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Preferential sites of tryptic cleavage on the major bovine caseins within the micelle

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Abstract — Caseins, major proteins in bovine milk, are structured in micelles within which the detailed casein organisation remains unclear. We have used limited proteolysis to find the most exposed regions of the caseins within the micelle. Under hydrolysis conditions, only 9% of colloidal calcium and 15.6% of the colloidal inorganic phosphate were removed from the micelle after 4 h. The released peptides during tryptic hydrolysis were separated from the residual micelle by ultracentrifugation. They were subsequently analysed and characterised by reversed-phase HPLC coupled online with electrospray ion source mass spectrometry (ESI-MS). After 4 h of hydrolysis, the β-casein had completely disappeared while about 40% of α_{s1}-casein and 37% of κ-casein remained undigested. The initial distribution, i.e., 91% caseins and 9% peptides prior to hydrolysis, was modified after hydrolysis to give 64% peptides remaining within the micelles together with 13% caseins and 22% of the peptides produced were released from the micelles. Among the 61 peptides identified, 34 arose from β-casein, 16 from α_{s1}-casein, 11 from α_{s2}-casein and none from κ-casein. For the latter casein, the failure to detect peptides was due to its lower concentration in the large micelles compared with that of the β and α_{s1}-caseins. The peptides derived from β-casein were mainly released from the micelle, while peptides from both α_{s1}- and α_{s2}-caseins were preferentially retained within the residual micelles, suggesting that all the caseins are accessible to trypsin but are differentially involved in the micellar framework. Our results suggest that α_s-caseins play a significant role in the micellar cohesion via salt binding with colloidal calcium phosphate and hydrophobic interaction, with itself and with the other caseins. © Inra/Elsevier, Paris.

casein / micelle / limited proteolysis / trypsin / reversed-phase chromatography / mass spectrometry / peptide / accessibility

Résumé — Sites privilégiés de coupure sur les caséines bovines majeures par trypsinolyse des micelles. Les caséines, constituants majeurs des protéines du lait de vache, sont présentes sous forme de micelles dont l'organisation interne est encore mal connue. Nous avons utilisé la protéolyse limitée pour déterminer les régions des caséines les plus accessibles au sein de la micelle. Dans les conditions utilisées, seuls 9 % du calcium colloïdal et 15,6 % du phosphate inorganique colloïdal sont

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éliminés de la micelle après 4 h d'hydrolyse. Afin de différencier les peptides qui sont libérés de la micelle de caséine au cours de l'hydrolyse par la trypsine de ceux qui restent situés à l'intérieur, nous avons ultracentrifugé les hydrolysats avant de caractériser les peptides. Leur caractérisation était ensuite réalisée par couplage direct entre la CLHP de phase inverse et la spectrométrie de masse à source d'ionisation electrospray. Après 4 h d'hydrolyse, la caséine β avait complètement disparu tandis qu'il restait encore 40 % de caséine α_{s1} et 37 % de caséine κ non dégradées. La répartition des composés azotés au sein de la micelle est passée d'une composition initiale de 91 % de caséines et de 9 % de peptides à 13 % de caséines et 64 % de peptides après hydrolyse, alors que seulement 22 % des peptides produits étaient libérés de la micelle. Parmi les 61 peptides identifiés, 34 provenaient de la caséine β, 16 de la caséine α_{s1}, 11 de la caséine α_{s2} et aucun de la caséine κ. Pour cette dernière, sa plus faible concentration dans les grosses micelles comparée à celle des caséines β et α_{s1} ne nous a pas permis de les détecter. Les peptides issus de la caséine β étaient presque tous libérés dans la solution tandis que ceux provenant des caséines α_{s1} et α_{s2} étaient majoritairement retrouvés dans les micelles résiduelles. Ceci suggère que tous les types de caséines sont accessibles à la trypsine mais que leur rôle dans l'assemblage micellaire serait différent. Ainsi, les caséines α_s participeraient de manière significative à la cohésion micellaire en faisant intervenir des liaisons salines avec le phosphate de calcium colloïdal et des interactions intermoléculaires hydrophobes avec elle-même et avec les autres caséines.

caséine / micelle / protéolyse limitée / trypsine / chromatographie de phase inverse / spectrométrie de masse / peptide / accessibilité

1. INTRODUCTION

The major protein component of bovine milk is represented by the caseins, made up of four phosphoproteins (α_{s1}, α_{s2}, β, and κ) which are present as a colloidal dispersion called casein micelles. They are complexed with colloidal calcium phosphate [37], which has a crucial role in maintaining casein micelle integrity [30, 33]. The clearly defined micellar composition and its size-dependence has led to various structural models. These have been progressively refined as more information on micellar structure has become available (for a review, see [15] and [36]). The preferential location of the κ-casein, on the micellar surface, is to date generally accepted whereas the distribution of the other caseins still remains an issue of debate.

The proteolytic approach has often been used to investigate the distribution of the individual caseins within the micellar structure. By the combined use of free and immobilised carboxypeptidase A [5, 6, 34] all the casein types were found to be distributed not only within the micelles but also on the surface. Additional information was acquired on casein accessibility within the micelle using endoproteinase. Thus, Leaver and Thomson [27] showed that β-casein is one of the most accessible phosphoproteins and they suggested that most of the β-and κ-caseins may be located in the coat region of the micelle. However, this was not in agreement with the calculations made from the ultracentrifugation separation of micelles by Dalgleish et al. [8], showing that up to 10% of the β-casein might be located at the surface. Finally, in a previous study [13], using extended trypsinolysis of casein micelles, we showed that the α_{s1}-casein directly interacts with the colloidal calcium phosphate, constituting the so-called micellar backbone, whereas α_{s2}-, and β-caseins are only partially implicated within this backbone.

We have herein extended our previous study to establish the most exposed regions of the caseins in the micelle under condi-

tions of limited trypsinolysis, avoiding colloidal mineral depletion. The released peptides were separated from the residual micelle by ultracentrifugation. They were then analysed and characterised by reversed-phase HPLC coupled on-line with electrospray ion source mass spectrometry (ESI-MS), a powerful method for peptide characterisation even for complex hydrolysate systems [12].

2. MATERIALS AND METHODS

2.1. Preparation of large micelles

Milk from the morning milking (Inra farm, Rennes, France) was ultracentrifuged (100 000 *g*, 1 h, 37 °C). The whole caseins were resuspended in milk ultrafiltrate, arising from the same initial milk, and prepared by ultrafiltration on a DC 10 LA module (Amicon Inc, Beverly, MA, USA) equipped with a hollow fibre cartridge (cut off 10 kDa) (Romicon, Woburn, MA, USA). Sodium azide (0.2 $g \cdot L^{-1}$) was added as a preservative. The casein micelles were equilibrated at 37 °C, with stirring, for 2 h prior to fractionation by ultracentrifugation [32]. Due to the heterogeneity of the casein micelles in terms of size and composition only one type of micelle, the larger ones (with a diameter over 100 nm and representing from 60 to 75% by mass of all the casein micelles present in milk), was used in this study. Large micelles were obtained in the pellet after centrifugation (15 000 *g*, 1 h, 37 °C).

2.2. Determination of trypsin (EC.3.4.21.4.) activity

Tosyl-phenylalanine-chloromethyl-ketone treated trypsin (TPCK-trypsin, Serva, Heidelberg, Germany) activity was measured by spectrophotometry at 247 nm using 10 $mmol \cdot L^{-1}$, 4-toluene-sulphonyl-arginine-methyl-ester (TAME, Sigma, St.-Quentin Fallavier, France), as reported by Hummel [18]. One unit (1 U) was defined as the number of micromoles of TAME hydrolysed per min at 25 °C and at pH 6.7, per mg of protein. The trypsin activity in this case was 32.3 $U \cdot mg^{-1}$.

