Identification of Peptides Released from Casein Micelles by Limited Trypsinolysis

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Bovine casein micelles were treated with low concentrations of trypsin before and after heating. Peptides present in the supernatant solutions of time course samples after high-speed centrifugation were separated by reversed-phase HPLC and identified principally by mass spectrometry. β-Casein was hydrolyzed rapidly, the initial cleavages occurring in the region of the plasmin-sensitive bonds. α_{s1}-Casein was hydrolyzed more slowly, the initial cleavage points being in the N-terminal region, but some trypsin-sensitive bonds in this part of the molecule appeared to be shielded. α_{s2}-Casein was hydrolyzed slowly from the C-terminal. Hydrolysis of κ-casein was insignificant. No peptides containing phosphoserine clusters were released. Although heating increased the rate of hydrolysis of all of the caseins, the HPLC profiles were generally similar to those obtained with unheated micelles. The results are discussed in relation to the possible structure of the surface of the casein micelle.

Keywords: *Caseins; micelles; milk; trypsin*

INTRODUCTION

Milk is white as a result of light scattering caused mainly by the submicrometer-sized colloidal particles into which the bulk of the milk proteins are packaged. These particles have been named micelles and the proteins contained within them, the caseins. Electron microscopy shows micelles to be roughly spherical, with a surface which has been likened to that of a raspberry. Most of the casein is located in micelles with diameters ranging between 50 and 300 nm that in addition to protein contain high levels of colloidal calcium phosphate (CCP). The caseins consist of four major protein species designated α_{s1}-, α_{s2}-, β-, and κ-caseins whose chemistry has been recently reviewed (Swaisgood, 1992). All are relatively lacking in secondary structure and, with the exception of κ-casein, which usually has a single phosphorylation site, contain a number of phosphoserine residues which tend to be located in clusters. The proteins are believed to interact with the CCP via their phosphoserine residues.

The rheological properties of milk are crucially dependent upon the integrity of the micellar structure. Addition of the enzyme chymosin, or reduction of the pH of the milk as a result of fermentation of lactose to lactic acid by bacteria, causes the micelles to aggregate and gelation to occur. These are the initial steps in cheese and yogurt manufacture, respectively.

The organization of the various caseins within the micelles is still a matter of some debate. Several models for the structure of casein micelles have been proposed [see reviews by Rollema (1992) and Holt (1992)]. However, in general, these models do not describe all aspects of micellar properties and the substructure of the micelles remains unclear. In particular, information regarding the organization of the various classes of caseins within the micelles is vague, and much of it is contradictory.

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A number of techniques have been used to investigate the distribution of the individual caseins within the micellar structure. For example, differential centrifugation has shown that the composition of micelles is size dependent, with the proportion of κ-casein being higher in smaller micelles (Donnelly *et al.*, 1984; Dalgleish *et al.*, 1989). These results, together with the knowledge that chymosin-catalyzed aggregation is due to hydrolysis of the κ-casein component, have been interpreted as showing that the micelle consists of a core of equimolecular amounts of β- and α_s-caseins together with only small amounts of κ-casein and a surface composed of κ- and α_s-caseins and small amounts of β-casein (Dalgleish *et al.*, 1989). A variety of proteolytic enzymes have also been used in order to analyze the distribution of the caseins in the micelle. Hydrolysis by carboxypeptidase A (Cheryan *et al.*, 1975) and polymerized papain (Ashoor *et al.*, 1971) suggested that all of the casein fractions are located on the micellar surface. Mehaia (1984) treated micelles with immobilized chymosin, pepsin, and neuraminidase and reported a surface location of most of the κ-casein. Trypsinolysis of whole milk (Leaver and Thomson, 1993) suggested that most of the β- and κ-caseins may be located in the coat region of the micelle.

In the present paper, trypsinolysis has again been used to investigate the organization of proteins within the micelle. Peptides released from the micellar caseins by enzymatic hydrolysis were separated by reversed-phase (RP-) HPLC and identified principally by N-terminal and C-terminal sequencing using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), a technique suitable for the analysis of peptide mixtures resulting from enzymatic protein digestion (Thiede *et al.*, 1995). In this way, it was hoped to obtain information on the accessibility of specific regions of the micellar caseins.

