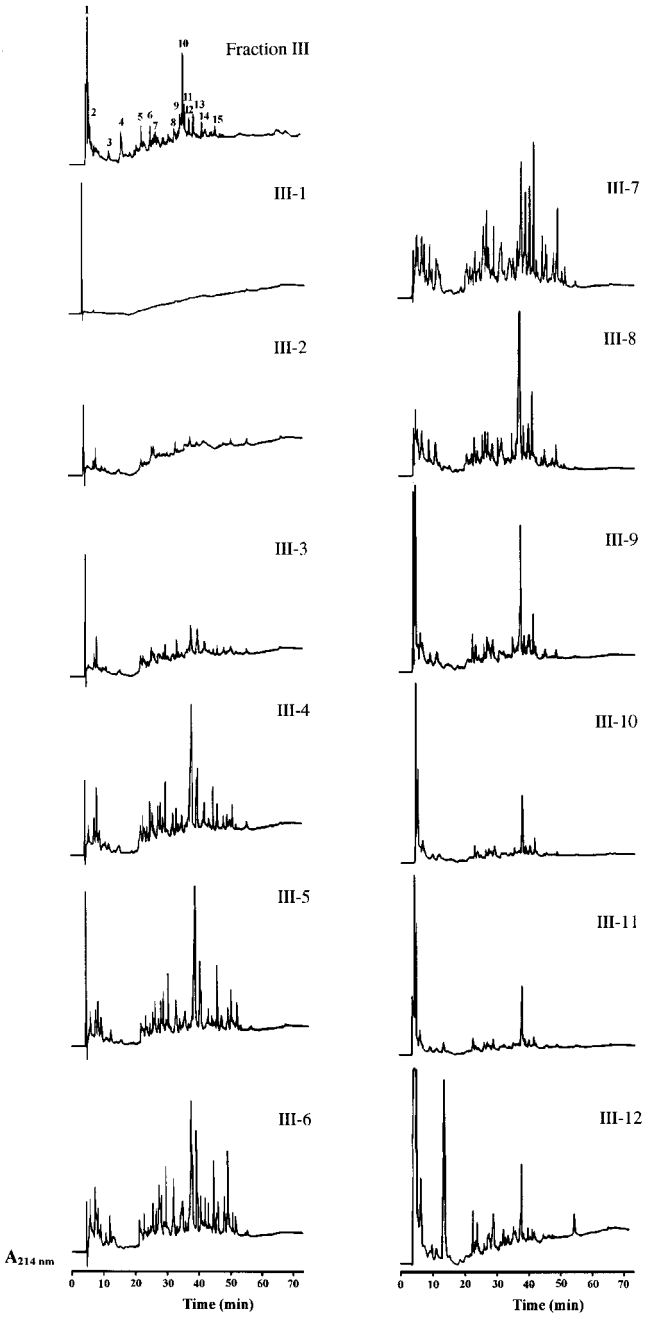

Figure 3. RP-HPLC chromatograms of fraction III (Figure 1) of WSE-DFP of Cheddar cheese (indicating peaks isolated for identification) and of subfractions III-1−12 from Sephadex G-25 (Figure 2).

bond Phe^{23}−Phe^{24} of α_{s1}-casein is hydrolyzed rapidly by chymosin during the early stages of ripening (Hill et al., 1974), resulting in the formation of small, α_{s1}-CN f1−23, and large, α_{s1}-CN f24−199, fragments. α_{s1}-CN f1−23 is hydrolyzed rapidly by starter CEP. In solution, α_{s1}-CN f1−23 is hydrolyzed by CEP-I at the bonds His^8−Gln^9, Gln^9−Gly^{10}, and Gln^{13}−Glu^{14} and by CEP-III at Leu^{16}−Asn^{17}, Asn^{17}−Glu^{18}, and Leu^{21}−Arg^{22} (Exterkate et al., 1991; Exterkate and Alting, 1995). These differences in specificity between CEP-I and -III are observed at pH 6.5 and low ionic strength buffer. In the cheese environment, with a low pH and a high ionic strength, these differences in specificity disappear, due mainly to elimination of electrostatic forces involved in substrate binding (Exterkate and Alting, 1993). Hydrolysis of bonds Gln^9−Gly^{10} and Gln^{13}−Glu^{14} by CEP results