2.3. Hydrolysis of casein micelles

Casein micelles were resuspended in milk ultrafiltrate at 37 °C for 2 h, under stirring, prior to hydrolysis. Tryptic digestion of casein micelles (31.6 $gTN \cdot kg^{-1}$) was performed at 37 °C in milk ultrafiltrate at an enzyme concentration of 0.021 $U \cdot mL^{-1}$. Aliquots were withdrawn at intervals and the reaction stopped by adding soyabean trypsin inhibitor type I (Sigma), in a molar inhibitor/enzyme ratio of 3, followed by ultracentrifugation (100 000 *g*, 1 h, 37 °C). The supernatants, containing the peptides released from the micellar structure, and the pellets, corresponding to the residual micelles, were stored frozen for further analyses. A control experiment, containing all the reagents except the enzyme, was prepared to take into account the endogenous plasmin activity. This activity was found to be negligible under our hydrolysis conditions.

2.4. Determination of the casein micelle size

The size of the casein micelles, prior to and during hydrolysis, was determined by photon correlation spectroscopy with a Coulter N4MD (Coultronics France, Margency, France), as described by Gagnaire et al. [13].

2.5. Analysis of calcium, inorganic phosphorus and nitrogenous contents

Analytical determinations were performed on the whole casein micelle solution, on the peptides released from the structure and on the residual micelles prior to and during the tryptic hydrolysis. The residual micelles were resuspended in the same volume of milk ultrafiltrate as the original micelle solution before determination of calcium, inorganic P and N.

Analysis of calcium and inorganic phosphorus content was carried out as described by Gagnaire et al. [13].

Total N (TN) analysis was performed by a micro-Kjeldahl method [31], using a factor of 6.38 to convert to N fraction concentration. The NCN fractions were obtained by precipitation at pH 4.6 with 10% (v/v) acetic acid according to Achaffenburg and Drewry [1]. The residual

micelles led to a soluble and insoluble fraction at pH 4.6 in contrast to the samples containing the peptides released from the structure.

2.6. HPLC analyses

The residual caseins present in the fractions precipitated at pH 4.6 were analysed on a Vydac C_4 column (150 x 4.6 mm ID, Touzart et Matignon, Vitry sur Seine, France). They were dissolved in 8.75 mol·L^{-1} urea, then reduced with 16 mmol·L^{-1} dithiothreitol (Serva) and incubated for 1 h at 37 °C. At a flow rate of 1 mL·min^{-1}, samples (100 µL) diluted to 1/20 with buffer A (0.106% (v/v) trifluoroacetic acid (TFA, Pierce, Touzart et Matignon)) in MilliQ water (Millipore, St Quentin en Yvelines, France) were injected onto the column maintained at 40 °C and the absorbance was measured at 214 nm. A linear gradient was performed in 37.5 min from 37 to 57% buffer B (0.1% TFA (v/v), 80% acetonitrile (v/v) (Carlo Erba, Nanterre, France) and 20% (v/v) Milli Q water).

Purity of the peaks corresponding to each casein was checked, at each hydrolysis time studied, by collecting the peak and analysing it by urea-PAGE according to Andrews [2]. The α_{s1} and β-caseins peaks were pure throughout the hydrolysis period, whereas only the non-glycosylated part of the κ-casein fraction was pure (corresponding to the main peak) after 2 h of hydrolysis. α_{s2}-casein was contaminated early on with peptides which have the same chromatographic elution time (data not shown).

The peptides released from the micelle, together with those present within the residual micelles and separated from the caseins by precipitation at pH 4.6 (NCN soluble fractions), were analysed on a Vydac C_{18} column (250 x 4.6 mm ID, Touzart et Matignon) after 1/10 dilution with buffer A. A linear gradient from 0 to 37% buffer B in 28 min followed by 37 to 57% B in 37.5 min, was applied for the elution under the conditions described above.

2.7. Estimation of the proportion of peptides present within the residual micelles during hydrolysis

We needed an estimation of the concentration of the micellar casein for each period of hydrolysis to estimate, by difference, the peptide concentration present within the residual micelles. For that purpose, we first determined the whole micellar casein concentration by micro-Kjeldahl (TN-NCN). Second, to differentiate the proportion of each casein we have taken into account, on the one hand, that α_{s1}-casein represents 37.7% of the whole caseins in bovine milk, β-casein 37.9%, and κ-casein 8.7% and, on the other hand, that the proportion of each casein changed with each period of hydrolysis, as determined by chromatography. The α_{s2}-casein was not taken into account in the estimation due to contamination.

2.8. Electrospray mass spectrometry

Released peptides and those present in NCN fractions were initially separated by reversed-phase HPLC on a Vydac C_{18} column (250 × 2.1 mm ID, Touzart et Matignon). For the caseins, the separation was performed on Vydac C_4 (150 × 2.1 mm, Touzart et Matignon). The elution conditions corresponded to those described above except for the flow rate, which was reduced to 0.25 mL·min^{-1}. Eluted peaks were detected by absorbance at 214 nm and analysed by electrospray source ionisation-mass spectrometry (ESI-MS). The conditions of the mass analysis have been previously described by Gagnaire et al. [13]. Assignment of individual sequences from the molecular masses of peptides was based on: 1) the known sequence of bovine caseins arising, in our case, from Holstein cows milk containing the main genetic variants α_{s1} B 97%, α_{s2} A 100%, βA^1 53%, βA^2 45%, κA 71% and κB 29% [14] and, as a precondition, 2) the presence of a regular tryptic cleavage site at each end of the sequence. The mass assignment was confirmed either from the amino acid composition by the Pico-tag method or by amino acid sequence determination using tandem mass spectrometry (MS-MS) analysis, both previously described [13].

3. RESULTS

3.1. Limited proteolysis of the casein micelles

Since the colloidal calcium phosphate is mainly responsible for maintaining the integrity of the micelle [30, 33], the release of the colloidal minerals into solution during

hydrolysis could be considered as an index of casein micelle disruption. With a trypsin concentration of 0.021 U·mL^{-1} of micellar solution containing 31.6 gTN·kg^{-1}, we have determined that only 9% (2.8 mmol·L^{-1}) of the colloidal calcium and 15.6% (2.1 mmol·L^{-1}) of the colloidal P_i were removed from the micelles after 4 h of hydrolysis. The percentage of P_i, which could be removed without any significant casein micelle dissociation, was lower than that determined by Holt et al. [17] (30% of P_i). The low level of casein micelle disruption occurring under our hydrolysis conditions was also supported by the small decrease in the average micellar diameter, from 240 for the control experiment to 207 nm observed at the end of hydrolysis. These proteolysis conditions, based on a very low colloidal mineral decrease, were therefore chosen for further studies.

