Viscoelastic Properties of Oil-Water Interfaces Covered by Bovine β-Casein Tryptic Peptides

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ABSTRACT

A combination of proteolysis and dilational rheology has been used to study the behavior of films of β-casein (β-CN) and of peptides spread at the oil-water interface. Identification of the peptides produced by trypsin hydrolysis of β-CN in emulsion at 37°C provided information on the structure of β-CN adsorbed at the oil-water interface. Good interface properties were observed for β-CN or its peptides, probably because of the amphipathic nature of β-CN or a synergistic effect between hydrophilic and hydrophobic peptides. Remarkable surface activity was found for the amphipathic peptide β-CN (f114–169). Rheological studies had shown that interface films made with peptide fractions or with β-CN were elastic rather than viscous. Film made with the purified peptide β-CN (f114–169) was merely elastic at the triolein-water interface. A decrease of the viscoelastic modulus was observed for aging β-CN film but not for aging peptide films; The β-CN decrease was related to the flexibility of its structure. When the interface is increased by the dilation of an aqueous droplet plunged into oil, β-CN may expose new polypeptide trains to cover the increased interface, unlike peptides with simpler structures.
(**Key words:** oil-in-water emulsion, casein hydrolysis, bovine milk, viscoelasticity)

Abbreviation key: AP = adsorbed peptides, $\mathbf{A_s}$ = specific surface area, $\mathbf{d_{3,2}}$ = mean droplet diameter, ε' = viscoelastic modulus, $\mathbf{F_{ads}}$ = fraction of adsorbed proteins/peptides, γ = surface tension, Γ = concentration of proteins/peptides loading the oil droplet surface, **SP** = soluble peptides generated by trypsin from β-CN in solution, **TP** = total peptides generated by trypsin from β-CN in emulsion, **TPCK** = L-1-tosylamide-2-phenylethyl chloromethyl ketone.

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INTRODUCTION

In food applications, e.g., the dairy industry, emulsions must be stabilized to prevent flocculation, aggregation, or phase separation during storage. Emulsions can be stabilized by a very thin monolayer of adsorbed protein at the oil-water interface of an emulsion. These highly interacting polymer molecules (16) form a protective steric or electrostatic barrier around the dispersed droplets. Interface properties of proteins are influenced by structural factors such as molecular flexibility, steric hindrance, amphipathic structure, hydrophobicity, molecular size, and secondary structure (41) and by physical factors such as pH, ionic strength, and temperature (19). Properties of emulsions stabilized by milk proteins and competitive displacement of proteins from the emulsion droplet surface have been widely reported in the literature (for review, see 17).

The two most abundant proteins in bovine milk are α_{s1}-CN and β-CN. Investigations of competitive adsorption of individual caseins in oil-in-water emulsions showed that β-CN preferentially adsorbs at the oil-water interface when a mixture of α_{s1}-CN and β-CN is used (18). The β-CN added to an emulsion made with α_{s1}-CN is able to replace the latter at the interface, whereas the inverse occurs rarely (18). This preferential adsorption of β-CN over α_{s1}-CN was also observed at the air-water interface (1). The emulsifying properties of β-CN modified by glycosylation (10), dephosphorylation (25), or proteolysis (7, 8, 31) have been investigated. Glycosylation enhances the emulsifying properties of β-CN (10). The results of dephosphorylation of β-CN demonstrate that the phosphorylated groups of the N-terminal region of β-CN are important for stabilizing emulsions against coalescence. Because of its reduced N-terminal charge, dephosphorylated β-CN adopts a different interface structure, resulting in lost or reducted electrostatic or steric repulsion or both between oil droplets (25). In the same way, the N-terminal peptide β-CN (f1–105/107) was more amphipathic than peptide β-CN (f29–105/107) and was reported to be more emulsion stabilizing than the latter (7).

Knowing the functional properties of peptides generated by proteolysis of milk proteins is of interest for developing new applications (i.e., dairy-based infant formulas, sports nutritive supplements, and dietary supplements for patients with serious digestive troubles) or for understanding the structure-function relationships of proteins (31, 41). Peptides should be of minimum length (>20 residues) and have distinct zones of hydrophobic and hydrophilic residues for good emulsifying and interface properties (7, 31, 41).

The objectives of the present work were to study interface and dilational rheological properties of β-CN and its peptide fractions generated by trypsin action at the oil-water interface. Hydrolyses were carried out in solution or at an oil-water interface at 37°C, where β-CN is predominantly polymer. Trypsinolysis at 0°C of β-CN in emulsion has been reported (30). At 37°C, the degree of association and structure of β-CN are different than at 0°C, and hydrophobic interactions with oil are favored. To achieve these objectives, an n-dodecane-in-water emulsion was made by high-pressure homogenization in the presence of pure β-CN. The rates of the appearance of tryptic peptides were investigated as a function of time. Adsorbed peptides at the alkane-water interface were selectively recovered and identified. Their surface active and viscoelastic properties at a triolein-water interface were studied in comparison with those of β-CN.

MATERIALS AND METHODS

Isolation of Bovine β-CN

Sodium caseinate was obtained from raw skimmed milk by successive isoelectric precipitation at pH 4.6 and dissolution at pH 7 by the addition of sodium hydroxide. This process of precipitation and solubilization was repeated twice, and then the sodium caseinate solution was dialyzed and freeze-dried.

A β-CN-enriched fraction was first prepared by batchwise chromatography (38) with a weak ion-exchanger DEAE cellulose DE 23 (Whatman Biosystems Ltd, Springfield, UK). Pure β-CN was obtained after separation of the β-CN-enriched fraction by ion-exchange fast protein liquid chromatography on a preparative column TSK-DEAE 5PW (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the conditions of ionic strength gradient optimized by Andrews et al. (2).

Emulsion Formation

Oil-in-water emulsions (oil phase: 30 g in 100 g; aqueous phase: 70 g in 100 g) were prepared with a jet homogenizer (Labplant, Huddersfield, UK) operating at 10 MPa (5). Before we made the emulsion, the oil and aqueous phases and the two stainless steel chambers of the homogenizer were tempered at 37°C. The aqueous phase of the emulsion was a 20 m*M* Tris buffer, pH 7 containing 0.5 g of β-CN in 100 g of solution. The oil phase was n-dodecane (Sigma Chemical Co., St. Louis, MO) or triolein (Trisun 80-high oleic sunflower oil supplied by Danisco Ingredients, Brabrand, Denmark). Triolein was previously silica treated to remove any surface-active impurities (e.g., monoacylglycerols and fatty acids) according to the method described by Gaonkar (23). The emulsion was centrifuged at 15,000 × *g* for 15 min at 20°C, and cream was washed three times with the 20 m*M* Tris buffer, pH 7, to remove protein not bound at the interface. Protein concentrations were determined in the aqueous phases before and after emulsion formation (32), and the fraction of adsorbed protein ($\mathbf{F_{ads}}$) was expressed in percentage (wt/wt) of the total protein (13).