Table 1. Amino Acid Composition (Relative Percentage) of RP-HPLC Peaks 1 and 2 of Fraction III and Peaks 1−3 of Fraction IV from the WSE-DFP of a 9-Month-Old Cheddar Cheese

	fraction III		fraction IV		
amino acid	1	2	1	2	3
cysteic acid	0.47	10.89	0.06	0.30	0.33
aspartic acid	3.40		3.66	0.01	
threonine	1.88		9.04	0.49	
serine	0.45		16.82	0.27	1.33
glutamic acid	40.70		27.03	17.97	
glycine	0.26		10.21	0.25	
alanine	3.22		9.67	1.14	
cysteine	0.70	4.59	0.59	0.48	0.01
valine	9.72	37.81	11.06	27.47	7.14
methionine		1.37	0.13	8.22	24.14
isoleucine		16.44	0.03		6.56
leucine		13.42			60.37
tyrosine				0.16	0.14
phenylalanine					
histidine	1.40	0.96	2.30	0.19	
lysine	32.08	3.42	3.49	1.03	
arginine			1.53		
proline	5.73	10.96	4.40	42.01	

Table 2. Peptides Isolated from Gel Permeation Fractions III and IV of the DF Permeate of WSE from a 9-Month-Old Cheddar Cheese

fraction	peak	N-terminal residues	mol wt theor	mol wt exptl	peptide identification
III	3	Asp-Ile-Lys-Gln-Met			α_{s1}-CN (56-?)[a]
		Lys-Asn-Thr-Met-Glu			α_{s2}-CN (1−?)[a]
	4	Lys-Val-Asn-Glu-Leu			α_{s1}-CN (36−?)[a]
		Gly-Leu-Pro-Gln-Glu			α_{s1}-CN (10−?)[a]
	5	Gly-Tyr-Leu-Glu-Glu			α_{s1}-CN (93−?)[a]
	6	Asn-Glu-Asn-Leu-Leu			α_{s1}-CN (17−?)[a]
		Glu-Asn-Leu-Leu-Arg			α_{s1}-CN (18−?)[a]
		Lys-Pro-Trp-Ile-Gln	896.08	896.10	α_{s2}-CN (191−197)
	7	Val-Ala-Pro-Phe-Pro	756.88	757.20	α_{s1}-CN (25−31)
		Leu-Gly-Tyr-Leu-Glu			α_{s1}-CN (92−?)[a]
	8	Asp-Val-Pro-Ser-Glu	864.89	863.10	α_{s1}-CN (85−91)
		Leu-Pro-Gln-Tyr-Leu			α_{s2}-CN (176−?)[a]
	9	Leu-Pro-Gln-Glu-Val			α_{s1}-CN (11−?)[a]
		Ala-Leu-Pro-Gln-Tyr	933.10	931.00	α_{s2}-CN (175−182)
	10	Gly-Tyr-Leu-Glu-Gln			α_{s1}-CN (93−?)[a]
		Leu-Gln-Asp-Lys-Ile	997.14	995.10	β-CN (45−52)
	11	Phe-Val-Ala-Pro-Phe	804.93	804.5	α_{s1}-CN (24−30)
	12	Ala-Pro-Phe-Pro-Gln	804.93	804.8	α_{s1}-CN (26−32)
		Ala-Pro-Phe-Pro-Phe	990.15	989.30	α_{s1}-CN (26−34)
IV	6	Val-Arg-Tyr-Leu	549.65	548.60	α_{s2}-CN (204−207)

[a] Peptides failed to give data on mass spectrometer.

