Functionality of β-Casein Peptides: Importance of Amphipathicity for Emulsion-Stabilizing Properties

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To investigate structure−function relationships with regard to emulsion-stabilizing properties, peptides from bovine β-casein (βCN), obtained by plasmin hydrolysis and fractionation of the hydrolysate, were isolated and identified on the basis of their masses determined by electrospray ionization mass spectrometry, the primary structure of the intact protein, and the known specificity of the enzyme. An amphipathic peptide fraction was fractionated further by ion-exchange chromatography and subsequent hydrophobic interaction chromatography resulting in the components βCN[f 1−105/107] and βCN[f 29−105/107]. The latter peptides had poor emulsion-stabilizing properties compared to the former ones, and the stability of an emulsion formed with βCN[f 29−105/107] was also more sensitive to hydrophobic impurities than that of an emulsion formed with βCN[f 1−105/107]. The highly charged N-terminal part appeared to be important for the emulsion-stabilizing properties of these peptides. A hypothesis for the structure−function relationship is given.

Keywords: *β-Casein peptides; plasmin; emulsion; mass spectrometry; structure−function relationship*

INTRODUCTION

Milk proteins are known for their ability to form and stabilize foam and emulsions (Walstra, 1988; Wong et al., 1996). Enzymatic hydrolysis can be used to modify such functional properties (Arai and Fujimaki, 1991; Chobert et al., 1996; Panyam and Kilara, 1996). Shimizu et al. (1984, 1986) investigated the effect of hydrolysis on the emulsifying properties of α_{s1}-casein. They demonstrated that the emulsifying activity of a nonpure fraction containing mainly the α_{s1}-casein fragment 1−23 decreased after subsequent purification of the sample. It was, therefore, concluded that this functionality was in fact formed by a so-called synergistic effect with coexisting peptides present in the sample (Shimizu et al., 1986). Lee et al. (1987) reported similar synergistic effects between the β-casein fragment 193−209 and the κ-casein fragment 106−169 (glycomacropeptide). No information on the emulsion stability was presented by Shimizu et al. (1986) and Lee et al. (1987).

One of the major caseins present in milk is β-casein (βCN), a 24 kDa protein with a distinct distribution of charged groups. The N-terminal part of the sequence is highly hydrophilic and contains five phosphoseryl residues (residues 15, 17−19, and 35), whereas the C-terminal part contains mainly hydrophobic side chains, carrying almost no net charge at neutral pH (Swaisgood, 1982).

In a previous paper we described the foam- and emulsion-forming and -stabilizing properties as well as the surface-active properties of βCN peptide mixtures, which were produced by plasmin hydrolysis of βCN (Caessens et al., 1997). By applying a stepwise fractionation of the hydrolysate we were able to test the influence of removing certain peptides on the functional properties of the fractions. The final fractions obtained could be categorized as hydrophilic, amphipathic, and hydrophobic fractions showing differences in functional properties. Moreover, differences were observed among the emulsion-stabilizing properties of the various amphipathic fractions. The results might be explained by the above-described so-called synergistic effects among the different peptides present in the fractions. However, this functionality may also be accomplished by one or more specific peptides present in some peptide fractions but absent in others (Caessens et al., 1997).

The objective of the present study is to investigate the possibility of either synergistic effects or specific functional properties of certain peptides. To this end the previously described βCN peptide fractions were identified to establish structure−function relationships, an amphipathic fraction was fractionated further, and the peptides obtained were analyzed for their composition and emulsion-forming and -stabilizing properties.

MATERIALS AND METHODS

Materials. Bovine βCN (90% w/w βCN, 95% βCN based on nitrogen, w/w), containing mainly the genetic variants A^1 and A^2, was purchased from Eurial (Rennes, France). Bovine plasmin (EC 3.4.21.7) and aprotinin were obtained from Sigma (St. Louis, MO; article no. P-7911 and A-6012, respectively). Unless stated otherwise, all other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany) or BDH (Poole, U.K.).

Preparation of β-Casein Peptides. The preparation of the βCN peptide fractions has been described earlier (Caessens et al., 1997). Briefly, the preparation was as follows. Bovine

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βCN was hydrolyzed at pH 6.8 by bovine plasmin with repeated intermediate pellet separation, giving a total pellet fraction (PEL-1) and a total supernatant fraction (SUP-1). SUP-1 was fractionated by means of selective precipitation based on pH and solvent composition (resulting in SUP-2 and SUP-3) and by ultrafiltration (resulting in RET-1 and PER-1). PEL-1 was dissolved at pH 10, after which part of it was hydrolyzed further at pH 8 with plasmin. Hydrolysis was terminated by adding aprotinin to the reaction mixture. The hydrolysate of the PEL-1 fraction was fractionated by centrifugation (resulting in PEL-4 and SUP-4).