MATERIALS AND METHODS

Reagents. L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin from bovine pancreas (12 000 BAEE units mg^{-1} of solid; 12 200 BAEE units mg^{-1} of protein),

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trypsin inhibitor from soybean, phenylmethanesulfonyl fluoride (PMSF), sequencing grade carboxypeptidase Y, trifluoroacetic acid (TFA), bovine insulin, kemptide, substance P, and most of the N-terminal sequencing reagents (Bartlet-Jones *et al.*, 1994) were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Trifluoroethylisothiocyanate (TFEITC) was provided by the Protein Sequencing Laboratory, Imperial Cancer Research Fund, London, U.K. α-Cyano-4-hydroxycinnamic acid (ACH matrix) was from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. HPLC grade solvents were from Fisons Ltd., Loughborough, Leics., U.K.

Micelle Preparations. Milk, collected from the bulk tank of the Institute farm after morning milking of the Friesian herd, was skimmed by centrifugation (2000*g*, 40 min, 4 °C). Skimmed milk (120 mL) was incubated at room temperature for 2 h. The milk was then centrifuged at high speed in order to pellet the micelles (28000*g*, 2 h, 20 °C), and the pellet was resuspended in milk ultrafiltrate to the original volume. A portion (18 mL) of this micellar suspension was removed and incubated at 80 °C for 30 min. This sample was again centrifuged to pellet the micelles and resuspended to the original volume in ultrafiltrate. Milk ultrafiltrate was prepared by filtration through a PM10 membrane using an Amicon ultrafiltration cell Model 420 (Amicon, Glos, U.K.) at a pressure of 380 kPa. This process removed all of the globular whey proteins from the serum phase.

Kinetics of Peptide Formation. Aliquots (2 mL) of raw and heated micellar suspensions were removed as zero time course samples and added to 40 μL of a solution of trypsin inhibitor (2 mg mL^{-1} in water) and 20 μL of a solution of PMSF (8.7 mg mL^{-1} in methanol). A freshly prepared solution of TPCK-treated trypsin in water (40 μg, 500 BAEE units) was added to the remaining 16 mL (containing approximately 500 mg of casein) of each of the micelle preparations. The casein to trypsin molar ratio was approximately 12000:1. Aliquots (2 mL) of the reaction mixtures were removed at various times and trypsin activity was inhibited as above. In early experiments, 1 mL of chloroform/methanol (2:1 v/v) was added immediately to 500 μL of the time course samples, and after shaking, the samples were centrifuged (2600*g*, 10 min, 20 °C) in order to separate the phases. The upper methanol/water layer was removed and used as a source of peptides. In later experiments, after addition of 20 μL of a 10% sodium azide solution to each time course sample in order to inhibit bacterial growth, the tubes were centifuged at 30000*g* for 40 min at 20 °C. The clear supernatant solution was used as a source of soluble peptides.

RP-HPLC. Tryptic peptides were separated by RP-HPLC on an APEX ODS WP column (25 cm × 4.6 mm i.d.; 7 μm) (Jones Chromatography, Hengoed, Mid Glamorgan, U.K.). Samples (200 μL) were loaded onto the column and peptides eluted using a linear gradient of acetonitrile in 0.1% TFA. The gradient used with the water-soluble peptides was 0−50% acetonitrile over 60 min. Flow rate was 1.5 mL min^{-1}, and detection was at 214 nm. Individual peptides were collected manually, freeze-dried, and stored at −15 °C.