3.2. Disappearance of the micellar caseins

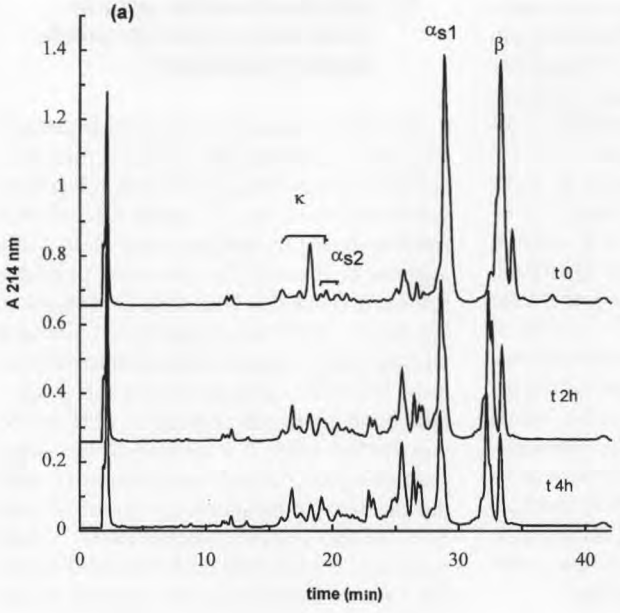
The casein composition of the residual micelles was analysed by reversed-phase HPLC and corresponded closely with that reported by Davies and Law [9]. The chromatographic profiles at different hydrolysis times are presented in *figure 1a*. Each type of casein diminished during incubation time, with a complete disappearance of β-casein at the end of hydrolysis. The kinetics of the disappearance of β-casein, α_{s1}-casein and the major peak of unglycosylated κ-casein are given in *figure1 b* except for α_{s2}-casein, which was contaminated early on by co-eluted peptides as observed by urea-PAGE (data not shown). β-casein was hydrolysed about twice as rapidly as α_{s1}- and κ-caseins. After 4 h of hydrolysis, when the β-casein had disappeared, only 40.2% and 37% of α_{s1}- and κ-caseins, respectively, remained in the micelles. Concomitantly, we observed a significant increase in peptide content within the residual micelles during the period of hydrolysis (*figure 1a*).

3.3 Distribution of the peptides inside and outside the micelle during trypsinolysis

In order to quantify the peptide content still present within the residual micelles, and also those released from the structure, we determined the TN and NCN of each fraction during hydrolysis (*table I*). In a first approach, it could be seen that peptides released from the structure increased to 6.3 g·kg^{-1}, which was a high level compared with the NCN content found within the residual micelles, amounting to 0.6 g·kg^{-1}. However, from the content in (TN-NCN) reported in *table I*, it appeared that some peptides precipitated concomitantly with the caseins. Consequently, with 40.2% and 37% of the initial content of α_{s1}- and κ-caseins, respectively and no more β-casein after 4 h of hydrolysis, the micellar casein concentration reached only 3.9 g·kg^{-1} instead of the 21.8 g·kg^{-1} determined value. This shows that the peptides represented the majority of the nitrogenous components in the residual micelles. Indeed, the initial distribution, i.e., 91% caseins and 9% peptides before hydrolysis, was modified, after 4 h of hydrolysis to reach 64% peptides remaining within the micelles together with 13% caseins, whereas only 22% peptides were released from the micelles. This latter percentage indicates that the peptides were mainly entrapped within the residual micelles. Thus, although this residual micelle was mainly composed of peptides, the micellar cohesion seemed to be maintained as shown above.

3.4. Identification of the peptides produced during casein micelle trypsinolysis

To establish the preferential cleavage sites of the caseins within the micelles, it was necessary to characterise all the peptides produced, those entrapped and those released. The mass determination (*tables II, III* and *IV*)



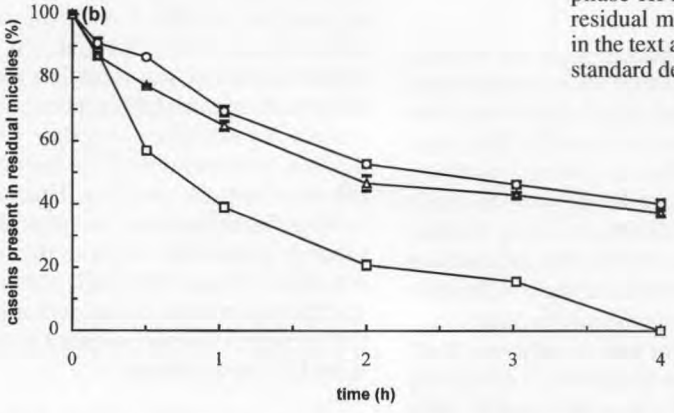


Figure 1. (**a**) Chromatographic profiles of the micellar residual caseins during trypsinolysis (0.021 U·mL^{-1} of micellar solution, containing 31.6 gTN·kg^{-1}). Reversed-phase HPLC analysis on Vydac C_4 column. Samples were diluted to 1/20 with 1 mL·L^{-1} trifluoroacetic acid (TFA). A linear gradient was performed in 37.5 min from 37 to 57% buffer B (1 mL·L^{-1} TFA, 800 mL·L^{-1} of acetonitrile and 200 mL·L^{-1} of Milli Q water) under the following conditions: 1 mL·min^{-1}, at 40 °C, absorbance measured at 214 nm and 100 µL injected. "t0" corresponds to the native caseins and 2 h and 4 h to the time of tryptic hydrolysis. (**b**) Kinetics of disappearance of the α_{s1}- (○), β- (□) and κ- (△) caseins during hydrolysis of the casein micelle suspension. The percentage of remaining casein during hydrolysis was determined from reversed-phase HPLC analysis of the residual micelles as described in the text and represented with standard deviation.

Figure 1. (**a**) Profils chromatographiques des caséines micellaires résiduelles obtenues après hydrolyse trypsique (0,021 U·mL^{-1} de solution micellaire contenant 31,6 g azote total·kg^{-1}). L'analyse CLHP en phase inverse était réalisée sur colonne Vydac C_4. Les échantillons étaient dilués au 1/20^e avec 1 mL·L^{-1} d'acide trifluoroacétique (TFA). Un gradient linéaire était effectué en 37,5 min entre 37 et 57 % tampon B (1 mL·L^{-1} de TFA, 800 mL·L^{-1} d'acetonitrile et 200 mL·L^{-1} d'eau Milli Q) dans les conditions suivantes: 1 mL·min^{-1}, à 40 °C et une détection des pics à une longueur d'onde de 214 nm. « t0 » correspond à l'analyse des caséines micellaires natives, 2 et 4 h aux prélèvements réalisés après 2 et 4 h d'hydrolyse trypsique. (**b**) Cinétique de disparition des caséines α_{s1}- (○), β- (□) et κ- (△) pendant l'hydrolyse trypsique des micelles. Les résultats sont exprimés en pourcentage de caséines restantes et ont été établis à partir des chromatogrammes CLHP en phase inverse des micelles résiduelles. Les résultats sont présentés avec les écarts types.

Table I. Distribution of the nitrogenous components between the fractions released from the casein micelle and those entrapped within.

Tableau I. Répartition des composés azotés entre les fractions libérées ou non de la micelle.

Time (h)	Nitrogen content			
	Residual micelles ($g \cdot kg^{-1}$)		Fraction released from the micelle structure ($g \cdot kg^{-1}$)	Peptide fraction insoluble at pH 4.6 and retained within residual micelle[b]
	(TN-NCN)	NCN (t-t0)	NCN[a] (t-t0)	
0.0	27.3	0.0	0.0	2.5
0.2	23.5	0.1	0.2	6.2
0.5	26.1	0.2	1.5	10.6
1.0	24.6	0.5	2.3	13.4
2.0	23.0	0.7	2.9	15.7
3.0	19.9	1.6	5.1	14.3
4.0	21.8	0.6	6.3	17.9

[a] Values of total nitrogen of the fraction released from the micelle were equivalent to those present in the soluble samples after precipitation to pH 4.6 and are not presented in this table. [b] Values estimated according to the following data: $N_{\text{peptide residual micelles}} = (TN\text{-}NCN) - N_{\text{caseins } (\alpha_{s1}, \beta \text{ and } \kappa)} + NCN_{\text{residual micelles}}$ with $N_{\text{caseins}} = (TN\text{-}NCN) \times$ casein proportion in initial bovine milk (as reported in the materials and methods section) x native casein proportion during hydrolysis (as determined by reverse-phase HPLC analysis from *figure 1*).