Ion-Exchange Chromatography (IEC). Preparative IEC was performed on an ÄKTA-explorer, controlled by a UNICORN-control system (Pharmacia Biotech, Uppsala, Sweden) using a SourceQ column (280 mL bed volume; Pharmacia) at 20 °C. Solvent A (20 mM Tris-HCl buffer, pH 8) and solvent B (20 mM Tris-HCl buffer, 1 M NaCl, pH 8) formed the eluent in the following linear gradient steps: 5 min isocratic elution at 100% A; 2 min sample injection (25 mL/min); to 50% B over 10 min; to 100% B over 3 min followed by 3 min isocratic elution at 100% B; finally to 100% A over 3 min. Except where stated otherwise, a flow rate of 60 mL/min was applied. Appropriate IEC fractions were pooled, desalted by ultrafiltration (OMEGA membrane, 5 kDa MWCO, Pall Filtron Corp., Northborough, MA), lyophilized, and stored at 4 °C prior to analysis or further purification. Analytical IEC runs were performed on an FPLC system (Pharmacia) using a ResourceQ column (1 mL, Pharmacia) at 20 °C. Solvents and gradient for the analytical runs were similar to those for the preparative runs, except for the flow rate, which was 1 mL/min, and the injection volume, which was 200 μL. Generally, the protein load of the column was 1 mg/mL of bed volume for both preparative and analytical runs, and detection was at 220 and/or 280 nm.

Hydrophobic Interaction Chromatography (HIC). HIC was performed on a High-Load system (Pharmacia) using a Phenyl-Sepharose high-performance column (150 mL bed volume, Pharmacia) at 4 °C. Solvent A (50 mM potassium phosphate buffer containing 1.7 M ammonium sulfate, pH 7.0) and solvent B (50 mM potassium phosphate, pH 7.0) formed the eluent in the following linear gradient steps: 2 min isocratic elution at 100% A; to 48% B over 19.4 min; to 85% B over 26.5 min; to 100% B over 2.1 min, and finishing by 4 min isocratic elution at 100% B. A flow rate of 28 mL/min was applied, and detection was performed at 280 nm. The samples to be purified by HIC were collected from the preparative IEC runs and diluted 3-fold in the sample solvent (75 mM potassium phosphate buffer containing 1 M ammonium sulfate, pH 7.0). The protein load of the column was ~1 mg/mL of bed volume. Prior to loading, the column was equilibrated with solvent A for 5 min at 28 mL/min. Appropriate HIC fractions were pooled, desalted (ultrafiltration, 5 kDa MWCO), lyophilized, and stored at 4 °C prior to analysis.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). The peptide composition of the βCN peptide fractions was analyzed by RP-HPLC. The equipment used was described by Visser et al. (1991). For the separation of the several peptide fractions two different gradients, formed with solvent A [0.1% trifluoroacetic acid (TFA) in 10% aqueous acetonitrile, v/v] and solvent B (0.07% TFA in 90% aqueous acetonitrile, v/v), were used. The different concentrations of TFA in solvents A and B were used to avoid a rise of the baseline due to the absorption if increasing amounts of acetonitrile. Gradient RP-1 was used for the total βCN peptide fractions and has been described previously (Caessens et al., 1997). Gradient RP-2 was used for the analysis of amphipathic βCN peptide fractions, and the linear gradient steps to form gradient RP-2 were as follows: from 25 to 30% B over 3 min followed by 6 min isocratic elution; to 36% B over 12 min; then to 70% B over 5 min, and finally 5 min isocratic elution at 70% B. For both gradients, a flow rate of 0.8 mL/min was applied. Generally, 50 μL of a 1 mg/mL protein solution was injected onto the column for the analytical runs and 100 μL of a 2 mg/mL solution for the semipreparative runs. Peak detection and quantitation were performed at 220 nm using Turbochrom data acquisition and processing software (Perkin-Elmer, Ueberlingen, Germany).

Electrospray Ionization Mass Spectrometry (ESI-MS). All βCN peptides were collected by semipreparative RP-HPLC and identified by ESI-MS on a Quattro II triple-quadrupole instrument (Micromass, Cheshire, U.K.). Samples were dissolved in water, and this solution was diluted in a mixture of acetonitrile/water/formic acid (100:100:1, v/v/v); 20 μL of a 0.5−1 mg/mL (estimated concentration) solution was injected into a flow of acetonitrile/water (1:1, v/v, 4 μL/min). The potential at the capillary tip was maintained at 3.2 kV; the cone voltage was 30 V. Nitrogen was used as nebulizing and drying gas. Calibration was performed using horse heart myoglobin. The raw mass spectral data were processed and transformed with Masslynx software version 2.2 (Micromass). Peptides were identified from the molecular masses determined combined with sequence data of the protein and the known specificity of plasmin (Arg-X and Lys-X; Bastian and Brown, 1996).