Mass Spectrometry. Mass spectra were recorded using a MALDI time-of-flight mass spectrometer (Lasermat 2000, Finnigan MAT Ltd., Paradise, Hemel Hempstead, U.K.) equipped with a nitrogen laser (337 nm wavelength). The acceleration voltage was 20 kV, and the spectra were obtained by summing 50 laser pulses. Freeze-dried fractions were dissolved in 10 μL of deionized water. Samples (0.5 μL) were mixed with an equal volume of ACH matrix (10 mg mL^{-1} in 70% acetonitrile/water v/v) on a stainless steel sample slide and dried at room temperature. The accuracy of the mass determination of peptides was improved by means of internal standard calibration using solutions of kemptide (6.43 μg mL^{-1} in water), substance P (2.4 mg mL^{-1} in water), and bovine insulin (0.57 mg mL^{-1} in 10 mM HCl). Peptide mass search of the different casein peptides was performed using the Protein Abacus program version 2.02 (Lighthouse Data, Finnigan MAT Ltd.). The primary structures of the caseins were taken from Swaisgood (1992). Peptides released during the initial tryptic hydrolysis of micellar caseins were identified on the basis of their molecular weights, lysine content, and C-terminal and/or N-terminal sequences.

C-Terminal Sequence Analysis. C-Terminal sequencing was performed using carboxypeptidase Y. Peptides were dissolved in 25 μL of digestion buffer (50 mM ammonium acetate, pH 5.5), and 21 μL was digested using a carboxypeptidase Y solution (2 μL; 1 μg μL^{-1} in water). Aliquots (3 μL) were withdrawn at time intervals and the digestions terminated by addition of 1 μL of glacial acetic acid. Time course samples (0.3 μL) were applied to sample slides and dried at room temperature. ACH matrix (0.3 μL) was added, and after drying, the samples were analyzed by mass spectrometry.

N-Terminal Sequence Analysis. N-Terminal sequencing was carried out according to a modification of the method described by Bartlet-Jones *et al.* (1994), which involved mass spectrometric analysis of peptides at the end of each Edman-type degradation cycle without addition of aliquots of the original sample at the beginning of the cycle. The number of cycles made depended on the information necessary to characterize each peptide. ϵ-NH_2 groups of any lysine residues in the peptide also react with the TFEITC reagent, resulting in a mass increase of 141 Da per lysine residue, yielding additional information concerning the number of lysine residues present in the peptides. Unusual results were obtained when histidine and proline occupied adjacent positions in a peptide molecule, due to a precleavage of the histidine residue during removal of the volatile buffer in vacuo after the coupling reaction (Allen, 1989). The presence of glutamine at the second position caused an additional perceived mass decrease of 17 Da when the acid cleavage step of the N-terminal residue was made. This was the result of the exposed glutamine cyclizing under the acid conditions to a pyrrolidonecarboxylyl (pyroglutamyl) residue, with the concomitant release of an ammonia molecule. Partial blockage to further degradation was observed when glutamine residues were terminal (Allen, 1989).

Amino Acid Analysis. Some of the larger peptides could not be identified by terminal sequencing, because the decrease in their molecular weight resulting from the removal of individual residues could not be determined with a sufficiently high degree of accuracy. These peptides were therefore subjected to gas-phase acid hydrolysis in 6 M HCl and after preparation and separation, by RP-HPLC, of the phenylthiocarbamyl amino acids, the peptides were identified on the basis of their molecular mass and amino acid composition.

Kinetics of Hydrolysis of Micellar Caseins. After trypsinolysis of the micelles as described above, but at the higher casein to trypsin molar ratio of approximately 6000:1, casein separation, precipitation, and sample preparation was carried out according to Leaver and Thomson (1993). Ion-exchange FPLC on a MonoQ HR5/5 anion-exchange column (Pharmacia Ltd., Milton Keynes, Bucks, U.K.) was used to monitor the trypsin-catalyzed hydrolysis of the various micellar casein fractions in heated and unheated micelles, in order to check the effect of heating on micellar caseins as previously described (Davies and Law, 1987).

RESULTS

Rates of Hydrolysis of Micellar Caseins. The relative rates of hydrolysis of the α_s-, β-, and κ-casein components of raw and heated micelles are shown in Figure 1. The results are similar to those obtained previously with whole milk (Leaver and Thomson, 1993). In unheated micelles, β-casein was hydrolyzed rapidly at a rate which approached that of β-casein in solution. The rate of hydrolysis of the α_s-caseins was slower, and the breakdown of κ-casein was very slow. Unlike the situation in heated milk, where the rate of hydrolysis of κ-casein was reduced and the rates of hydrolysis of the other caseins were increased, heating micelles increased the rates of hydrolysis of all of the micellar components.