Determination of Droplet Size and Aggregation

The droplet size distribution of the emulsions was determined with a light scattering apparatus (Malvern Mastersizer MS 20; Malvern Instruments Ltd, Malvern, UK). Droplet size was measured in triplicate on the freshly made emulsion dispersed in water and after 240 min of incubation at 37°C. Another measurement was made after the emulsion had been left in the presence of a dissociating medium containing 1% (wt/vol) SDS for 30 min at 30°C to disperse the aggregates formed during emulsification (40).

Tryptic Hydrolysis of β-CN in Solution and in Emulsion

L-1-Tosylamide-2-phenylethyl chloromethyl ketone (**TPCK**)-treated trypsin (EC 3.4.21.4) from bovine pancreas (Sigma) was used. Tryptic digestion was performed for 5 to 240 min at 37°C on β-CN either dissolved at 5 g L^{-1} in 20 m*M* Tris buffer, pH 7, or adsorbed at the oil-water interface of an emulsion. The trypsin to CN molar ratio was 1/100. Kinetics of hydrolysis were followed by titration (12) of the free amino groups generated by the trypsin activity. Incubation was stopped by heating the sample at 100°C for 5 min in the case of β-CN in solution. Reaction was inhibited by adding trypsin inhibitor from white egg (Sigma) at an inhibitor/enzyme molar ratio of 4:1 in the case of β-CN in emulsion to avoid destabilization of the emulsions consecutively to thermal inhibition.

Soluble peptides (**SP**) released after hydrolysis of β-CN in solution for 240 min were freeze dried. The total (adsorbed and unadsorbed) peptides (**TP**) obtained by

trypsin hydrolysis of β-CN in emulsion for 240 min were extracted by adding two volumes of a mixture of chloroform and methanol (2:1, vol/vol, respectively). After centrifugation ($8000 \times g$, 10 min, 20°C), the upper methanol and water layer was taken and used for peptide analysis (30). To selectively prepare the tryptic peptides adsorbed (**AP**) at the oil-water interface, we first discarded the unadsorbed tryptic peptides in two successive centrifugation steps ($5000 \times g$, 30 min, 20°C). Then, the cream was dispersed in Tris buffer, and the adsorbed peptides were extracted by two volumes of a mixture of chloroform and methanol (2:1, vol/vol, respectively). Trace amounts of peptides (estimated to 2% of the total peptides) were present in the chloroform layer after extraction (estimated by counting free α-amino groups that reacted with o-phthaldialdehyde and β-mercaptoethanol).

Finally, the SP, TP, and AP fractions were freeze-dried and stored at 4°C until analyzed.

Reversed-Phase HPLC and Identification of the Peptides

Tryptic peptides were separated by reversed-phase HPLC (model Alliance 2690; Waters, Milford, MA) on a LichroCart C18 column (250×4 mm i.d., 5-μm particle size, 30 nm porosity) obtained from Merck (Darmstadt, Germany). Peptides were eluted with a gradient from 5 to 45% acetonitrile (Rathburn, Walkerburn, UK) in pure water containing 0.1% (vol/vol) trifluoroacetic acid (Sigma) for 80 min at a flow rate of 1 ml min^{-1}. Peptides that were only partially purified were separated again under isocratic conditions.

Two peptide fractions were prepared by reversed-phase HPLC. The hydrophilic peptide fraction corresponded to peptides eluted between 5 and 45 min (7.5 to 27.5% acetonitrile), whereas the hydrophobic peptide fraction contained peptides eluted between 45 and 75 min (27.5 to 42.5% acetonitrile).

Four methods were combined to identify the peptides separated by HPLC—derivative UV spectrometry, retention time prediction, AA composition analysis, and amino-terminal microsequencing, as described earlier (36). For the UV spectral analysis, a photodiode-array detector model 996 (Waters) was connected to the HPLC system. During HPLC separation, UV spectra were recorded between 200 and 300 nm at a rate of one spectrum per second. First and second derivatives of the UV spectra were calculated by 2010 Millenium software (Waters), and the ratios of the three aromatic AA residues Phe, Tyr, or Trp in peptides containing at least one of them were calculated from the analysis of some characteristic absorption bands in the near UV of the aromatic AA residues according to the method of Miclo et al. (34) and Perrin et al. (35). Retention times of peptides separated by reversed-phase HPLC are correlated to the peptide length (number of AA residues) and AA residue composition. Predictive retention times of peptides were calculated according to the method of Guo et al. (24) modified by Mant et al. (33) and compared to the experimental retention times observed for the peptides eluted under the chromatographic conditions described above. The AA residue composition was performed by reversed-phase HPLC after acid hydrolysis of peptides and phenylisothiocyanate derivatization (4). Amino-terminal sequencing was carried out with an automated protein sequencer model 476A (Applied Bio-Systems, Foster City, CA) with online identification of the phenylthiohydantoin derivatives.

Surface Tension and Viscoelastic Modulus Measurements

A dynamic drop tensiometer (IT Concept, Longessaigne, France) was used (3). Figure 1 shows a diagram of the experimental setup. An integrating sphere light source [2], a thermostated cuvette [3] containing the oil phase and a camera [7] with a telecentric lens [6] were aligned on an optical bench [1]. A pendant drop of aqueous phase was delivered from a syringe [4], controlled by a motor drive [5], into the thermostated optical glass cuvette ($1 \times 2 \times 4.3$ cm). The syringe was connected to a laboratory pipetting canula with a flat-cut tip with external and internal diameters of 2 and 1 mm, respectively.