Lysinoalanine (LAL) Determination. Determination of the LAL content of the samples was performed as described elsewhere (Pellegrino et al., 1996).

Emulsion Properties. Emulsion-forming and -stabilizing properties of the peptides were tested in a screening test at pH 6.7, calculated ionic strength 0.075 mM, and 20 °C, as described earlier (Caessens et al., 1997). Briefly, the screening test was as follows. The emulsions (tricaprylin oil/water 1:9 v/v; 0.44% w/v protein solution) were made by homogenizing in a laboratory high-pressure homogenizer at 60 bar. At several times after homogenizing (0, 1, and 24 h), emulsion samples were diluted in a 0.1% w/v SDS solution to delay the instability process. The emulsion-forming property was determined by measuring the droplet-size distribution immediately after homogenizing using the Malvern MasterSizer X (Malvern Instruments Limited, Malvern, U.K.). The emulsion-stabilizing property against mainly coalescence was determined by measuring, after appropriate dilution, the turbidity at 500 nm immediately after homogenizing and again after 1 and 24 h. The turbidity measurement was favored over the light scattering measurements using the Malvern MasterSizer because of a higher sensitivity of the former. The presence of flocs and/or aggregates in the emulsion samples taken was examined using a light microscope.

RESULTS

Identification of Peptides. Table 1 shows the ESI-MS results of the plasmin-derived βCN peptides. The code of the peaks refers to Figure 1, which shows the RP-HPLC chromatograms of the βCN fractions described previously (Caessens et al., 1997). The results (Table 1) show that the differences between the measured and calculated mass values are mostly within 2 Da. Therefore, the identification can be based on the masses determined, the primary structure of βCN, and the specificity of the enzyme. Nearly all peptides present in the fractions (Figure 1) could be identified, and the complete βCN sequence could be recombined from the peptide sequences present in the several fractions. The mass spectrum of peak V-x was difficult to interpret; the results displayed a complex mixture of different masses. This peak was also present in the starting material (Caessens et al., 1997). Further analysis showed that the material present in this peak contained ~900 ppm of LAL (unpublished results), and hence it contained some cross-linked protein material, probably formed during the purification of the protein. On the basis of the molecular mass found it is likely that the fraction contained "βCN- and γCN-like material" (Table 1).

Purification of Amphipathic Peptide Fractions. The several amphipathic peptide fractions SUP-1, RET-

Table 1. ESI-MS Results for the RP-HPLC Collected Peak Components of βCN/Plasmin Hydrolysates (Figure 1)

RP-HPLC group[a]	peak	measd value (Da)	peptide sequence	calcd value (Da)
I	a	283.7	106−107	283.3
	b	779.3	170−176	780.0
	c	1012.5	106−113	1013.2
		747.4	108−113	747.9
		1949.4	−[b]	
II	a	3478.0	1−28	3478.4
III	x	8716.0	A^2 (29−105)	8716.9
		8755.7	A^1 (29−105)	8756.9
		8980.9	A^2 (29−107)	8982.2
		9021.3	A^1 (29−107)	9022.3
		12175.1	A^2 (1−105)	12177.3
		12216.0	A^1 (1−105)	12217.3
		12441.0	A^2 (1−107)	12442.6
		12480.0	A^1 (1−107)	12482.6
IV	a	4483.4	170−209	4484.4
	b	3721.3	177−209	3722.5
	c	7356.4	106−169	7357.6
	d	8118.3	106−176	8119.5
	e	4483.0	170−209	4484.4
		6360.7	114−169	6362.3
		7122.5	114−176	7124.3
		7852.5	108−176	7854.2
		3721.3	177−209	3722.5
		7090.0	108−169	7092.2
V	a	11823.0	106−209	11824.0
	b	10827.1	114−209	10828.7
	x	11556.7	108−209	11558.6
		24044.0	−	11558.6
		24056.9	−	
		23590.0	−	
		11555.0	108−209	
		6603.6	−	

[a] See Visser et al. (1989) for categorization of RP-HPLC groups I−V. [b] −, no sequence could be identified.

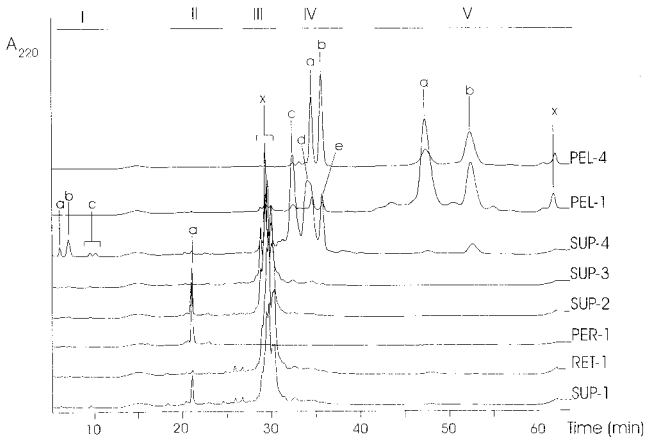

Figure 1. RP-HPLC chromatograms of the βCN hydrolysate fractions (Caessens et al., 1997). For abbreviations, see text; for conditions used, see Materials and Methods: RP-HPLC gradient RP-1, full scale detection 0.2 AU, maximal enlargement for representation 4×. Peptide composition of the fractions is categorized in groups I−V (Visser et al., 1989). Codes for the peaks refer to Table 1.