[a]Les valeurs obtenues lors de l'analyse de l'azote total des fractions libérées de la micelle étaient équivalentes à celles obtenues après précipitation à pH 4,6 et ne sont pas présentées dans ce tableau. [b]Les valeurs étaient obtenues à partir des données suivantes : $N_{\text{peptide residual micelles}} = (TN\text{-}NCN) - N_{\text{caseins } (\alpha_{s1}, \beta \text{ and } \kappa)} + NCN_{\text{residual micelles}}$ avec $N_{\text{caseins}} = (TN\text{-}NCN) \times$ pourcentage de la teneur en chacune des caséines dans le lait bovin (cf. Matériel et méthodes) × le pourcentage de caséines non hydrolysées (déterminé à partir des données CLHP de phase inverse de la *figure 1*).

was performed by an on-line coupling between the reversed-phase HPLC (*figure 2*) and ESI-MS. For identification, we used the amino acid composition of purified peptides in conjunction with their mass determination. The molecular masses of 61 peptides were determined and reported for the released peptides in *table II* and for those within the residual micelle in *tables III* and *IV*. Small and large peptides from 3 to 104 residues were produced. Most of them possessed several basic residues in the sequence, indicating they were intermediary products and consistent with our limited proteolysis conditions. Among the 61 peptides studied here, 34 arose from β-casein, 16 from α_{s1}-casein, 11 from α_{s2}-casein and none from κ-casein. For this latter casein, the absence of peptides could be explained by its lower concentration in casein micelles compared with α_{s1}- and β-caseins. The same was found during LC-MS since the intensity of the ion current produced by charged ions depends directly on their concentration in the chromatographic peaks.

The β-casein appeared to be almost completely hydrolysed by trypsin since only two among the 15 potential sites were not cleaved: Arg_1-Glu_2 and Arg_{202}-Gly_{203}. These sites were not hydrolysed even under extended trypsinolysis [13]. In addition, Arg 1 is known to be only weakly cleaved by trypsin [4, 35]. All the tryptic sites, except Arg_{183}-Asp_{184}, were observed amongst the peptides released from the micelle suggesting a great accessibility of this casein. Nevertheless,

Table II. Sequence assignment of peptides released from casein micelle during trypsinolysis.

Tableau II. Attribution de la séquence des peptides présents dans la fraction libérée de la micelle de caséine pendant l'hydrolyse trypsique.

Peak numbers	Mass (Da)		Sequence assignment	Number of basic residues
	Observed	Calculated		
1	516.60[a]	516.65	β-CN(29–32)	2
2	645.60[a]	645.79	β-CN(100–105)	1
3	872.84 ± 0.21	873.09	β-CN(98–105)	2
		or 872.08	or β-CN(26–32)	or 3
4	1 336.94 ± 0.22	1 337.46	α_{s1}-CN(80–90)	2
5	829.50[a]	829.96	β-CN(177–183)	1
6	779.70[a]	779.99	β-CN(170–176)	1
7	2 560.63 ± 0.22	2 560.62	β-CN(29–48)	3
	+ 1 012.79 ± 0.01	+ 1 013.24	+ β-CN(106–113)	2
8	3 133.48 ± 0.42	3 132.98	α_{s2}-CN(1–24)	3
9	974.99 ± 0.01	975.21	α_{s2}-CN(198–205)	2
10	2 716.18 ± 0.01	2 716.78	α_{s2}-CN(115–137)	3
	+ 1 097.84 ± 0.21	+ 1 098.39	+ α_{s2}-CN(189–197)	2
11	4 292.88 ± 0.87	4 293.47	α_{s2}-CN(115–150)	5
12	2 322.28 ± 0.43	2 321.66	α_{s1}-CN(84–102)	3
		or 2321.61	or α_{s1}-CN(101–119)	or 4
13	3 123.73 ± 0.21	3 122.96	β-CN(1–25)	2
14	3 606.58 ± 0.84	3 606.57	β-CN(1–29)	4
	+ 3 478.63 ± 0.22	+ 3 479.40	+ β-CN(1–28)	3
	+ 2 617.18 ± 0.01	+ 2 617.06	+ α_{s1}-CN(1–22)	4
15	1 267.34 ± 0.63	1 267.50	α_{s1}-CN(91–100)	1
	+ 1 642.19 ± 0.01	+ 1 641.94	+ α_{s1}-CN(23–36)	2
16	1384.19 ± 0.01	1 384.65	α_{s1}-CN(23–34)	1
17	2587.33 ± 0.21	2 586.94	α_{s1}-CN(80–100)	3
18	8258.56 ± 0.35	8 258.33	β-CN(33–105)	4
		or 8 257.33	or β-CN(26–97)	5
	+ 8130.16 ± 0.34	+ 8 129.20	+ β-CN(29–99)	5
	+ 7903.16 ± 0.65	+ 7 901.89	+ β-CN(29–97)	4
19	9113.36 ± 0.60	9 112.40	β-CN(26–105)	7
	+ 8758.33 ± 1.15	+ 8 756.96	+ β-CN(29–105)	6
	+ 8629.49 ± 1.49	+ 8 628.76	+ β-CN(30–105)	5
	+ 7774.56 ± 1.51	+ 7 773.71	+ β-CN(30–97)	3
20	11363.27 ± 0.80	11 362.27	β-CN(1–97)	7
21	3983.67 ± 0.42	3 983.69	α_{s1}-CN(1–34)	5
22	3721.93 ± 0.42	3 722.47	β-CN(177–209)	2
	+ 4485.57 ± 0.01	+ 4 484.44	+ β-CN(170–209)	3
23	7358.95 ± 1.08	7 356.57	β-CN(106–169)	3
	+ 8119.03 ± 1.13	+ 8 118.56	+ β-CN(106–176)	4
24	6363.07 ± 0.69	6 361.37	β-CN(114–169)	1
25	7855.76 ± 0.55	7 853.22	β-CN(108–176)	3
	+ 7093.40 ± 0.38	+ 7 091.25	+ β-CN(108–169)	2
26	11826.19 ± 0.80	11 822.98	β-CN(106–209)	6
27	11559.54 ± 0.91	11 557.66	β-CN(108–209)	5

[a] Singly charged molecular ions (no SD); CN, casein. Analysis performed by RP-HPLC-ESI MS after 4 h trypsinolysis of casein micelle solution and ultracentrifugation step as reported in text. The peak numbers refer to *figure 2a*.

[a] Ion moléculaire monochargé (pas d'écart type) ; CN, caséine. Analyse effectuée par couplage direct RP HPLC-ESI MS après 4 h d'hydrolyse des micelles de caséine par la trypsine puis ultracentrifugation, comme décrit dans le texte. Les numéros des pics renvoient à la *figure 2a*.

Table III. Sequence assignment of peptides present in the residual casein micelle during trypsinolysis. Analysis of the pH 4.6 supernatant.

Tableau III. Attribution de la séquence des peptides présents dans la fraction retenue dans la micelle de caséine résiduelle pendant l'hydrolyse trypsique. Analyse du surnageant de précipitation à pH 4,6.