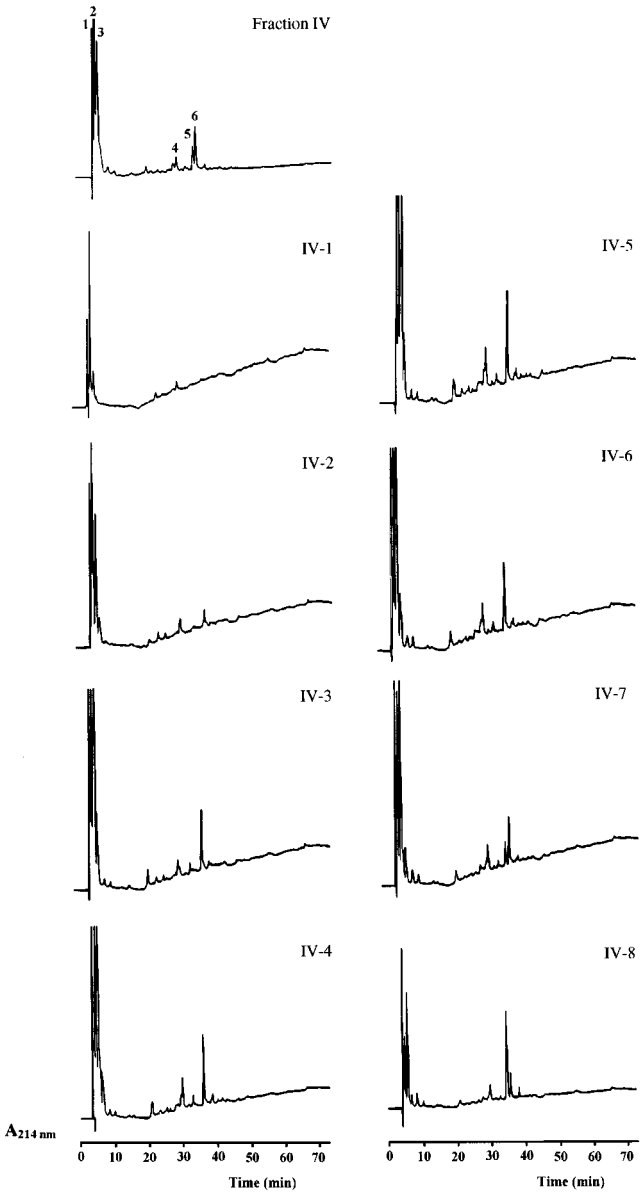

Figure 4. RP-HPLC chromatograms of fraction IV (Figure 1) of WSE-DFP of Cheddar cheese (indicating peaks isolated for identification) and subfractions IV-1−8 from Sephadex G-25 (Figure 2).

in the formation of peptides α_{s1}-CN f1−9 and f1−13, which were previously isolated and identified in WSE from the Cheddar sample used in this study (Singh et al., 1994). These two peptides, α_{s1}-CN f1−9 and f1−13, which elute in fraction I of the WSE-DFP (Singh et al., 1994), accumulate in cheese during ripening. In this study, four peptides originated from peptide 1−23 of α_{s1}-CN were identified. The N-terminal residue of these peptides, namely α_{s1}-CN f10−?, α_{s1}-CN f17−?, and α_{s1}-CN f18−?, correspond directly to the reported cleavage sites of CEP on α_{s1}-CN f1−23, that is, Gln^{9}−Gly^{10}, Leu^{16}−Asn^{17}, and Asn^{17}−Glu^{18}. Formation of the fourth peptide, α_{s1}-CN f11−?, would involve hydrolysis of bond Gln^{9}−Gly^{10} by CEP and removal of Gly^{10} by an aminopeptidase.

Four peptides, α_{s1}-CN f24−30, f25−31, f26−32, and f26−34, were originated from the large peptide, α_{s1}-CN f24−199, produced by chymosin. The formation of peptides α_{s1}-CN f25−31, α_{s1}-CN f26−32, and α_{s1}-CN f26−34 would involve removal of Phe^{24} or Phe^{24} and Val^{25}, respectively, by an aminopeptidase. Alternatively, chymosin is also reported to hydrolyze the bond Phe^{24}−Val^{25} (Pelissier et al., 1974), which could explain the N-terminal of α_{s1}-CN f25−31. The bonds Val^{31}−Phe^{32} and Phe^{32}−Gly^{33} are chymosin cleavage sites (McSweeney et al., 1993a), which would explain the C terminus of the peptides α_{s1}-CN f25−31 and α_{s1}-CN f26−32. The origin of peptide α_{s1}-CN f36−? cannot be explained on the basis of the known specificity of chymosin and CEP; the bond Lys^{35}−Val^{36} is a potential site of cleavage for plasmin but it is not one of those reported by LeBars and Gripon (1993) and McSweeney et al. (1993b). The bond Glu^{84}−Asp^{85} is a reported cleavage site of CEP, which would explain the N terminus of the peptide α_{s1}-CN f85−91. The presence of peptides α_{s1}-CN f85−91 and α_{s1}-CN f92−? indicates hydrolysis of the bond Tyr^{91}−Leu^{92} in the cheese environment, which is not a previously reported cleavage site. The peptide α_{s1}-CN f93−? could be formed by the release of Leu^{92} from α_{s1}-CN f92−? by an aminopeptidase. A mass was not obtained for either α_{s1}-CN f92−? or α_{s1}-CN f93−? The formation of the peptide α_{s1}-CN