1, SUP-2, and SUP-3 (Figure 1) had distinct differences in emulsion-stabilizing properties (Table 2). In our previous study (Caessens et al., 1997) it was shown that fraction SUP-3 (having poor emulsion-stabilizing properties) contained fewer peptides than fraction RET-1 (having fairly good emulsion-stabilizing properties). Therefore, RET-1 was further fractionated by IEC,

Table 2. Summary of the Emulsion Properties of βCN Hydrolysate Fractions Described by Caessens et al. (1997)

sample	process	emulsion[a−c] formed at pH 4		emulsion[a−c] formed at pH 6.7	
β-casein	formation	nd	floc.	2.2	
	stabilization			+++	
SUP-1	formation	nd	floc.	1.5	
	stabilization			+	
RET-1	formation	nd		1.4	
	stabilization		floc.	++	
PER-1	formation	3.0		1.5	
	stabilization	−	cr/floc.	− −	coal.
SUP-2	formation	nd	floc.	1.9	
	stabilization			+	
SUP-3	formation	nd	floc.	1.7	
	stabilization			− −	coal.
PEL-1	formation	nd	floc.	nd	floc.
	stabilization				
SUP-4	formation	1.7		nd	floc.
	stabilization	+			
PEL-4	formation	nd	floc.	nd	floc.
	stabilization				

[a] − and + indicate the extent of emulsion stabilization, based on turbidity measurements (from stable [+++] to complete phase separation within 8 h [− −]). [b] coal., coalescence; floc., flocculation; cr, creaming. [c] Average droplet size (μm) immediately after homogenizing. [d] nd, not determined because of flocculation.

resulting in IEC1, IEC2, and IEC3 (Figure 2a). Analytical IEC separation of SUP-3 (results not shown) demonstrated that this fraction contains IEC1 and IEC2 but lacks the IEC3 peak (identified as βCN[f 1−105/107]). Figure 2b shows the RP-HPLC results of the IEC fractions in combination with the ESI-MS-identified sequences of the amphipathic peptides. From the results it appears that IEC1 was a mixture of peptides from the C-terminal, hydrophobic part of βCN (RP-HPLC groups IV and V, Table 1). The main peptides present in IEC2 were βCN[f 29−105/107], whereas the main peptides in IEC3 were βCN[f 1−105/107].

The RP-HPLC chromatograms of IEC2 and IEC3 (Figure 2b) still showed peaks with a retention time of ∼35 min (∼5% of the total RP-HPLC peak area determined at 220 nm), which is similar to the retention time of the hydrophobic peptides in the IEC1 fraction. To remove these last impurities from the IEC2 and IEC3 fractions, parts of these fractions were further purified by HIC (Figure 3a), resulting in a separation of the peptides into two fractions: IEC2HIC + HIC-R and IEC3HIC + HIC-R, respectively. Figure 3b shows the RP-HPLC chromatograms of IEC−HIC-purified fraction. From the chromatograms it appears that after HIC purification most of the material eluting in the RP-HPLC chromatogram at 35 min was removed from IEC2 and IEC3 with the HIC-R fractions. The HIC-R fraction accounted for ∼20% of the total HIC peak area determined at 280 nm, being ∼4 times higher than that analyzed at 220 nm (Figure 2b). This could indicate a relatively high content of aromatic residues in these fractions. The protein material with a retention time of ∼35 min was collected from IEC2, IEC3, and HIC-R (see Figures 2b and 3b) by semipreparative RP-HPLC and analyzed by ESI-MS. The mass spectra were difficult to interpret. Mass ranges were from 16055 to 16855 Da in IEC2 and from 19515 to 24070 Da in IEC3. Masses