The interface tension was determined from the drop profile obtained by image analysis [8], using the fundamental Laplace equation (3). The sinusoidal fluctuation

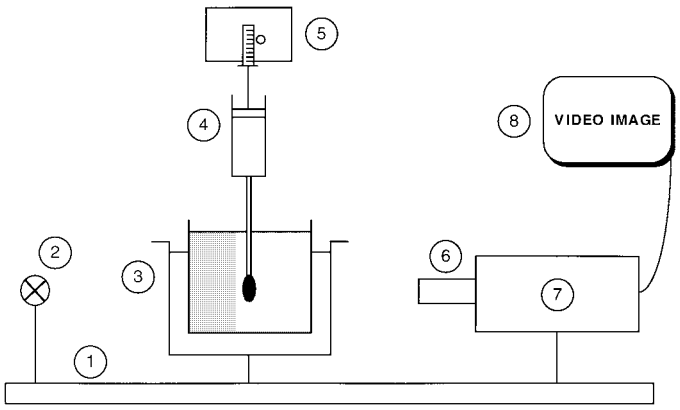

Figure 1. Dynamic drop tensiometer (side view; adapted from Benjamins et al., 3). [1] Optical bench, [2] integrated sphere light source, [3] drop formation device with thermostated cuvette, [4] syringe containing drop phase, [5] motor driving the piston of the syringe, [6] telecentric gauging lens, [7] charge-coupled device camera, [8], video monitor and computer.

of the drop volume at a chosen amplitude and frequency allows one to measure the surface viscoelastic modulus (ε) as defined by

$$\varepsilon = d\gamma/d\ln A$$

where $d\gamma$ was the interface tension variation and dA the area variation of droplet surface. It was possible to determine both the absolute value of the complex modulus $|\varepsilon|$, and the phase angle ϕ between the changes in interface tension and the changes in interface area. The elastic component ε' and the viscous component ε'' were calculated from $|\varepsilon|$ and the phase angle ϕ by

$$\varepsilon' = |\varepsilon| \cos \phi$$
$$\varepsilon'' = |\varepsilon| \sin \phi.$$

RESULTS

Characterization of the Emulsion Stabilized by β-CN

Emulsions were made with pure β-CN solubilized in Tris buffer (aqueous phase) and with n-dodecane or triolein as oil phase. The droplet size distribution was determined by light scattering, and the apparent and real mean droplet diameters ($\mathbf{d_{3,2}}$) were obtained in the absence or in the presence of SDS, respectively (40). When the oil phase was n-dodecane, the apparent and real mean diameters of the freshly made emulsion were identical ($d_{3,2} = 0.88 \pm 0.05$ μm) which meant no aggregates of oil droplets were detected. After incubation at 37°C for 240 min, the apparent and real mean diameters remained unchanged ($d_{3,2} = 0.91$ μm). When the emulsion was made with triolein, the apparent and real mean droplet diameters of the fresh emulsion were identical ($d_{3,2} = 1.67$ μm) but higher than the mean diameters observed for the n-dodecane droplets. The mean droplet diameters remained unchanged after incubation at 37°C for 240 min. This meant less recoalescence of oil droplets during incubation with the alkane than with the triacylglycerol. The better stabilization of the n-dodecane droplets could be explained by the higher hydrophobicity of the alkane than of triolein; the protein layer may be formed more quickly after homogenization. Droplet size measurements and microscopic observations have shown that coalescence of the triolein droplets begins after 240 min of incubation at 37°C. Destabilization of emulsion was not observed in the case of n-dodecane droplets (results not shown).

The specific surface area per unit volume of dispersed phase in freshly made emulsions ($\mathbf{A_s}$) was higher for emulsion made with n-dodecane ($A_s = 6.8 \pm 0.1$ m^2 ml^{-1}) than for emulsion made with triolein ($A_s = 3.6$ m^2 ml^{-1}). The protein surface concentration (Γ) was higher for the emulsion made with triolein ($\Gamma = 2.16 \pm 0.05$ mg m^{-2}) than for the emulsion made with n-dodecane ($\Gamma = 1.28$ mg m^{-2}) because the specific surface area was twice as large in the case of n-dodecane than in the case of triolein, while the adsorbed protein fractions were high and close one to another ($F_{ads} = 90\%$ in the case of n-dodecane and 75% in the case of triolein).

Tryptic Hydrolysis of β-CN and Identification of Peptides

Pure β-CN in solution or in emulsion made with n-dodecane was hydrolyzed by trypsin at pH 7 and 37°C for 5, 10, 20, 60, and 240 min, and the enzymatic reaction was followed by titration of the amino groups released by trypsinolysis. The extent of hydrolysis of β-CN in emulsion was only about 10% of that observed in solution for an incubation of 240 min. Intact β-CN adsorbed onto the oil-water interface could not be extracted by the mixture of chloroform and methanol into the aqueous phase. As was previously observed for β-CN (30) or β-lactoglobulin (21) in emulsion, β-CN appeared in our experimental conditions as a white band of insoluble material between the chloroform-oil and methanol-water layers during extraction and was discarded. Trace amounts of peptides in the chloroform phase were not analyzed by HPLC and were discarded, too.

Peptides from the SP fraction were separated by reversed-phase HPLC and identified according to the method of Perrin et al. (36). Fifteen peptides were identified without ambiguity (Figure 2) and 12 specific and three nonspecific (bonds Phe52-Ala53, Asn68-Ser69, and Phe190-Leu191) trypsin cleavage sites were determined. The nonspecific cleavage sites at positions 52 to 53, 68 to 69, and 190 to 191 had previously been identified (9, 14).

The overall HPLC peptide profiles after hydrolysis of β-CN in solution (SP) or in n-dodecane emulsion (TP) were almost similar after hydrolysis for 240 min (Figure 2): all of the peptides identified in the SP fraction were also present in the TP fraction, but with some variations in their amount (shown by variations in peak areas). Hydrophilic peptide β-CN (f1–25) was released in totality after 5 min of trypsinolysis in the aqueous phase of the emulsion and was therefore used as an internal standard; each peptide peak area was divided by the area of β-CN (f1–25), and plots of relative peak areas over time were reported on Figure 3. In emulsion, the content of peptide β-CN (f191–202) was half of that observed in solution. In contrast, the content of β-CN (f184–202) was higher in the emulsified system than