Peak numbers	Mass (Da)		Sequence assignment	Number of basic residues
	Observed	Calculated		
1'	872.84 ± 0.21	873.09	β-CN(98–105)	2
		or 872.08	or β-CN(26–32)	or 3
2'	779.70[a]	779.99	β-CN(170–176)	1
3'	3 149.08 ± 1.27	3 149.48	α_{s1}-CN(106–132)	3
4'	3 137.18 ± 0.84	3 132.98	α_{s2}-CN(1–24)	3
	+ 2 747.83 ± 0.22	+ 2 747.56	+ α_{s2}-CN(1–21)	2
5'	1 097.74 ± 0.22	1 098.39	α_{s2}-CN(189–197)	2
6'	795.90[a]	–	NI	
7'	747.60[a]	747.92	α_{s1}-CN(194–199)	0
8'	3 123.43 ± 0.63	3 122.96	β-CN(1–25)	2
9'	3 607.18 ± 0.01	3 606.57	β-CN(1–29)	4
	+ 2 617.18 ± 0.01	+ 2 617.06	+ α_{s1}-CN(1–22)	4
	+ 3 478.63 ± 0.22	+ 3 479.40	+ β-CN(1–28)	3
	+ 3 977.23 ± 0.22	+ 3 977.02	+ β-CN(1-32)	5
10'	2 332.02 ± 0.22	2 321.66	α_{s1}-CN(84–102)	3
		or 2 321.61	or α_{s1}-CN(101–119)	or 4
11'	3 857.07 ± 0.79	3 859.50	α_{s1}-CN(4-36)	4
12'	4 742.59 ± 0.44	4 741.54	α_{s2}-CN(151–188)	9
	+ 4 512.27 ± 0.79	+ 4 512.26	+ α_{s2}-CN(153–188)	8
13'	5 822.88 ± 0.37	5 821.90	α_{s2}-CN(151–197)	11
	+ 5 950.38 ± 1.02	+ 5 950.07	+ α_{s2}-CN(150–197)	12
14'	2 829.18 ± 0.70	2 828.28	α_{s1}-CN(80–102)	4
	+ 1 763.84 ± 0.21	+ 1 762.05	+ α_{s2}-CN(153–166)	4
15'	8 129.56 ± 0.70	8 129.20	β-CN(29-99)	5
16'	7 902.56 ± 1.19	7 901.89	β-CN(29-97)	4
17'	12 483.88 ± 1.80	12 482.65	β-CN(1–107)	10
	+11 591.50 ± 1.49	+ 11 589.57	+ β-CN(1-99)	8
	+12 219.43 ± 0.87	+ 12 217.33	+ β-CN(1–105)	9
	+11 363.47 ± 0.83	+ 11 362.27	+ β-CN(1-97)	7
18'	3 984.72 ± 0.63	3 983.69	α_{s1}-CN(1-34)	5
19'	4 484.68 ± 0.31	4 484.44	β-CN(170–209)	3
	+ 3 722.83 ± 0.63	+ 3 722.47	+ β-CN(177–209)	2
20'	7 359.31 ± 1.08	7 356.57	β-CN(106–169)	3
	+ 8 121.05 ± 0.40	+ 8 118.56	+ β-CN(106–176)	4
21'	6 363.07 ± 0.76	6 361.37	β-CN(114–169)	1
22'	7 092.64 ± 0.79	7 091.27	β-CN(108–169)	2
23'	7 855.08 ± 0.86	7 853.24	β-CN(108–176)	3

[a] Singly charged molecular ions (no SD); CN, casein, NI, not identified. Analysis performed by RP-HPLC-ESI MS after 4 h trypsinolysis of casein micelle solution and ultracentrifugation step as reported in material and methods section. The pellet was resuspended in the same volume as original and precipitated at pH 4.6 according to the method of Aschaffenburg and Drewry [1].The peak numbers refer to *figure 2b*.

[a] Ion moléculaire monochargé (pas d'écart type) ; CN, caséine, NI, non identifié. Analyse effectuée par couplage direct RP HPLC-ESI MS après 4 h d'hydrolyse des micelles de caséine par la trypsine puis ultracentrifugation comme décrit dans le texte. Le culot d'ultracentifugation était resolubilisé dans le même volume qu'initialement puis précipité à pH 4,6 selon Aschaffenburg et Drewry [1]. Les numéros des pics renvoient à la *figure 2b*.

Table IV. Sequence assignment of peptides present in the residual casein micelle during trypsinolysis. Analysis of the pH 4.6 pellet.

Tableau IV. Attribution de la séquence des peptides présents dans la fraction retenue dans la micelle de caséine résiduelle pendant l'hydrolyse trypsique. Analyse du culot de précipitation à pH 4,6.

Peak numbers	Mass (Da)		Sequence assignment	Number of basic residues
	Observed	Calculated		
1"	4 240.22 ± 1.34	4 240.98	α_{s1}-CN(1–36)	6
2"	3 984.02 ± 0.49	3 983.69	α_{s1}-CN(1–34)	5
3"	11 365.44 ± 1.44	11 362.27	β-CN(1–97)	7
4"	4 484.87 ± 0.15	4 484.44	β-CN(170–209)	3
	+ 3 723.48 ± 0.28	+ 3 722.47	+ β-CN(177–209)	2
5"	2 910.08 ± 0.15	2 910.53	β-CN (184–209)	1
6"	11 169.00 ± 1.28	11 169.46	α_{s1}-CN(104–199)	6
7"	14 110.41 ± 1.23	14 107.88	α_{s1}-CN(80–199)	1
8"	8 638.96 ± 0.65	8 638.63	α_{s1}-CN(125–199)	3
9"	23 703.05 ± 5.10	23 694.79	α_{s1}-CN(+1 PSer)	9
	+23 620.21 ± 3.34	+ 23 614.79	+ α_{s1}-CN	8
10"	11 825.88 ± 1.43	11 824.97	β-CN(106–209)	6
11"	11 560.22 ± 0.83	11 559.66	β-CN(108–209)	7

CN, casein. Analysis performed by RP-HPLC-ESI MS after 4 h trypsinolysis of casein micelle solution and ultracentrifugation step as reported in text. The pellet was resuspended in the same volume as original and precipitated to pH 4.6 according to the method of Aschaffenburg and Drewry [1]. The precipitation pellet was dissolved in 8.75 mol·L^{-1} urea, then reduced with 16 mmol·L^{-1} dithiothreitol for 1 h at 37 °C before chromatographic analysis as described in text. The peak numbers refer to *figure 2c*.

CN, caséine. Analyse effectuée par couplage direct RP HPLC-ESI MS après 4 h d'hydrolyse des micelles de caséine par la trypsine puis ultracentrifugation comme décrit dans le texte. Le culot d'ultracentifugation était resolubilisé dans le même volume qu'initialement puis précipité à pH 4,6 selon Aschaffenburg et Drewry [1]. Le culot de précipitation était solubilisé dans l'urée 8,75 mol·L^{-1}, puis réduit en présence de 16 mmol·L^{-1} de dithiothreitol pendant 1 h à 37 °C avant analyse chromatographique, comme décrit dans le texte. Les numéros des pics renvoient à la *figure 2c*.

when we compared the origin of the peptides (*table V*) it appeared that, of the 30 β-casein peptides released, 18 were also partially retained within the residual micelle whereas the intermediary peptides of high molecular masses (1–99, 1–105 and 1–107) were completely retained within the pellet, together with the fragment 184–209. The occurrence of intermediary products, found either free in solution or inside the residual micelle, such as 1–25, 1–28, 1–29, 1–32, 1–97, 1–99, 1–105, 1–107, 106–209 and 108–209 showed that the N-terminus and the middle part of the β-casein were highly sensitive and accessible to trypsin. The kinetics of some peptides are illustrated in *figure 3*. The bonds 25–26, 105–106 or 99–100 (*figure 3a*) were cleaved almost simultaneously since the peptides 1–25, 100–105, 1–107/1–105 and 1/97 were all produced as soon as the hydrolysis started. Concerning the C-terminal part of the β-casein, the fragment 106–209 (*figure 3b*) was formed before 106–169, 106–176 and 114–169, which may result from the subsequent hydrolysis of the first intermediary product.