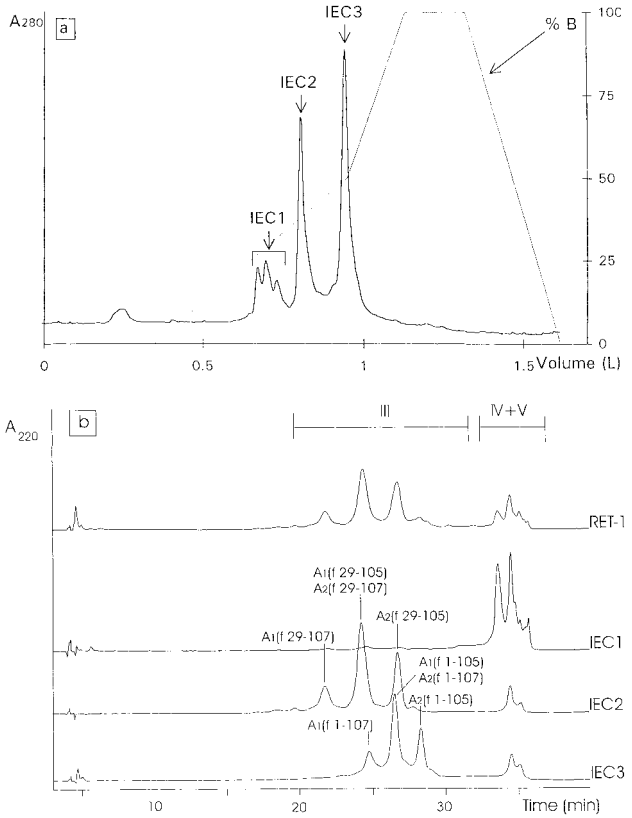

Figure 2. IEC chromatogram of RET-1 (a) and RP-HPLC chromatograms of the IEC-purified fractions (b). For abbreviations, see text; for conditions used, see Materials and Methods: RP-HPLC gradient RP-2, full scale detection 0.2 AU, maximal enlargement for representation 4×. Peptide composition of the fractions is categorized in groups III−IV/V (Visser et al., 1989).

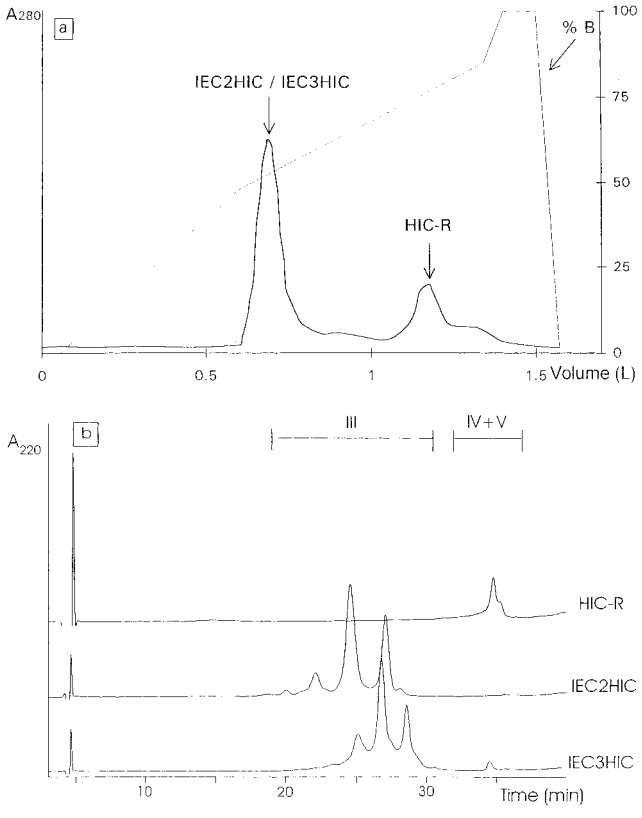

Figure 3. HIC chromatogram of IEC2 and IEC3 (a) and the RP-HPLC chromatograms of the HIC-purified fractions (b). For abbreviations, see text; for conditions used, see Materials and Methods: RP-HPLC gradient RP-2, full scale detection 0.2 AU, maximal enlargement for representation 4×. Peptide composition of the fractions is categorized in groups III−IV/V (Visser et al., 1989).

deduced from the ESI-MS spectrum of HIC-R were 5366 D and from 16058 to 16860 Da. The results obtained with these fractions did not allow a determination of βCN sequences. Before IEC purification, these masses were not found among peptides of similar retention times (IEC1), probably because the amounts present were too low to detect them among the other peptides present. Analytical reinjection of the removed HIC-R on the IEC column resulted in two peaks with retention times similar to those of IEC2 and IEC3 (see Figure 2), indicating that the protein material present in HIC-R resembles the IEC fractions mentioned (results not shown). The material in this fraction appeared to contain 1300 ppm of LAL. Apparently, the LAL content present in the starting material was somewhat concentrated in IEC2 and IEC3 and, after further purification, in HIC-R.