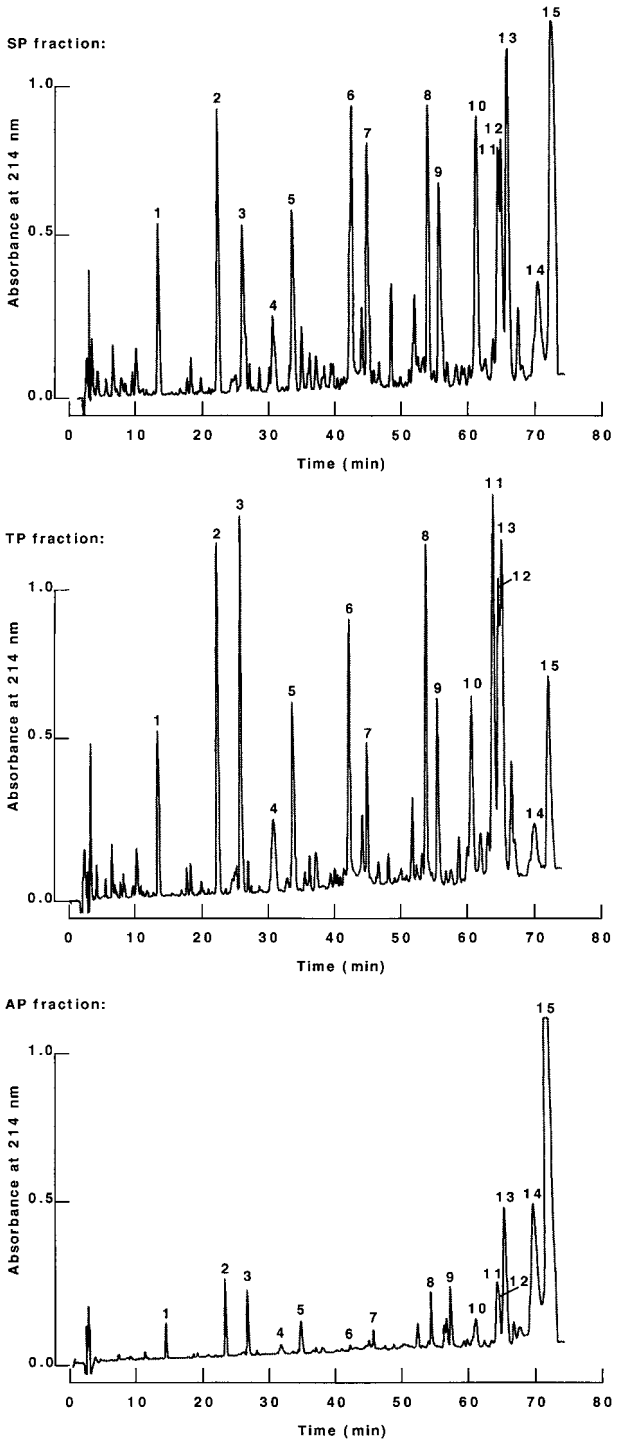

Figure 2. Separation of tryptic hydrolysates of soluble and emulsified β-CN by reversed-phase HPLC (hydrolysis time: 240 min). See Materials and Methods for the conditions. SP = Peptides resulting from β-CN trypsinolysis in solution; TP = total peptides resulting from β-CN hydrolysis in emulsion; AP = adsorbed peptides. The tryptic peptides were identified as follow: peak 1, β-CN (f100–105); 2, 177–183; 3, 33–48 (72%) + 170–176 (28%); 4, complex mixture containing 106–113 (77%); 5, 108–113; 6, 1–25; 7, 191–202; 8, 49–68; 9, 203–209; 10, 69–97; 11, 53–97 (70%) + 33–68 (30%); 12, 184–202; 13, 49–97; 14, 114–169; 15, 114–169.

in solution. Trypsin hydrolyzed bond Phe190-Leu191 with more difficulty in emulsion than in solution. In the literature (9), it was found that the nonspecific cleavage Phe190-Leu191 occurred in solution with a yield of 50%, whereas the two other nonspecific cleavages Phe52-Ala53 and Asn68-Ser69 were complete. In our solution and emulsion systems, the appearance of β-CN (f191–202) over time (peak 7, Figure 3) followed a linear regression, suggesting that bond Phe190-Leu191 was not cleaved in totality after 240 min of trypsin action. This bond could be slowly hydrolyzed by trypsin due to its low affinity to the enzyme. The longest peptide β-CN (f114–169) was the major component of the AP fraction and was highly hydrophobic, as shown by its elevated retention times (70 and 72 min). This peptide of 56 AA residues was characterized by two peaks. According to Dong and Ng-Kwai-Hang (20), differences in elution times of fragment 114–169 would be due to a change in hydrophobicity caused by AA residue substitution at position 137 (Pro for variants A1 or A2 and Leu for variant G identified by the authors in Holstein cow's milk).

Peptides β-CN (f49–68), (f69–97), (f184–202), (f191–202), and (f203–209) appeared more slowly in emulsion than in solution (Figure 3), which suggested that the two bonds 68–69 and 202–203 were hydrolyzed by trypsin with more difficulty in emulsion than in solution. Moreover, the relative peak areas of β-CN (f49–97) and β-CN (f53–97) were maximal for less than 5 min of hydrolysis in solution or 10 min of hydrolysis in emulsion and, then, decreased but more slowly in emulsion than in solution.

Conversely, bonds 48–49 and 183–184 seemed to be more resistant to trypsin hydrolysis in solution than in emulsion, as shown by the relative contents of β-CN (f33–48), (f49–97), (f177–183), and (f184–202), which were lower in solution than in emulsion. Resistance of bond 48–49 to trypsin either in solution or in emulsion was previously reported (30).

Surface Tension and Viscoelastic Modulus

Both surface tension and viscoelastic measurements at a triolein and water interface were carried out with a dynamic drop tensiometer in the presence of β-CN or of its peptide fractions. The initial drop volume was 30 μl, temperature 20°C, and pH 7. Sinusoidal fluctuation characteristics consisted in a frequency of sinusoidal area variations of 0.1 Hz with an amplitude of 10%. Under these conditions, and for a concentration of β-CN or peptides of 11 mg L^{-1}, the interface was fully covered, and only a very small amount of protein remained in the aqueous phase.