Sites of Tryptic Hydrolysis. RP-HPLC profiles of the high-speed centrifugation supernatant solutions of

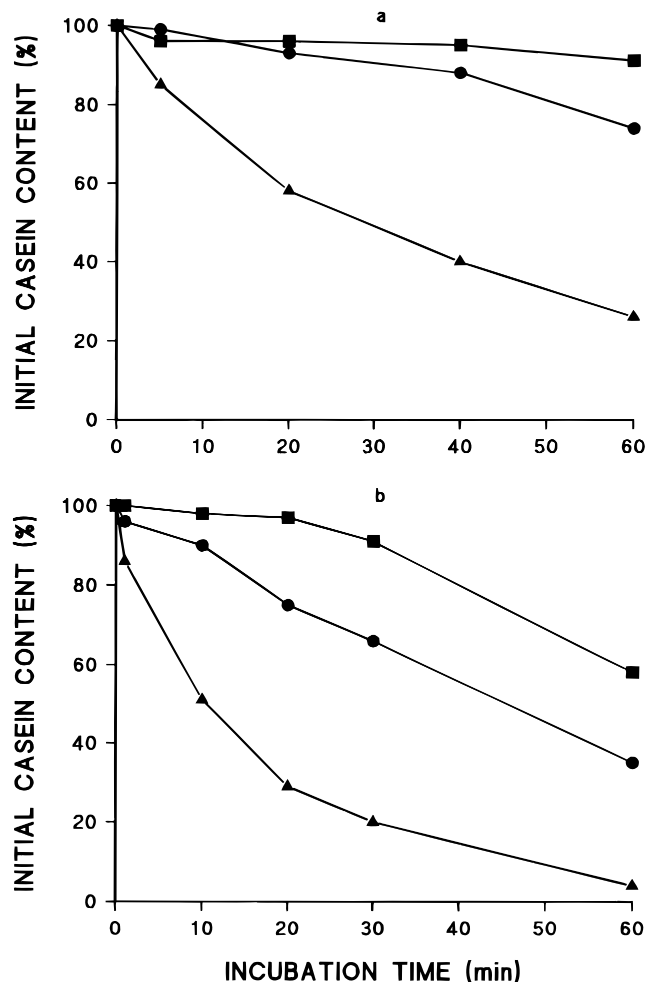

Figure 1. Time course of the hydrolysis of caseins by trypsin in unheated (a) and heated (b) micelles: ●, α_s-casein; ▲, β-casein; ■, κ-casein.

untrypsinized micelles and of raw and heated micelles trypsinized for 90 min are shown in Figure 2. The small number of background components present at low levels in the zero-time samples were unaffected by protease activity, indicating that all of the peptides formed were derived from micellar caseins. Table 1 shows the identity of those peptides formed early in the hydrolysis. Specific regions of the various caseins appeared to be susceptible to trypsinolysis. In the case of α_{s1}- and α_{s2}-caseins, peptides detected during the early stage of the hydrolysis were all derived from the terminal regions of the molecules: from the N-terminal region of α_{s1}-casein and the C-terminal region of α_{s2}-casein. β-Casein