Emulsion-Stabilizing Properties. Table 3 shows the results of the emulsion screening test with intact βCN, the crude fractions RET-1 and SUP-3, the IEC-fractionated IEC2 and IEC3 fractions, and the HIC-purified IEC2HIC and IEC3HIC fractions. The emulsion-forming properties of the peptide fractions were similar and were superior to those of intact βCN, but the stabilizing properties varied. Normally, smaller emulsion droplets cause slower coalescence (Walstra, 1996). However, the results in Table 3 show that, despite the smaller droplet size, the emulsions formed with the peptides are less stable against coalescence than the emulsion formed with intact βCN. RET-1 had fair stabilizing properties, whereas SUP-3 showed poor stabilizing properties, similar to results obtained earlier (Caessens et al., 1997). IEC2 had poor stabilizing properties as well, whereas IEC3 had fairly good stabilizing properties, similar to those of RET-1 (both somewhat less than those of intact βCN). Because IEC2 and IEC3 are both fractions originating from RET-1, these results demonstrate the role of a specific peptide sequence rather than that of some synergistic effect between fragments in the emulsion stability. However, in this respect the results of IEC2HIC and IEC3HIC are less clear; the emulsion formed with IEC2HIC is only slightly less stable than that formed with IEC3HIC, which was comparable to that formed with IEC3. Hence, the removal of traces of impurities by HIC improved the stabilizing properties of IEC2 (βCN[f 29−105/107]-enriched). Possibly, the material that was removed by

Table 3. Screening-Test Results of Emulsions Made with βCN Hydrolysate Fractions and IEC-Purified Fractions[a]

fraction[b]	sequence	d_{32}[c] (μm)	stability[d,e]	
βCN	1−209	2.2	+++	
RET-1	29−105/107	1.5	++	
SUP-3	1−105/107	1.7	− −	coal.
IEC2	29−105/107^{purified}	1.5	−	coal.
IEC3	1−105/107^{purified}	1.5	++	
IEC2HIC		1.7	+	
IEC3HIC		1.5	++	

[a] For conditions used, see Materials and Methods. [b] For abbreviations, see text. [c] Average droplet size of the emulsion droplets immediately after homogenizing. [d] − and + indicate the extent of emulsion stability against coalescence, based on turbidity measurements (from stable [+++] to complete phase separation within 8 h [− −]). [e] coal., coalescence.

Table 4. Stability of Emulsions Made with Mixtures of the Several Amphipathic Peptide Fractions and Hydrophobic Peptides[a]

fraction[b,c]	d_{32}[d] (μm)	turbidity after 1 h[e](%)	turbidity after 24 h[e,f] (%)
100% IEC2 + 0% IEC1	1.5	61	20
90.0% IEC2 + 10.0% IEC1	2.3	31	11
100% IEC3 + 0% IEC1	1.5	92	83
90.0% IEC3 + 10.0% IEC1	1.3	94	86
100% IEC2HIC + 0% IEC1	1.7	71	45
97.5% IEC2HIC + 2.5% IEC1	1.7	3	−[f]
95.0% IEC2HIC + 5.0% IEC1	2.7	2	−
92.5% IEC2HIC + 7.5% IEC1	4.1	2	−
90.0% IEC2HIC + 10.0% IEC1	4.1	2	−
100% IEC3HIC + 0% IEC1	1.5	100	93
97.5% IEC3HIC + 2.5% IEC1	1.5	94	67
95.0% IEC3HIC + 5.0% IEC1	1.6	85	51
92.5% IEC3HIC + 7.5% IEC1	1.7	69	−
90.0% IEC3HIC + 10.0% IEC1	2.3	1	−

[a] For conditions of emulsion test used, see Materials and Methods. [b] For abbreviations, see text. [c] Percent in w/w. [d] Average droplet size of the emulsion droplets immediately after homogenizing. [e] Percent of turbidity remaining; turbidity at 0 h of emulsion formed with the fraction without IEC1 added set at 100%. [f] −, complete phase separation of the emulsion.

HIC had a negative influence on the emulsion-stabilizing properties of the IEC fractions. Apparently (see Table 3), this destabilizing effect was noticed in IEC2 (βCN[f 29−105/107]-enriched), but not in IEC3 (βCN[f 1−105/107]-enriched).

To investigate the influence of hydrophobic impurities on the emulsion-stabilizing properties of the amphipathic peptides, βCN[f 1−105/107] and βCN[f 29−105/107], emulsions were made with IEC2, IEC3, IEC2HIC, and IEC3HIC, containing 2.5−10% IEC1, which is a mixture of hydrophobic peptides. The results are shown in Table 4. Making emulsions with only 75% of the normal protein/peptide concentration of the amphipathic fraction SUP-1 (a mixture of mainly βCN[f 1−28] and βCN[f 1/29−105/107], with some traces of hydrophobic peptides) did not have a clear influence on the emulsion stability (no further results shown). Therefore, we concluded that the up to 10% lower amount of amphipathic protein added in the experiments described will not have a major effect on the results. The stability of the emulsion formed with IEC2 (βCN[f 29−105/107]-enriched) was decreased even more after addition of IEC1. The addition of IEC1 to IEC3 (βCN[f 1−105/107]-enriched) had no negative influence on the emulsion stability against coalescence formed by this fraction. The fractions IEC2HIC and IEC3HIC were more sensitive to hydrophobic impurities. IEC2HIC with 2.5% w/w IEC1 addition was already unstable after 1 h, whereas IEC3HIC with 10% w/w IEC1 was unstable after 1 h. Despite this instability with 10% IEC1, IEC3HIC was rather stable during 1 h even with 7.5% w/w IEC1 addition. The emulsion-forming properties of the peptide mixtures decreased (i.e., average droplet size increased) when higher amounts of hydrophobic peptides were present. Possibly, the poor emulsion stability, caused by the hydrophobic peptides, influenced the determination of the average droplet size.