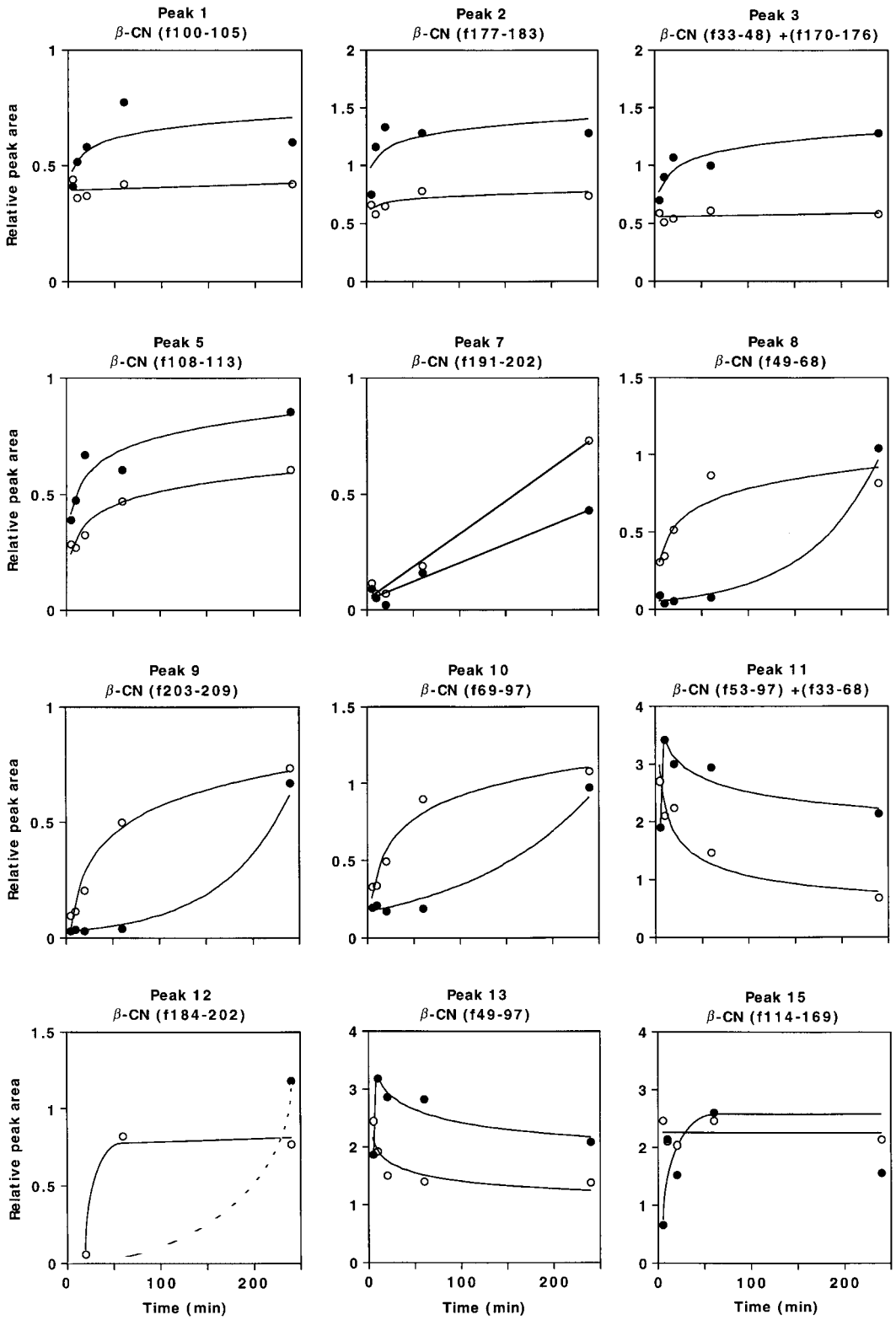

Figure 3. Time-course evolution of peptides from hydrolysis of β-CN in solution (○) and in emulsion (●). Each peptide peak area was divided by the area of β-CN (f1–25), and plots of relative peak areas over time were reported. Peak 12: for short hydrolysis times, the presence of β-CN (f184–202) was not obvious on the chromatograms, and its relative peak area could not be determined. Peak 15: in emulsion, the extraction yield of β-CN (f114–169) was low at 240 min because this peptide was partly recovered with β-CN in a white band of insoluble material.

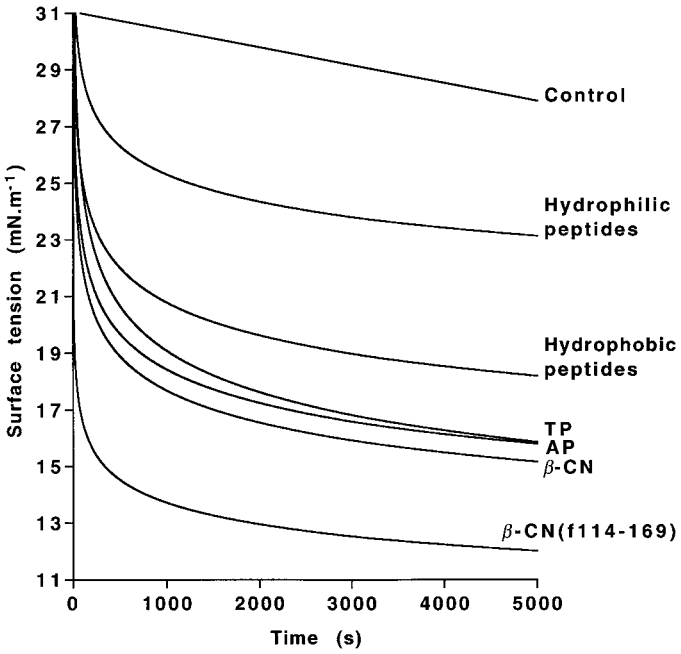

Figure 4. Surface tension of β-CN, β-CN (f114–169), hydrophilic and hydrophobic peptides, total peptides (TP), and adsorbed peptides (AP) as a function of time (conditions: aqueous phase was Tris buffer 20 m*M* at pH 7, oil phase was triolein, temperature 20°C, protein concentration 11 mg L^{-1}, initial drop volume 30 μl, frequency of sinusoidal area variations 0.1 Hz with an amplitude of 10%).

The equilibrium surface tension (γ) at the triolein and water interface and the surface tension derivative over time ($d\gamma/dt$ = initial adsorption velocity) were determined for β-CN, TP, AP, hydrophilic, and hydrophobic peptide fractions, and also for β-CN (f114–169) to provide information on the kinetics of adsorption at the interface (Figure 4). β-CN caused an important reduction in surface tension from 31.0 ± 0.5 mN m^{-1} (initial surface tension) to 15.5 mN m^{-1} (surface tension after 80 min). The AP and TP fractions showed a similar initial adsorption velocity as β-CN ($d\gamma/dt = -54\ 10^{-3} \pm 5\ 10^{-3}$ mN m^{-1} s^{-1} and $-66\ 10^{-3}$ mN m^{-1} s^{-1} for the AP and TP fractions, respectively, and $d\gamma/dt = -68\ 10^{-3}$ mN m^{-1} s^{-1} for β-CN) and also showed a similar surface tension after 80 min as β-CN (γ = 16.0 mN m^{-1} for the two peptide fractions), suggesting that these two fractions were as surface active as β-CN. The peptides of the hydrophobic fraction diffused more slowly onto the interface ($d\gamma/dt = -23\ 10^{-3}$ mN m^{-1} s^{-1}). The hydrophilic peptide fraction was the least surface active fraction ($d\gamma/dt = -14\ 10^{-3}$ mN m^{-1} s^{-1} and γ = 23.0 mN m^{-1}). The good surface tension of the TP, AP, and hydrophobic fractions after 80 min could be essentially due to the presence in these fractions of β-CN (f114–169). Indeed, after purification, the highly hydrophobic peptide β-CN (f114–169) exhibited the highest adsorption velocity ($d\gamma/dt = -250\ 10^{-3} \pm 10\ 10^{-3}$ mN m^{-1} s^{-1}) and a very low surface tension after 80 min (γ = 12.0 mN m^{-1}). The presence of a mixture of hydrophilic and hydrophobic peptides in the TP (or AP) fraction caused a larger surface tension decrease (from 31.0 to 16.0 mN m^{-1}) than in the case of the hydrophobic fraction (γ = 18.0 mN m^{-1} after 80 min), which contained only hydrophobic peptides as characterized by high retention times in HPLC.