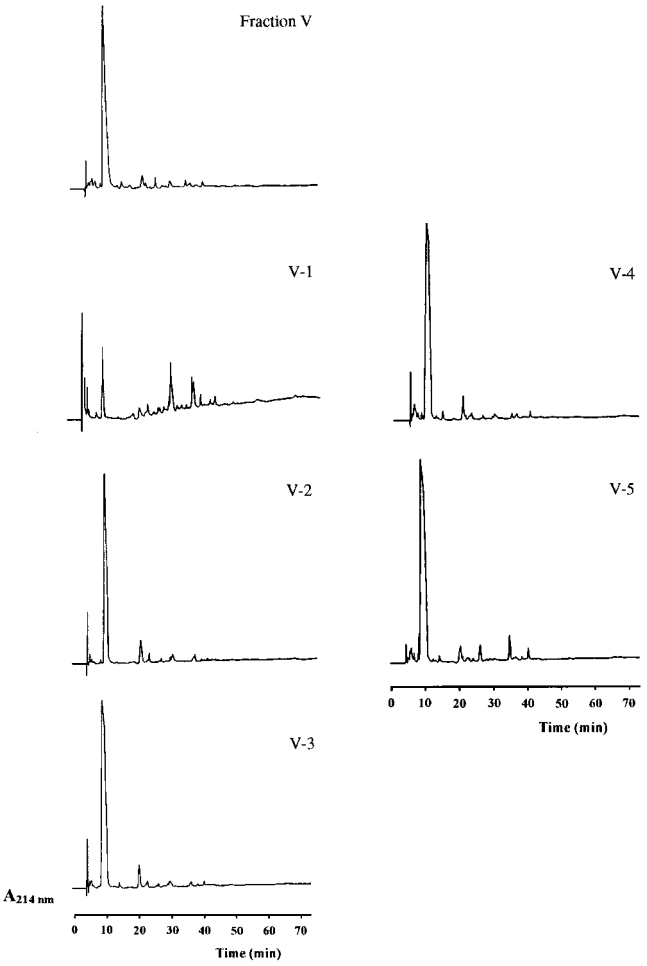

Figure 5. RP-HPLC chromatograms of fraction V (Figure 1) of WSE-DFP of Cheddar cheese and subfractions V-1−5 from Sephadex G-25 (Figure 2).

f56−? cannot be explained on the basis of known specificities and may involve activity of starter endopeptidases (Pep O, Pep F) or proteinases and peptidases from nonstarter lactic acid bacteria.

Five peptides produced from α_{s2}-casein were identified. Chymosin and CEP are reported to hydrolyze the bond Phe^{174}−Ala^{175}, which could explain the N terminus of the peptide α_{s2}-CN f175−182; the bond Thr^{182}−Val^{183} is a reported lactococcal CEP cleavage site, which would explain its C terminus. The formation of the peptide α_{s2}-CN f176? would require the removal of Ala^{175} from a peptide α_{s2}-CN f175−? by an aminopeptidase. The N terminus of the peptide α_{s2}-CN f191−197 cannot be explained on the basis of reported specificities of chymosin, plasmin, or lactococcal CEP. However, the bond Gln^{187}−Lys^{188} is a reported CEP cleavage site, whereas Lys^{188}−Ala^{189} is reported to be cleaved by plasmin; removal of three or two N-terminal residues from the resulting peptides could explain the N terminus of α_{s2}-CN f191−197. The bond Lys^{197}−Thr^{198} is a reported CEP cleavage site, which would explain the C terminus of the peptide α_{s2}-CN f191−197. Peptide α_{s2}-CN f204−207 represented the C-terminal residues (Val^{204}-Arg-Tyr-Leu^{207}) of the molecule; the bond Tyr^{203}−Val^{204} of α_{s2}-casein is a reported cleavage site of lactococcal CEP [see Fox et al. (1994)].