For the α_{s1}-casein, among the 20 potential tryptic sites, five were not cleaved: Arg_1-Pro_2, Lys_7-His_8, Lys_{42}-Asp_{43}, Lys_{58}-Gln_{59} and Arg_{151}-Gln_{152}. The region located between Val_{37} and Lys_{79}, insensitive to tryptic

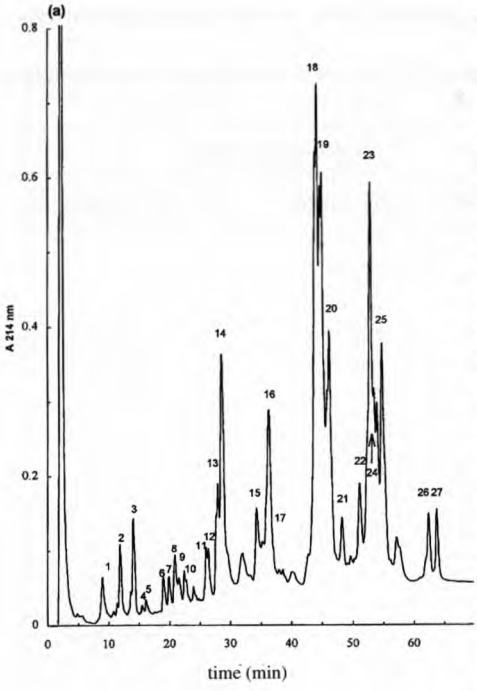
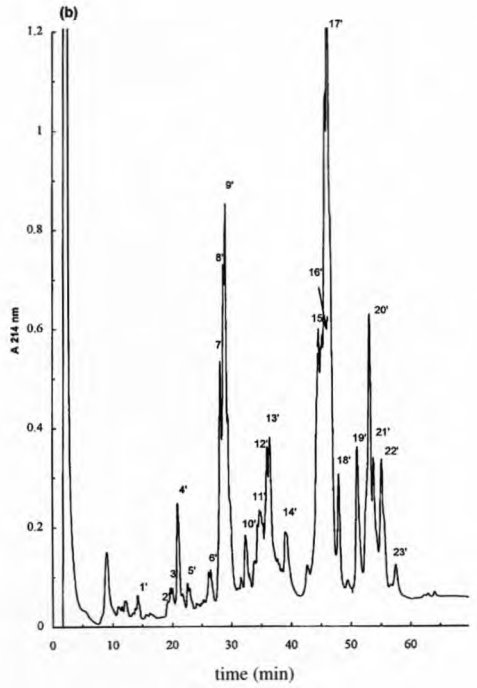


Figure 2. Reversed-phase HPLC coupled on-line with electrospray ion source mass spectrometry analysis of the components (**a**) released from the micellar structure; and those entrapped within the residual micelles and divided into two samples (**b**) NCN soluble fraction and (**c**) NCN insoluble fraction from hydrolysate of micelles after 4 h prepared as described in the text. Elution conditions are described in the text as well as the sample preparation and the columns used. Peak numbers refer, for the *figure 2a,* to *table II*, for *figure 2b* to *table III* and for *figure 2c* to *table IV*.

Figure 2. Analyse CLHP de phase inverse couplée en ligne au spectromètre de masse à source d'ionisation electrospray : (**a**) des composés libérés de la structure micellaire ; et de ceux présents au sein de la micelle résiduelle et dont l'analyse s'est effectuée sur les échantillons résultant de la précipitation pH 4,6 utilisée lors du dosage de l'azote non caséinique (NCN) : (**b**) fraction soluble et (**c**) fraction insoluble à pH 4,6. Le couplage a été réalisé sur les hydrolysats obtenus après 4 h d'hydrolyse trypsique des micelles de caséine dans les conditions décrites dans le texte. Les numéros des pics renvoient pour la *figure 2a* au *tableau II*, pour la *figure 2b* au *tableau III* et pour la *figure 2c* au *tableau IV*.



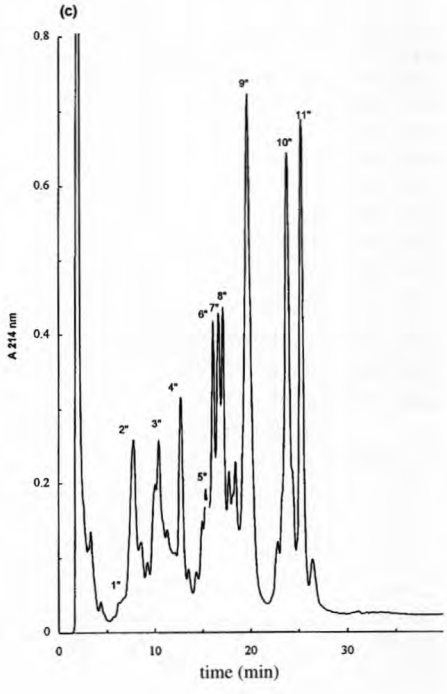


Table V. Distribution of the peptides between the solution and the residual micelle after 4 h trypsinolysis.

Tableau V. Répartition des peptides entre la solution et la micelle résiduelle après 4 h d'hydrolyse trypsique.

Peptide sequence	Peptides released	Residual micelles	
	from the micelles	NCN soluble fraction	NCN insoluble fraction
β-casein peptides			
1–25	+	+	–
1–28	+	+	–
1–29	+	+	–
1–32	–	+	–
1–97	+	+	+
1–99	–	+	–
1–105	–	+	–
1–107	–	+	–
26–32	+	+	–
26–97	+	–	–
26–105	+	–	–
29–105	+	–	–
29–32	+	–	–
29–48	+	–	–
29–97	+	+	–
29–99	+	+	–
30–97	+	–	–
30–105	+	–	–
33–105	+	–	–
98–105	+	+	–
100–105	+	–	–
106–113	+	–	–
106–169	+	+	–
106–176	+	+	–
106–209	+	–	+
108–169	+	+	–
108–176	+	+	–
108–209	+	–	+
114–169	+	+	–
170–176	+	+	–
170–209	+	+	+
177–183	+	–	–
177–209	+	+	+
184–209	–	–	+
α_{s1} *peptides*			
1–22	+	+	–
1–34	+	+	+
1–36	–	–	+
4–36	–	+	–
23–34	+	–	–
23–36	+	–	–
80–90	+	–	–
80–102	+	+	–

Table V. (continued) / **Tableau V.** (Suite).

Peptide sequence	Peptides released from the micelles	Residual micelles	
		NCN soluble fraction	NCN insoluble fraction
80–199	–	–	+
84–102	+	–	–
91–100	+	–	–
101–119	+	+	–
104–199	–	–	+
106–132	–	+	–
125–199	–	–	+
194–199	–	+	–
α_{s1}–	–	+	
α_{s2} peptides			
1–21	–	+	–
1–24	+	+	–
115–137	+	–	–
115–150	+	–	–
150–197	–	+	–
151–197	–	+	–
151–188	–	+	–
153–166	–	+	–
153–188	–	+	–
189–197	+	+	–
198–205	+	–	–

Characterization of the peptides by reverse–phase HPLC coupled on line ESI–MS as described in the text. Caractérisation des peptides par couplage direct entre la CLHP de phase inverse et la spectrométrie de masse à source d'ionisation electrospray, comme décrit dans le texte.

attack in spite of the presence of two lysyl residues at 42 and 58, is composed of phosphoserine residues which are in interaction with the colloidal calcium phosphate [13]. This probably reflects a weak accessibility of this part of the molecule. It appeared that of the 16 peptides arising from α_{s1}-casein (*table V*), four (23–34, 23–36, 91–100 and 80–90) were released, while five (1–22, 1–34, 80–102, 84–102 and 101–119) were distributed between solution and micelles and seven (1–36, 4–36, 80–199, 104–199, 106–132, 125–199 and 194–199) were present only within the residual micelle. As for β-casein, the most significant information about the accessibility of the α_{s1}-casein within the micellar structure was obtained by studying the residual micelle. Intermediary peptides 1–36 and 80–199 revealed that the most accessible part of the α_{s1}-casein was located on both sides of the phosphorylated region. The N- and C-terminal parts of the α_{s1}-casein, i.e., peptides 1–22 and 80–199 for which the kinetics of release are shown in *figure 4*, were produced from the initial hydrolysis periods. Cleavage of the other sites, located within both regions, was delayed as shown by the subsequent production of peptides such as 80–100 and 91–100 (*figure 4*).