The dilational rheological properties of the monomolecular films formed by β-CN, the TP and AP fractions, the hydrophilic and hydrophobic fractions, and β-CN (f114–169) at the triolein and water interface were studied by measuring the viscoelastic (ε) modulus as a function of the square root of time according to the method of Benjamins et al. (3). Films made with the TP, hydrophilic, or hydrophobic peptide fractions exhibited viscoelastic behavior similar to those made in the presence of the AP fraction and their rheological study was not reported. The rheological behavior of β-CN and peptide films was more elastic than viscous, as shown by the measurements of the elastic (ε') and viscous (ε'') components as a function of square root of time (Figure 5A, B, and C). Film made with the purified peptide β-CN (f114–169) was merely elastic, because the viscous component was negligible (Figure 5B). Nethertheless, different rheological properties were observed whether films were formed by β-CN or by peptides. Viscoelastic modulus of the peptide films increased in the first 200 s from a value close to 9 mN m^{-1} to about 14 mN m^{-1} and then remained constant with time (Figure 5A, B), whereas the viscoelastic modulus of the β-CN film first increased from 10 to 13 mN m^{-1} and, secondly, decreased to a value of about 6 mN m^{-1} (Figure 5C). A characteristic bell-shaped curve was obtained for the β-CN film but not for the peptide films.

DISCUSSION

Studies of rheological and tension properties of adsorbed protein films are numerous [for example, see Benjamins et al. (3) and Burgess and Sahin (6)], but there are very few studies of dilational properties of adsorbed peptides (i.e., viscoelastic properties) at the oil-water interface. The purpose of this study was to investigate first the susceptibility to trypsin of β-CN adsorbed at the surface of n-dodecane droplets dispersed in an aqueous medium, and second, the viscoelastic properties of films made with the generated peptides and their surface activity at triolein-water interface. To make emulsions stable for at least 240 min (kinetics of hydrolyses were determined from 0 to 240 min), n-dodecane was chosen instead of triolein. With triolein, the amount of peptides recovered in the ad-

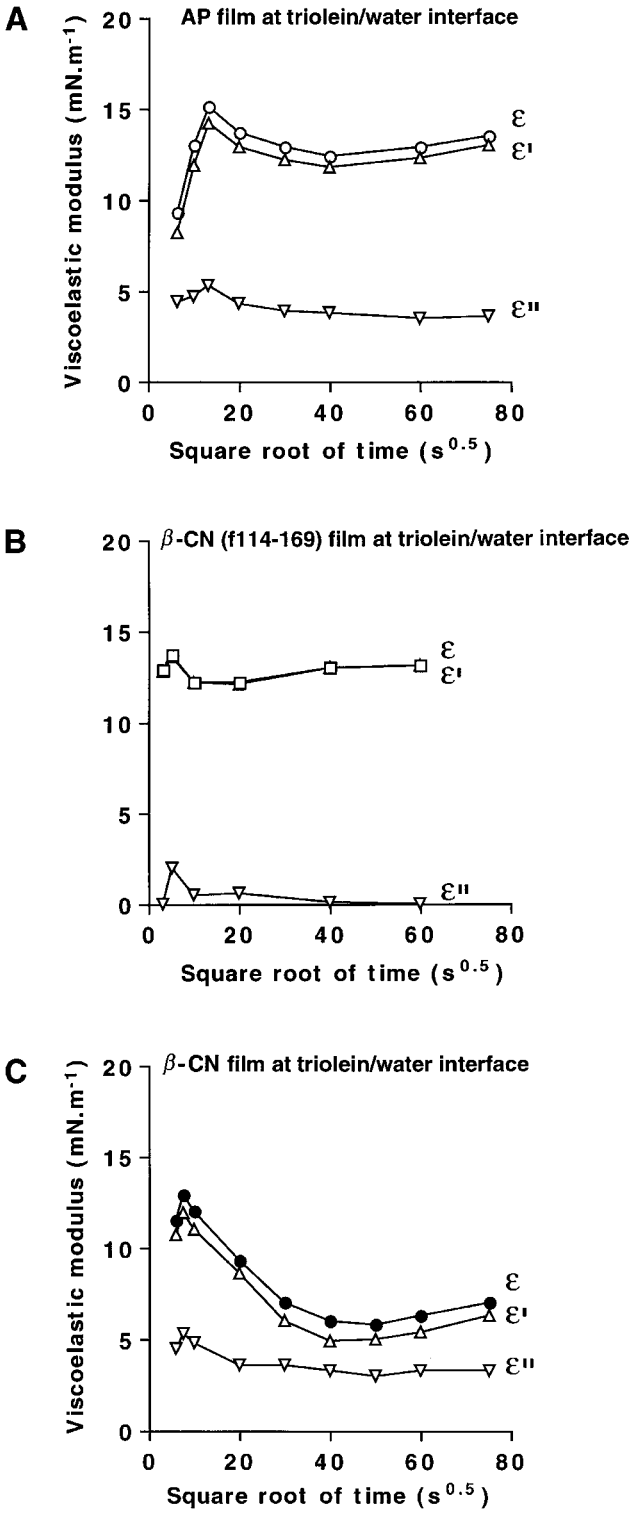

Figure 5. Viscoelastic modulus (ε) of (A) AP (adsorbed peptides; ○), (B) β-CN (f114–169) (□), and (C) β-CN films (●); elastic (ε'; △) and viscous (ε''; ▽); components as a function of square root of time. Conditions: aqueous phase was Tris buffer 20 m*M*, pH 7, oil phase was triolein, temperature 20°C, protein concentration 11 mg L^{-1}, initial drop volume 30 μl, frequency of sinusoidal area variations 0.1 Hz with an amplitude of 10%.

sorbed fraction AP was not sufficient for the rheological investigations. The surface active and viscoelastic properties of β-CN and its peptides were studied with triolein, an alimentary oil, instead of n-dodecane.

Interface rheology is complementary to surface tension measurements and provides information on molecular interface adsorption, interaction between adsorbed molecules, and film compactness.