Plasmin is mainly responsible for the primary hydrolysis of β-casein in cheese, resulting in the formation of γ-caseins and proteose peptones. Most of the peptides identified in the DF retentate of the WSE from Cheddar cheese originate from the N-terminal half of β-casein, especially from the sequence 29−105/107 (Singh et al., 1995, 1997). The concentration of γ-caseins increases during ripening of Cheddar, but the proteose peptones are further hydrolyzed by CEP (Singh et al., 1995). In solution, CEP preferentially hydrolyze peptide bonds in the C-terminal half of β-casein, but in the Cheddar cheese environment, it is the N-terminal half that is hydrolyzed preferentially. Only one β-casein-derived peptide (β-CN f45−52) was identified in this study, the origin of which can be explained by hydrolysis of bond Asp^{43}−Glu^{44}, a reported CEP cleavage site, and removal of Glu^{44} by an aminopeptidase. The C terminus of this peptide corresponds to a reported CEP cleavage site, that is, Phe^{52}−Ala^{53}. HPLC peak 10 of fraction III (the second largest peak in this fraction) contained β-CN f45−52, together with α_{s1}-CN f93-?

Two of the principal peptides, that is, α_{s1}-CN f1−9 and α_{s1}-CN f1−13, which accumulate in Cheddar during ripening have an N-terminal sequence X-Pro. . . (Arg-Pro-Lys-His-Pro. . .). Similarly, the N-terminal sequence of many of the peptides identified in this study was X-Pro. . . (e.g., α_{s1}-CN f11−?, α_{s2}-CN f176−?, α_{s2}-CN f191−197) or X-Pro-Y-Pro. . . (e.g., α_{s1}-CN f26−32 and f26−34). These peptides should be susceptible to hydrolysis by Pep X (X-prolyl dipeptidyl aminopeptidase), but their accumulation in cheese suggests that Pep X is either inactive in cheese or unable to hydrolyze these peptides in the cheese environment, that is, low a_w and high ionic strength. This conclusion is supported by the results of Booth et al. (1990), who reported that the Pep X of *L. lactis* subsp. *cremoris* AM2 was not active on peptides Arg-Pro-Pro or Arg-Pro-Pro-Gly-Phe. Unpublished results from this laboratory showed that Pep X failed to hydrolyze synthetic α_{s1}-CN f4−23 and α_{s1}-CN f1−23 in solution.

The principal free amino acids present in fractions III and IV of the WSE-DFP were Glu, Leu, Val, Pro, and Lys. It has been reported by many workers (Law et al., 1976; Hickey et al., 1983; Puchades et al., 1989; Wilkinson, 1993) that the most abundant amino acids in Cheddar cheese are Glu, Leu, Val, Ile, Lys, and Phe. Many of the bonds hydrolyzed by CEP expose Glu/Gln residues; the presence of high amounts of Glu indicates easy removal of N-terminal Glu residues from peptides by an aminopeptidase (Pep A). Activity of the general aminopeptidase (Pep N) can explain release of amino acids such as Leu, Val, and Lys. A considerable amount of Pro was present, especially in fraction IV, which, in the apparent lack of PepX activity, suggests considerable proline iminopeptidase activity in Cheddar cheese.

CONCLUSION

Most of the peptides in the WSE-DFP of Cheddar cheese have been isolated and partially identified. Characterization of these peptides facilitates further work on the enzymes responsible for the formation of small peptides and amino acids in cheese. This study and previously published work (Fox et al., 1994; Singh et al., 1994, 1995, 1997) from this laboratory demonstrate the important role played by the cell-envelope proteinase and peptidases from starter *Lactococcus* spp. in the degradation of the larger peptides produced by chymosin and plasmin from caseins. These results greatly extend our knowledge on proteolysis in cheese

and the mechanisms involved in the development of cheese flavor.

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