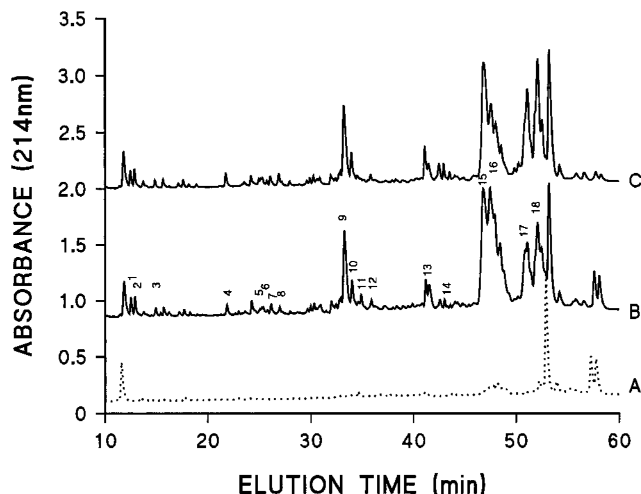

Figure 2. RP-HPLC profiles of the high-speed centrifugation supernatant fraction from (A) unheated, nontrypsinized micelles and (B) unheated and (C) heated micelles incubated with trypsin for 90 min. Labeled peaks: (1, 2, 3, and 4) β-casein f100−107, 100−105, 98−107, 170−176; (5, 6, 7, and 8) α_{s2}-casein f200−205, 198−205, 115−137?, 189−197; (9) α_{s1}-casein f1−22; (10) α_{s2}-casein f153−165/166; (11) α_{s1}-casein f1−21; (12) α_{s2}-casein f200−207; (13 and 14) α_{s1}-casein f23−36/42, 23−34; (15/16) β-casein f29/30/33−99/105; (17 and 18) β-casein f100/106−169, 170/177−209.

tryptic peptides resulted from hydrolysis in a variety of regions including those around the plasmin-sensitive bonds at positions 28−29, 105−106, and 107−108. β-Casein f191−209 contains the chymosin-sensitive bond at position 192−193. The Phe−Leu bond at position 190−191 of β-casein has previously been identified as a nonspecific trypsin cleavage site (Henle and Klostermeyer, 1993). Incubating micelles with the proteolytic enzymes chymosin and cathepsin D has been reported to result in the formation of α_{s1}-casein f24−199 and β-casein f1−189/192 and f1−165/167 (Kaminogawa *et al.*,1980; McSweeney *et al.*,1995), confirming the accessibility of these bonds to added proteases. As expected from the results shown in Figure 1, no peptides derived from hydrolysis of κ-casein were detected.

With the exception of those β-casein peptides containing the single phosphoserine residue at position 35, the major phosphopeptides containing the phosphate centers were not detected in the soluble fractions.

Kinetics of Peptide Formation. The rates of formation of various peptides from unheated micelles are shown in Figure 3. Two principal hydrolytic events occurred in the initial 10 min of reaction. These involved the release of α_{s1}-casein f1−22 and cleavage of some of the β-casein molecules at bonds in the region

Table 1. Peptides Released in the Early Stages of Trypsinolysis of Casein Micelles

α_{s1}-casein		α_{s2}-casein		β-casein	
peptide	mass[b]	peptide	mass[b]	peptide	mass[b]
1−13[a]	1536	200−207	1022	29/30/33−99/105	7630, 8000, 8128, 8257, 8628, 8756
1−16[a]	1877	200−205	746		
1−22	2617	198−205	975	98−107	1138
4−22	2236	198−207	1252	100−105	645
8−22	1760			100−107	911
10−22	1495			101−112[a]	1384
23−36	1642			100−169	7985
23−42	2313			170−176	780
				170−209	4484
				177−209	3422
				191−209[a]	2108
				194−209[a]	1718

[a] Nonspecific cleavage. [b] In daltons.