For α_{s2}-casein, we also noted that not all the tryptic sites were cleaved. Of the 30 potential sites, only 10 were cleaved. They are located in the N-terminal part of the casein at Lys_{21}-Gln_{22} and Lys_{24}-Asn_{25} and in the C-terminal moiety, but only 8 sites

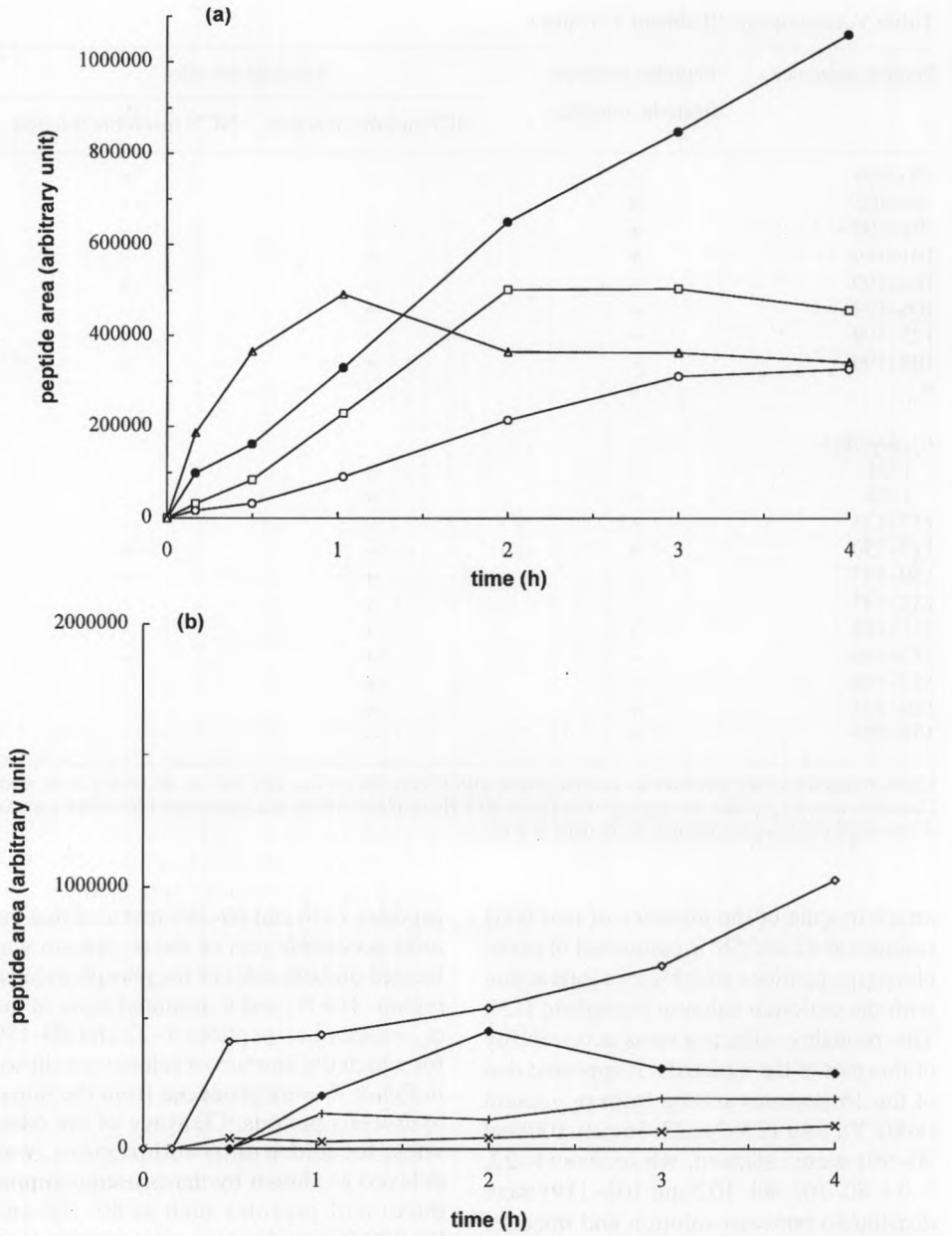


Figure 3. Kinetics of peptide formation from β-casein during micelle trypsinolysis. (**a**) (●) β-CN (1–25), (○) β-CN(100–105), (△) β-CN(1–105) + (1–107) + (1–99), (□) β-CN(29–97). (**b**) (◇) β-CN(106–209) area × 10, (◆) β-CN(114–169), (×) β-CN(106–169) + β-CN(116–176), (+) β-CN(106–113).

Figure 3. Cinétique de formation des peptides issus de la caséine β au cours de l'hydrolyse trypsique des micelles. (**a**) (●) β-CN (1–25), (○) β-CN(100–105), (△) β-CN(1–105) + (1–107) + (1–99), (□) β-CN(29–97). (**b**) (◇) β-CN(106–209) area × 10, (◆) β-CN(114–169), (×) β-CN(106–169) + β-CN(116–176), (+) β-CN(106–113).

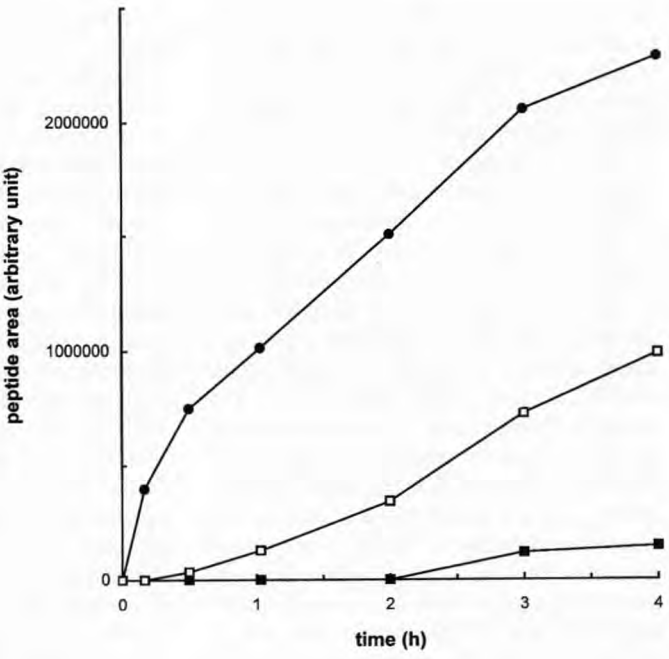


Figure 4. Kinetics of peptide formation from α_{s1}-casein during micelle trypsinolysis. (●) α_{s1}-CN(80–199), (□) α_{s1}-CN(80–100), (■) α_{s1}-CN(91–100) + α_{s1}- CN(23–36).