DISCUSSION

The emulsion results obtained (Table 3) with IEC2 (βCN[f 29−105/107]-enriched) and IEC3 (βCN[f 1−105/107]-enriched) suggest that the highly charged N-terminal end of the amphipathic peptide (1−28, containing four of the five phosphoserine residues present in βCN) is important for its stabilizing properties. Also, the results (Table 2) with the crude fractions RET-1 (a mixture of mainly βCN[f 29−105/107] and βCN[f 1−105/107], with some traces of smaller hydrophobic peptides) and SUP-3 (similar to RET-I, but lacking βCN[f 1−105/107]) can be explained in this manner. Of course, one might expect that the peptides present in IEC2 and IEC3 have different surface-active behaviors (due to the presence of the hydrophilic N-terminal in the latter) resulting in different emulsion properties. In Caessens et al. (1997) it was shown that the surface-active properties of the crude fractions RET-1 and SUP-3 were similar. Therefore, the results indicate the importance of the hydrophilic N terminus of the peptide βCN[f 1−105/107] for the emulsion-stabilizing effects. Following these results, a hypothesis for the emulsion-stabilizing effect of the highly charged N terminus of the amphipathic peptides can be postulated. Apparently, the N-terminal part projects into the continuous phase, and the phosphoseryl groups present in this part of the molecule cause both electric and steric repulsion between the oil droplets. As a result, this so-called electrosteric repulsion of the N-terminal part prevents rapid coalescence and produces stable emulsions. βCN [f 29−105/107] produces unstable emulsions, probably because it lacks the strong electrosteric repulsion of the N-terminal part. HIC purification of βCN[f 29−105/107] improves the stabilizing properties of this fraction (see Table 3). Before HIC treatment of the IEC fractions small traces of impurities are still present (∼5% of the total RP-HPLC peak area). It is assumed that, by analogy with the adsorption of the hydrophobic parts of intact proteins (Walstra and de Roos, 1993), these impurities adsorb to the interface with a higher preference than the amphipathic peptides, because of their somewhat higher hydrophobicity (the latter being judged by RP-HPLC retention time). Consequently, it can be hypothesized that less surface on the oil droplet is available for the amphipathic peptides. Apparently, the peptides with the highly charged N terminus can still cause a stable emulsion with a lower amount adsorbed, in contrast to the peptides with the shorter, less charged N terminus. The results in Table 4 also indicate that the emulsions formed with amphipathic peptides with additional hydrophobic peptides present are less stable. Although the hydrophobic peptides also had some negative influence on the emulsion stability of emulsions formed with IEC3HIC (βCN[f 1−105/107]) the influence in the case of IEC2HIC (βCN[f 29−105/107]) was once again more dramatic.

Figure 4 depicts the hypothesis described above; Figure 4a illustrates the difference in conformation of βCN[f 1−105/107] and βCN[f 29−105/107] when adsorbed onto the interface, and Figure 4b demonstrates the effect of this difference on the emulsion-stabilizing properties. The stronger amphipathic peptide βCN[f 1−105/107] causes more electrosteric repulsion between the emulsion droplets and is, therefore, better capable of preventing coalescence of the emulsion droplets than the less amphipathic peptide βCN[f 29−105/107] is. This effect is evident for the IEC fractions. Both the HIC-purified IEC fractions, IEC2HIC (βCN[f 29−105/107]) and IEC3HIC (βCN[f 1−105/107]), formed fairly stable emulsions, similar to IEC3. The removal of the impurities allows a higher surface load of the emulsion droplets