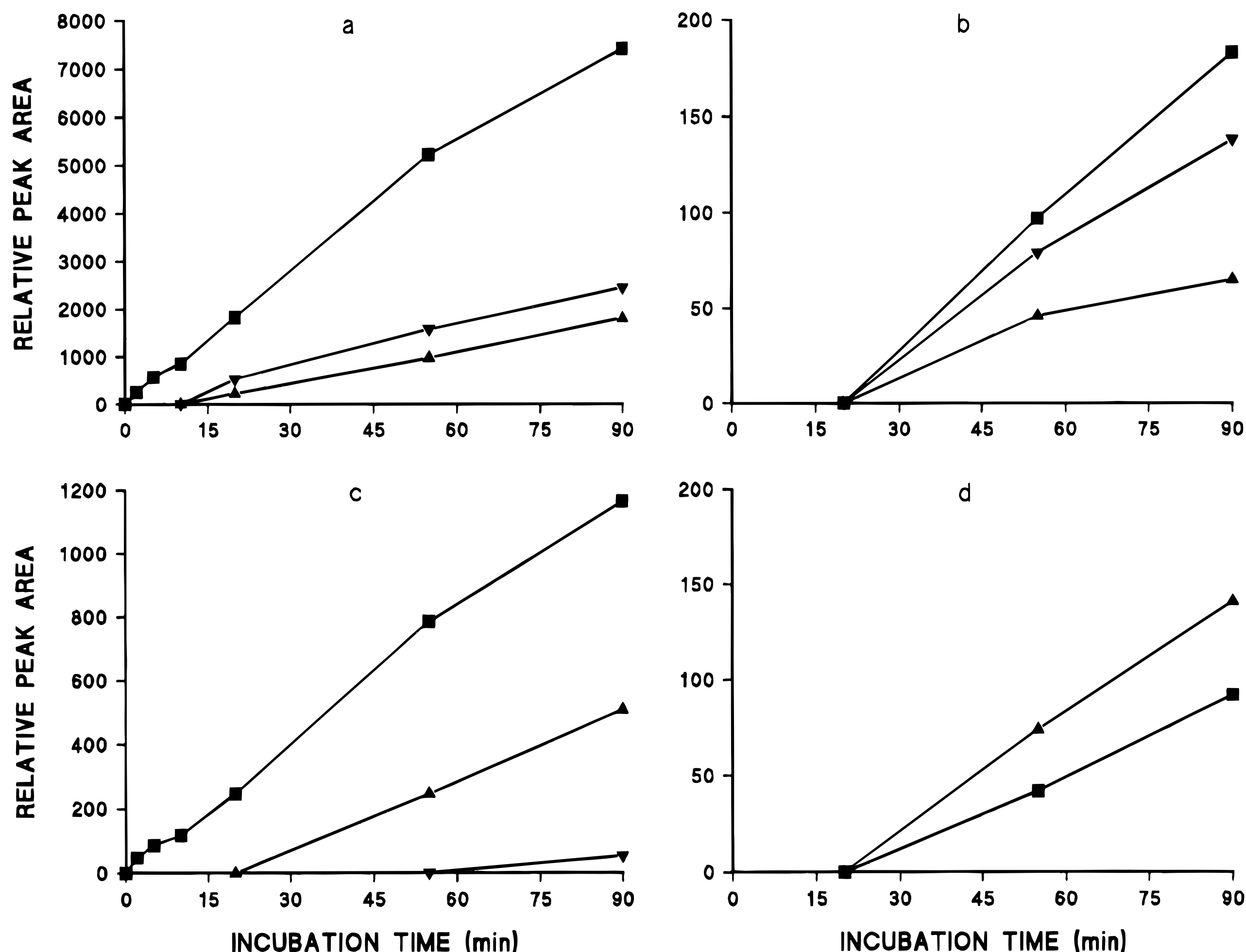

Figure 3. Rate of formation of selected peptides in unheated micelles. (a) β-Casein: (■) f29/33−99/105, (▲) f100/106−169, (▼) f170/177−209. (b) β-Casein: (■) f100−105, (▲) f98−107, (▼) f100−107. (c) α_{s1}-Casein: (■) f1−22, (▲) f23−36/42, (▼) f23−34. (d) α_{s2}-Casein: (■) f198−205, (▲) f200−205. Note the change in scale.

of the plasmin-sensitive site at position 28 and also in the center of the molecule around positions 99 and 105 (also a plasmin-sensitive site), resulting in the release of a variety of peptides with very similar retention times and structures (f29/30/33−99/105). Peptides resulting from hydrolysis of α_{s1}-casein at Lys3 and Lys7 were only detected much later in the experiment. The formation of α_{s1}-casein f23−36 and f23−42 gives an indication of the extent of the accessibility of the N-terminal part of the molecule. After about 15 min of hydrolysis, additional β-casein tryptic peptides were detected, apparently produced as a result of hydrolysis of the large C-terminal fragment at the lysine residues at positions 169 and 176 resulting in the formation of f170/177−209. Additional hydrolysis at lysine residues at positions 97, 99, 105, and 107 of the β-casein chain resulted in the formation of a number of short, hydrophilic peptides from the center of the molecule. At the same time, a number of relatively short peptides from the C-terminal end of α_{s2}-casein were detected.