Figure 4. Cinétique de formation des peptides issus de la caséine α_{s1} au cours de l'hydrolyse trypsique des micelles. (●) α_{s1}-CN(80–199), (□) α_{s1} CN(80–100), (■) α_{s1}-CN(91–100) + α_{s1}-CN(23–36).

out of 20 were hydrolysed. As for α_{s1}-casein, most of the peptides were retained within the residual micelle since only three peptides were completely released (115–137, 115–150 and 198–205).

4. DISCUSSION

Hydrolysis of individual bovine casein by various endoproteinases, such as trypsin and plasmin, is well documented [19, 23–26, 28, 29, 38], and shows that the isolated caseins behave as relatively floppy molecules [16]. In micelles, information about the preferential splitting sites are more scarce [10, 27], but they tend to show that there would be constraints on the flexibility of the polypeptide chains, as stated by Holt and Sawyer [16]. These constraints would arise from the linkage between caseins and colloidal calcium phosphate and from the association between individual caseins. From our previous study, we have actually shown that α_{s1}-casein is implicated more strongly in the casein-mineral linkage than the other caseins, especially β-casein [13].

In the present study, we observed only a low release of colloidal Ca and P_i, 9 and 15%, respectively, far from the 30% of P_i which can be removed without significant micellar casein disruption [17]. Therefore, micellar integrity seems to have been maintained until the end of hydrolysis. Nevertheless, significant modifications were observed in terms of casein composition because β-casein was entirely hydrolysed and only about 40% of the native α_{s1}- and κ-caseins remained, leading to a residual micelle made up mainly of peptides, comprising ca. 64% of the nitrogenous components.

We hypothesised that the nature of the peptides released from the micellar structure should provide information about the solvent exposed regions within casein micelles, with the aim of deducing the interactive regions between these chains and between caseins and mineral components.

However, we have observed that only a small part of the peptides produced (22% of the total nitrogen compounds) were released from the structure into the solution. These peptides were mainly derived from β-casein, overlapping all the sequence, thus suggesting that this casein molecule was entirely accessible in the casein micelle. This can be stated because our operating conditions (i.e., 37 °C) were chosen in order to maintain the β-casein in its native state inside the micelle structure and to avoid its strong tendency to migrate out of the micelle with decreasing temperature [7, 11]. The absence of this casein in the ultracentrifugation supernatant prior to hydrolysis was carefully checked. For the other caseins, fewer peptides were released. At first, this would tend to prove that α_{s1}-, α_{s2}- and κ-caseins were less accessible than β-casein, as if some regions of these molecules were hidden. However, most of the sites which are usually cleaved on individual caseins [23–25, 29] were also cleaved within the micelle but were not released from the structure. Therefore, it can be hypothesised that, within the casein micelle, hydrophobic interactions as well as salt binding with colloidal calcium phosphate can modify the release of peptides from the macrostructure. Identification of only those peptides released from the micelle appears to give partial information about the casein region accessible to enzymes and should undoubtedly be completed by identifying peptides remaining within the residual micelles. This is all the more significant because the micellar structure is open [34] rendering the cleavage of endoproteinases easier compared with globular proteins. Therefore, differences between the very accessible regions and the other regions are not so marked in casein micelles, in contrast with data obtained on globular proteins which have very structured domains.

Regarding β-casein, its fast hydrolysis as well as the splitting of almost all the tryptic sites suggest that it could be present in the casein micelle as a loose molecule. This is in agreement with the presence of numerous proline residues in the sequence which impair the formation of the organised structure of this protein in solution [20, 21]. According to Leaver and Thomson [27], this might indicate that β-casein is present at the micellar surface. Like carboxypeptidase A [34], trypsin is probably able to penetrate within the casein micelle structure. Consequently, it could be concluded that this casein also might be present within the structure, as suggested by Dalgleish et al. [8]. Because we observed a fast release of the phosphopeptide β-CN (1–25) consistent with our previous study [13], we assumed that β–casein interacts weakly with the colloidal calcium phosphate. Moreover, it appeared that of the 30 β-casein peptides released, 18 were also partly retained within the residual micelle whereas the intermediary peptides of high molecular masses (1–99, 1–105 and 1–107) were completely retained within the pellet, together with the fragment 184–209. We hypothesise that the β-casein initially interacts with the other caseins because the first peptides produced remained within the micelles. Nevertheless, such interaction seems to be relatively weak because the subsequent hydrolysis products became free in solution. However, the C-terminal moiety appears to be mainly retained in the residual micelle, i.e., β-CN (106/108–209), or even completely retained within, β-CN (184–209). This is in agreement with the results of Yoshikawa et al. [39] showing that β-casein interacted with α_{s1}-casein through the hydrophobic region β-CN (108–209).

Even if α_{s1}-casein offers a relative resistance to proteolysis with 40% native casein remaining at the end of hydrolysis, an observation which could be partly explained by its involvement in the interaction with colloidal calcium phosphate [13], nearly all the potential tryptic sites were split. However, unlike β-casein, few peptides resulting from these cleavages were released from the micellar structure into the solution. They arose from the N-terminal moiety of the molecule

containing the first 36 amino acid residues and from the middle part including amino acid residues 80 to 119. Notably, these regions are more hydrophilic than the C-terminal half moiety of the molecule, which could be partly responsible for the pronounced self-association of the α_{s1}-casein by hydrophobic interactions [22]. We observed that the C-terminal region of α_{s1}-casein remained within the residual micelles even after tryptic cleavage. Therefore, α_{s1}-casein might be strongly implicated in hydrophobic interactions with itself but also with the C-terminal region of β-casein as underlined above.

For α_{s2}-casein, information from peptides released into solution was more scarce. As for α_{s1}-casein, we showed that most of the peptides remained within the micelles because only three peptides, amongst the 11 produced, were released. Therefore, we found that not only the C-terminus was sensitive to trypsin, as shown by Diaz et al. [10], but also the N-terminus and the middle of the molecule suggesting a relative accessibility of the whole α_{s2}-casein. As regards κ-casein, the fact that peptides were not produced in this study, as found by Diaz et al. [10], or were only produced in small quantities even under extended proteolysis [13], could be explained by their low concentration in the solution compared with α_{s1}- and β-caseins. Hence, the problem in detecting these peptides, it seems that proteolysis within the micelle is not dependent upon prior proteolysis of κ-casein as shown in *figure 1b*.

In conclusion, our results suggest that all the caseins were accessible in the micellar structure. The faster rate of hydrolysis of β-casein, compared with the other caseins may reflect a much lesser structural constraint for β-casein than for the others in the micellar organisation. Several results in the literature lead to speculation about the role of each casein in the micelle, except for κ-casein which is implicated in the steric stabilization of the micelle and mainly located at its surface [40]. Probably due to its loose association with other micellar components, β-casein may be involved in the dynamic equilibrium of the micelle in order to contend with the physico-chemical modifications of the environment and it might also serve as a steric stabilisation agent at the micellar surface [40]. In contrast, the α_{s1}-casein appeared to be held more firmly. Its lower flexibility could be explained by the hydrophobic linkage leading to the self association of this casein; the interaction between this casein and the other caseins; and the interaction between this casein and the colloidal calcium phosphate, via the phosphoseryl residues which are present in a greater number than for β-casein [3, 13, 17]. This casein appears to be the keystone of micellar cohesion. As α_{s2}-casein also contains a high number of phosphoserine residues, it is probable that it plays a similar role to that of α_{s1}-casein in the micellar structure, although analytical difficulties (partial identification of peptides and the absence of disappearance quantification) have prevented us from confirming this hypothesis.

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