Trypsinolysis of β-CN in Solution and in Emulsion

Susceptibility to trypsin of β-CN peptide bonds was compared between a solution system when β-CN was present as a soluble form at a concentration of 5 g L^{-1} and an emulsion system when β-CN was adsorbed at the surface of oil droplets. β-CN is known to have good emulsifying properties (15, 30). Its N-terminal region is hydrophilic, whereas its C-terminal region is hydrophobic. β-CN is, therefore, considered to be a good substrate to obtain peptides of various hydrophobicities. According to Leaver and Dalgleish (30), the crucial initiating step of the hydrolysis of β-CN adsorbed at an oil-water interface is the cleavage of the N-terminal peptide 1–25 from the protein. After removal of this peptide, the remaining macropeptide can reorient and other regions become accessible to the proteinase. The former work (30) was done at 0°C when the β-CN fragments are less likely to associate. The present work performed at 37°C confirmed that the hydrophilic peptide 1–25 was rapidly generated by trypsinolysis and released in totality in the aqueous phase of the emulsion. Figure 6A summarizes the cleavage sites of trypsin action on the polypeptide chain of β-CN. Two cleavage sites (bonds 68–69 and 202–203) were hydrolyzed with more difficulty in emulsion than in solution. Based on the hypothetical model of the tertiary structure of β-CN (Figure 6B; 28) and on the hydropathy profile of β-CN (Figure 7; 29), both sites would be located in two distinct hydrophobic regions. The hydrophobic C-terminal region (residues 196 to 206) forms a β-sheet-spiral structure involved in the aggregation process of β-CN in solution. At room temperature, in a sodium phosphate buffer 20 m*M*, pH 6.7, hydrophobic self-association of β-CN monomers occurs at a concentration superior to 1 g L^{-1} and can be described by a model based upon "micelle" formation (27, 39). Moreover, the β-CN polymer is in dynamic equilibrium with its monomer. Thus, in an emulsified system, hydrophobic trains of β-CN monomers would be in contact with the oil phase and could be less accessible to trypsin attack, especially at 37°C. On the contrary, the interaction of β-CN at the oil-water interface at 37°C led to better cleavage of bonds 48–49 and 183–184 located in hydrophilic regions of the tertiary structure (Figures 6B and 7). Bond 48–

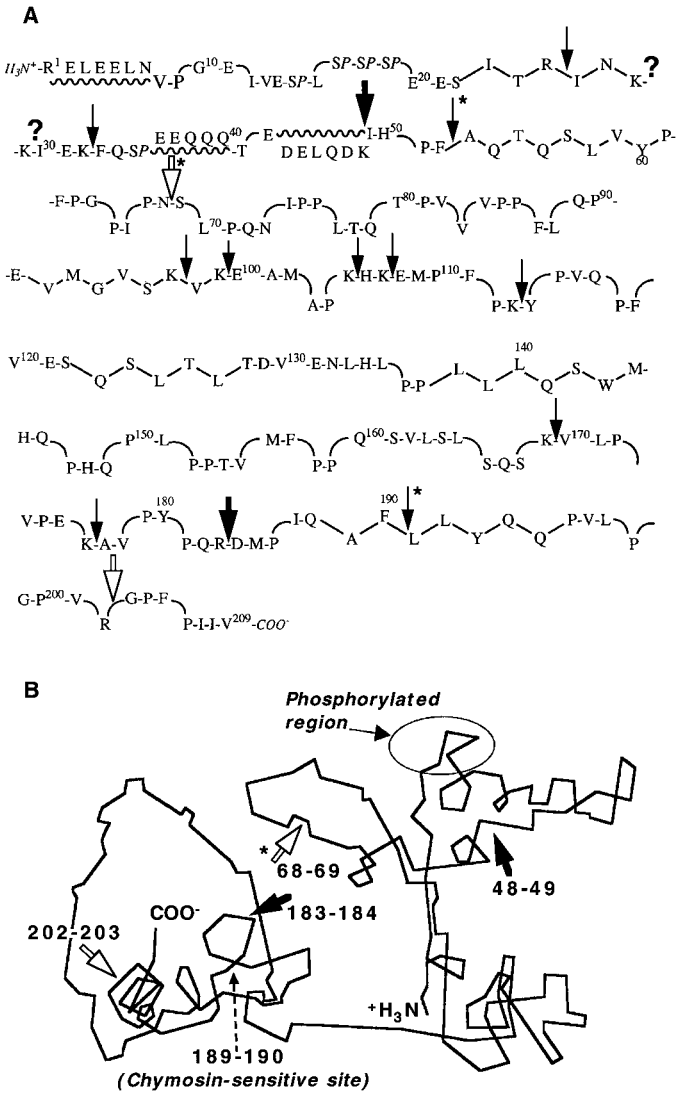

Figure 6. Schematic representation of primary and secondary structures (A; 22, 37) and of the tertiary structure hypothetical model of bovine β-casein A2 (B; 28) reproduced by permission of Cayot and Lorient (Structures et Technofonctions des Protéines du Lait, Ed. Lavoisier, Paris, France) and adapted. Arrows alone show specific trypsin cleavage sites and arrows with asterisk, nonspecific cleavage sites identified in this study. Open arrows indicate the positions cleaved with more difficulty in emulsion than in solution and bold arrows the reverse. Question marks indicate potential cleavage sites not studied. Dashed arrow shows the cleavage site sensitive to chymosin. SP: phosphoserine residue. Graphic symbols for secondary structure: potential α-helices; waved lines, β-sheets: dashes, β-turns: bent lines.

49 is included in one of the two hydrophilic arms (residues 28 to 55) of the "crablike" appearance of the hypothetical model (28). According to the secondary structure of β-CN (22), the bond 48–49 is at the end of a hydrophilic α-helix (Figure 6A). The second site is near the well-known chymosin-sensitive site 189–190. The bond 189–190 is accessible to enzyme action in the monomer of β-CN, but not when hydrophobic self-association occurs (42). In solution, the specific folding of β-CN or micelle formation could generate steric hindrance and slow down the enzyme cleavage of bond 189–190, but also bonds 183–184 and 48–49. The latter is known to be resistant to trypsin attack at 0°C, where less hydrophobic β-CN-oil interation would occur and for a low β-CN-to-enzyme ratio of 1/500 either in solution or in emulsion (30). In emulsion, unfolded β-CN is constituted by hydrophobic trains oriented towards the oil phase and hydrophilic loops protruding in the aqueous phase. Thus, unfolding of β-CN could lead to better accessibility of the bonds located in the hydrophilic loops at 37°C.