With the exception of some of the smaller peptides, which may have been formed as a result of secondary hydrolysis of larger fragments after they had been cleaved from the intact micellar caseins, e.g., α_{s1}-casein f4−22, 8−22, etc., the pattern of hydrolysis in different micellar preparations was very consistent.

DISCUSSION

A typical bovine micelle with a diameter of 150 nm contains approximately 10^4 polypeptide chains and 3×10^3 microgranules of CCP with an average diameter of 2.5 nm. The micelle may be composed of submicelles, although this is still an area of some debate, and is surrounded by a "hairy" layer of polypeptide chains which, through a combination of steric and electrostatic repulsion, prevents individual micelles from aggregating. Addition of ethanol above a critical concentration causes this hairy layer to collapse, resulting in aggregation of the individual micelles. Similarly, addition of the proteolytic enzyme chymosin, which specifically cleaves κ-casein at the Phe−Met bond at position 105−106, decreases micellar size and results in aggregation, indicating that some, if not all, of these hairs are the highly electronegatively charged C-terminal caseinomacropeptide of κ-casein, which is released into the aqueous phase prior to aggregation. Whether all of the surface hairs are due to κ-casein is not known and the location and orientation of the various caseins within the micelle is unclear, the evidence arising from proteolytic digests and electron microscopy as reviewed by Holt (1992) being somewhat contradictory.

The rates of trypsinolysis of the various caseins as reported here and previously (Leaver and Thomson, 1993) show that much of the micellar β-casein is in a position readily accessible to the protease molecules since despite being present in an immoblized form within the micelle it is hydrolyzed at a rate similar to that in solution. Obviously, this may be on the surface of the micelles, but voluminosity measurements have shown that micelles have a very open structure with

only 25% of the total volume being occupied by protein molecules. It is therefore possible that the relatively small trypsin molecules, with a molecular weight (24 000) similar to that of the caseins, can rapidly penetrate into pores in the micellar structure and hydrolyze protein molecules not located on the circumference of the structure. The amount of trypsin used (one to two molecules per micelle) was selected in order to minimize destruction of the micellar structure, particularly in the early stages of hydrolysis, which are covered in the analysis of the kinetics of peptide release. Irrespective of the exact location of the β-casein, initial sites of hydrolysis are in those regions that are also attacked by the naturally occuring milk protease plasmin, which results in the formation of a series of proteose peptones and γ-caseins (Eigel *et al.*, 1984). It seems reasonable to conclude that some at least of the β-casein is probably present in the coat region as suggested by Walstra (1990) with these parts of the molecule exposed. After the initial cleavage, other parts of the β-casein polypeptide chain are hydrolyzed, including those in the region of the chymosin-sensitive bond at position 192−193.

α_{s1}-Casein was not hydrolyzed as rapidly as β-casein. The initial site of proteolysis is in the N-terminal region at the arginine residue at position 22, which is adjacent to the Phe−Phe bond at position 23−24, the most chymosin-sensitive bond in this protein, hydrolysis of which begins very soon after micellar aggregation during cheese manufacture. This also indicates that this region of the α_{s1}-casein molecule is in a position that is readily accessible to added soluble proteases. The failure to detect peptides resulting from tryptic hydrolysis at the lysine residues at positions 3 and 7 until later in the hydrolysis suggests that these positions may be protected in the intact micellar casein as a result of folding of the polypeptide chain to form an N-terminal loop.