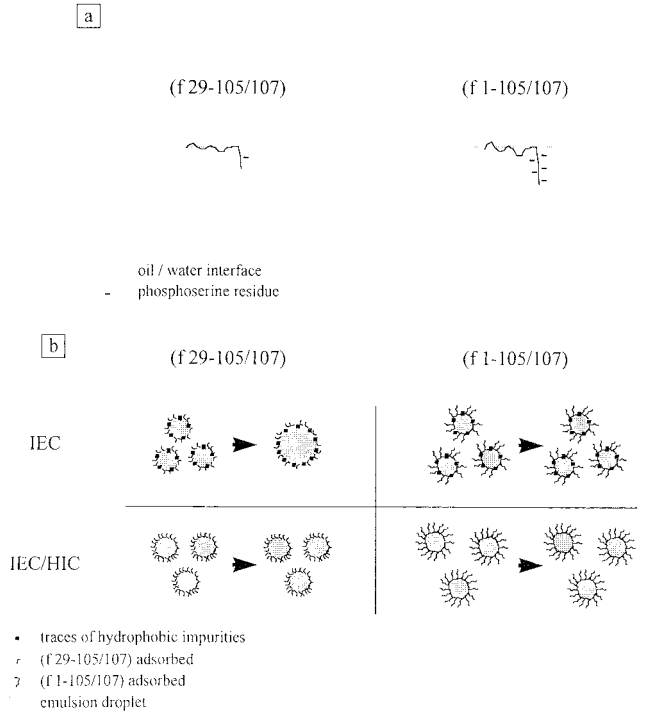

Figure 4. Schematic representation of the possible conformation of the amphipathic peptides at the oil/water interface (a) and schematic representation (not to scale!) of the hypothesis for the stabilizing properties of the several amphipathic peptide fractions IEC2, IEC3, IEC2HIC, IEC3HIC (b). For abbreviations, see text.

with the amphipathic peptides, which results in sufficient repulsion between the emulsion droplets covered with βCN[f 29−105/107]. It has to be realized that the real distribution on the interface of the different peptides present in the IEC fraction is unknown, and the distribution shown in Figure 4b is tentative. However, it is clear that the hydrophobic impurities do disturb the emulsion-stabilizing effect of the amphipathic peptides.

The emulsion properties of the amphipathic βCN peptides decreased with decreasing pH (e.g., pH 6.7 compared to pH 4.0; Caessens et al., 1997). Because at pH 4.0 these βCN molecules are less amphipathic than at pH 6.7, this result is in line with the hypothesis put forward above.

Comparison of our results with those of Shimizu et al. (1986) and Lee et al. (1987) with regard to the synergistic effects is not possible because their peptides are smaller than the ones presented in this study (about 2 and 10 kDa, respectively). Furthermore, our peptides are amphipathic, which appears to be of great importance, whereas the peptides described in the other studies are not amphipathic, but strongly hydrophilic or hydrophobic.

The hypothesis concerning the electrosteric repulsion between the emulsion droplets caused by the amphipathic βCN peptides concurs with previous publications on the adsorption of intact βCN. It has, for instance, been reported (Leaver and Dalgleish, 1990; Ter Beek et al., 1996) that when βCN is adsorbed onto an oil/water interface, its highly hydrophilic N terminus is flexible and protrudes into the water phase. Furthermore, neutron reflectivity studies revealed that the layer thickness of a βCN monolayer at *n*-hexane/water and/or at air/water interfaces is ∼7−9 nm, which is larger than the layer thickness of 2−3 nm of some globular proteins such as β-lactoglobulin (βLg; Dickinson et al., 1993; Atkinson et al., 1995). Combining these results with the amount of protein adsorbed onto the interfaces [approximately 2.5−3.8 and 1.5−2 mg m^{-2} for βCN and the globular protein βLg, respectively (Dickinson et al., 1993; Atkinson et al., 1995; Smulders et al., 1998)], they can be explained by assuming that βCN adsorbs in a "loop and train" conformation, with a condensed layer directly at the interface (trains) and a more diffuse layer of the highly charged N-terminal part (loop and/or tail) further away from the interface (Dickinson et al., 1993; Nylander and Wahlgren, 1994).

Although the highly charged N-terminal part of the peptide appears to be critical for its emulsion-stabilizing properties, the emulsion formed with the strongly amphipathic βCN peptide is less stable than that formed with intact βCN. Another study (Smulders et al., 1998) showed that the surface load of intact βCN on emulsion droplets is significantly higher than that of the amphipathic peptides (3.8 and 2.4 mg/m^2, respectively). The higher surface load found for intact βCN will probably be caused by a condensed packing of the hydrophobic, C-terminal part of the molecule. Evidence for this is the high surface load of the peptide fraction PEL-1 (Table 2) on emulsion droplets (Smulders et al., 1998). The results in Table 1 show that the hydrophobic peptide fraction PEL-1 (Figure 1) contains peptides from the C-terminal half of βCN. Probably, this close packing of the C-terminal part of βCN is the reason for the better emulsion-stabilizing properties of the intact protein compared to the amphipathic peptides, despite the somewhat larger droplet size.

In conclusion, our results show that specific peptides having emulsion-stabilizing properties rather than a synergistic effect of several peptides cause the emulsion stability. The highly charged N-terminal part of the amphipathic βCN peptides is important for the stability against coalescence of emulsion droplets, especially when small traces of hydrophobic impurities are present. Currently we are investigating the secondary structure of (dephosphorylated) βCN peptides, both in solution and adsorbed at interfaces, to elucidate the structure−function relationships in more detail.

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