Interface and Viscoelastic Properties of β-CN and Peptide Films

The two peptides β-CN (fl-25) and (fl93-209), which correspond to the hydrophilic N-terminal and hydrophobic C-terminal regions of β-CN, respectively, have a low emulsifying activity at neutral pH, but are more surface active at pH 3. Both chain length and degree of hydrophobicity do not explain this result (31). Structural changes of secondary or spatial structures of peptides as well as peptide-peptide association at the oil-water interface must be considered for emulsification according to the pH. Interface properties of peptides generated by proteolysis of milk proteins such as

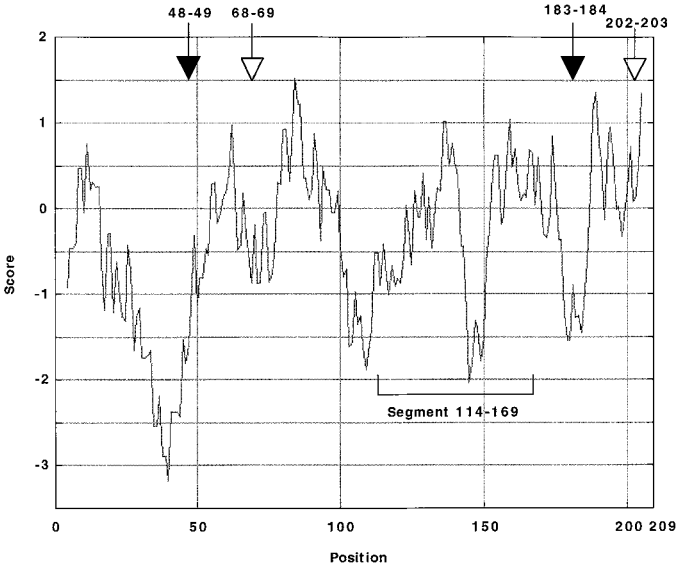

Figure 7. Hydropathy scan of the amino acid sequence of β-CN and of β-CN (f114–169). A moving segment of nine amino acid residues was used (29). Open arrows indicate the positions cleaved by trypsin with more difficulty in emulsion than in solution and bold arrows the reverse.

α_{s1}-CN or β-lactoglobulin have been studied (26, 41). Good interface properties of peptides are conferred by the presence of hydrophilic and hydrophobic distinct zones and by synergistic effects between peptides adsorbed at the interface. Similar conclusions were also deduced from the investigations of emulsifying properties of peptides obtained by plasmin hydrolysis of β-CN (7, 8). In the present work, the TP or AP fractions, containing a mixture of hydrophilic and hydrophobic peptides, behaved the same as β-CN regarding their surface activity at the oil-water interface. The formation of peptide-peptide interactions occurred after the unfolding of the peptides adsorbed at the interface. The two hydrophilic and hydrophobic fractions, however, gave smaller effective decreases in surface tension than the TP and AP fractions. These observations suggested a synergistic effect between hydrophilic and hydrophobic peptides adsorbed onto the oil-water interface. The major component of the AP fraction, β-CN (f114–169), possesses an amphipathic character of first order, i.e., with distinct hydrophobic and hydrophilic zones. The hydropathy profile of the region 114 to 169 shows a central hydrophilic domain flanked by two hydrophobic regions at N- and C-terminal sides (Figure 7). The excellent interface properties of β-CN (f114–169) were probably the consequence of both amphipathic character and a sufficiently long chain length.

Nevertheless, the results of dilational rheology were only partially explained in terms of hydrophilicity or hydrophobicity, because all the peptide fractions exhibited a similar rheological behavior. Viscoelastic moduli, particularly the elastic components, show clear differences between β-CN and the peptide fractions (e.g., the AP fraction presented in this work on Figure 5A), whereas the viscous components were similar. Whereas TP, AP, and β-CN have the same surface activity and equilibrium surface tension (Figure 4), they do exhibit different dilational behaviors: after film ages, peptide fractions give high viscoelastic moduli, while β-CN gives a lower elastic modulus. Conversely, peptide fractions have different surface activities while they all give a similar viscoelastic modulus. So, some samples can behave similarly in terms of surface activity or equilibrium surface tension and exhibit different dilational behaviors. Dilational rheology could be a very useful tool for determining the composition of an interface mixed film of peptides and protein. The bell-shaped curve observed in the case of β-CN was not found in the case of the peptide fractions, for which the viscoelastic modulus or the elastic component remained constant at a high value (close to 14 mN m^{-1}) during the film aging. Although already noticed in some previous studies with other proteins (sodium caseinate, BSA, ovalbumin; 3), there is yet no explanation for the bell-shaped curve of viscoelasticity observed for β-CN. At the initial stage of adsorption, the increase of viscoelastic modulus with time is due to protein adsorption. The subsequent decrease in viscoelastic modulus suggests a change in film properties and either diffusional relaxation or a rearrangement of proteins upon area variations, thus attenuating the changes in surface tension. This was also suggested by Williams and Prins (43). Strongly adsorbed at the interface, peptides would be unable to encounter these mechanisms. We can hence assume that the protein film is able to change its structure upon aging, whereas rearrangements would not occur with peptides. This suggests differences in the formation of protein-oil (or peptide-oil) or protein-protein (or peptide-peptide) interactions at the interface whether the intact protein (β-CN) or the protein hydrolysates (corresponding to the TP or AP fractions) was used. Upon dilation of the interface area, protein would be able to expose new trains at the increased interface, which would thwart an increase of surface tension. Peptides, e.g., β-CN (f114–169), would be able to more efficiently cover the interface but would be less able to cover the interface newly formed by dilation. Peptides have simpler structures than proteins (fewer tertiary structures and less rigid structure; 31), which could explain the higher value of viscoelastic modulus, primarily elastic component, observed in the presence of peptides than in the presence of β-CN, for an aged film (old of 400 to 6400 s or more). In other words, oil-water interface film appears to be more elastic with peptides than with protein. This difference in viscoelasticity between β-CN and peptide films might explain the lower stability of emulsion made with peptides, although some peptides have been reported to be good emulsifying agents (26, 41).

CONCLUSIONS

The structure of adsorbed proteins or peptides determines the properties of the emulsions. In the present work, identification of the peptides produced during the proteolysis of β-CN in solution or in emulsion provided information on the structure of β-CN at the oil-water interface. It was assumed that trypsin-sensitive bonds 68–69 and 202–203 could belong to hydrophobic regions oriented towards the oil phase, whereas bonds 48–49 and 183–184 could be a part of hydrophilic regions pointed towards the aqueous phase. These observations were in agreement with the hypothetical "trains-loops" model proposed by Leaver and Dalgleish (30) for β-CN adsorbed at a soya oil-water interface. The amphipathic structure conferred good interface properties to β-CN and also to the peptide β-CN (f114–169), whose structure has a central hydrophilic zone and two hydrophobic

zones at each N- and C-terminal side. Nevertheless, films made with peptides and β-CN had different rheological behaviors. Peptides were completely unfolded at the oil-water interface on the contrary of β-CN. The protein could expose more trains towards the oil phase when the interface area increased by dilation and, therefore, could stabilize emulsions with more efficiency than peptides, while, from surface tension measurements, β-CN and its peptides appear to be very active at the surface, their dilational behavior differs. It would be interesting to determine if this difference is related to emulsion stabilization. Understanding structure-viscoelasticity relationships in order to improve emulsion stability by peptide films is now in progress.

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