α_{s2}-Casein is hydrolyzed more slowly than the α_{s1}-casein component as judged by release of peptides derived from this protein, indicating that it is less accessible in the micelle. When hydrolysis does begin, it is from the C-terminal region, and in contrast to α_{s1}-casein hydrolysis, there is no evidence of protected residues in this tail.

The very low rate of hydrolysis of κ-casein may be due to the paucity of arginine and lysine residues in the exposed C-terminal region of the molecule. The trypsin-sensitive bond closest to the C-terminal end is the lysine residue at position 116, which is 53 residues into the molecule. Other trypsin cleavage points are located at positions 112, 111, and 97, but despite their proximity to the Phe−Met bond at 105−106 which is readily hydrolyzed by chymosin, proteolysis by trypsin occurs much less readily. This may be due to polymerization of κ-casein molecules through the formation of intermolecular disulfide bridges shielding these trypsin-sensitive sites. Whatever the reason, proteolysis of the other caseins is not dependent upon prior proteolysis of κ-casein.

Trypsinolysis of β-casein either in solution or adsorbed at oil/water interfaces results in rapid formation of the 1−25 and 1−28 phosphopeptides which, due to their hydrophilicity, were very soluble or readily extracted into the methanol/water phase (Leaver and Dalgleish, 1990). In this study, no phosphopeptides from any of the caseins were detected in either the aqueous or methanol/water phases. Since hydrolysis of β-casein around position 28 was one of the initial proteolytic events as judged by the detection of the other peptides in the supernatant phase, which were formed in part by hydrolysis in this region, phosphopeptides were being formed early in the trypsinolysis but were retained within the micellar pellet. This would appear to confirm that caseins interact with the micellar CCP via their phosphate centers (Holt *et al.*, 1986; Aoki *et al.*, 1988), and as long as the CCP remains within the micelle, so too do the phosphopeptides.

Trypsinolysis of caseins was more rapid in micelles that had been heated and then cooled. In contrast to the situation in milk, where heating increased the rate of proteolysis of the β- and α_s-caseins but decreased that of the κ-casein (Leaver and Thomson, 1993), in micelles the rate of trypsinolysis of all of the caseins was enhanced. This confirms that the reason for the inhibition of κ-casein trypsinolysis in milk was due to interaction of this component with whey proteins, since in the micellar preparations, whey proteins had been removed in the ultrafiltration step. It also indicates that inhibition of renneting by heating must be due to specific interactions between the κ-casein and whey protein rather than general inhibition due to deposition of denatured whey protein on the micelles. The reason for the general increase in the rate of trypsinolysis resulting from heating is not known, but the rate of proteolysis of κ-casein by chymosin also increases when micelles are heated in the absence of whey proteins (Reddy and Kinsella, 1990) and both may result from a general loosening of micellar structure.

CONCLUSIONS

Determining the kinetics of proteolysis appears to be an effective way of measuring the accessibility and topography of proteins within the casein micelles. Using micelles rather than milk ensured that all peptides detected in the reaction mixture were derived from micellar caseins and not whey proteins. Heating increased the rate of hydrolysis of all of the micellar caseins and showed that the inhibition of κ-casein hydrolysis in heated milk which we reported previously was probably due to specific interactions between the κ-casein and whey proteins. Much of the β-casein component of the micelles was very accessible to soluble trypsin, with the regions around the plasmin-sensitive bonds at the center of the molecule and near the phosphate cluster being particularly exposed. Although α_{s1}-casein was not as accessible, the 1−22 fragment was formed very early in the hydrolysis, suggesting that the N-terminal region of some of the α_{s1}-casein molecules may also be in an exposed orientation within the micelle. Formation of peptides from α_{s2}-casein, in this case from the C-terminal region, was not detected until later in the reaction, indicating that this casein is not very accessible to the soluble protease. The failure to detect any of the hydrophilic phosphopeptides, particularly the β-casein phosphopeptides which are readily released by trypsinolysis of both soluble and emulsified β-casein, indicated that these regions of the various caseins probably interact with CCP. Further analysis of micellar structure in raw and processed milks using a variety of soluble and immobilized proteases should yield more information on the fine structure of these colloidal particles.

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