Improved Storage Stability of Model Infant Formula by Whey Peptides Fractions

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The purpose of this study was to evaluate the shelf-life stability (6 months) of model infant formula with whey protein hydrolysates or peptidic fractions as carrageenan replacers. Whey protein hydrolysates were prepared with trypsin and followed by ultrafiltration of the hydrolyzed mixture, and peptidic fractions were isolated from the ultrafiltered tryptic hydrolysate by anion- or cation-exchange chromatography. The stability of the model infant formula was evaluated using a stratification method based on fat content differences between the top and bottom strata of the samples. With protein hydrolysate-based formulations, the creaming rate of the fat in the product was slightly higher than in the standard formulation (with carrageenan), which is indicative of lower storage stability. The addition of cationic fractions to model infant formula also resulted in lower product stability, whereas the fat creaming rate was retarded in anionic fraction based formulations. The physicochemical characteristics of certain peptides combined with the reported high emulsifying properties of peptidic sequences found within these fractions may account for their ability to act as carrageenan replacers.

Keywords: *Whey proteins; β-lactoglobulin; peptide; tryptic hydrolysis; infant formula; storage stability*

INTRODUCTION

Concentrated dairy-based infant formulas are essentially protein-stabilized emulsions composed of emulsified oils, carbohydrates, and sparingly soluble minerals, vitamins, and minor ingredients (*1*). The nature of these products leads to physical separation, which can shorten their shelf life, normally established at 12−18 months. The major form of physical instability encountered in infant formulas is creaming, in combination with aggregation of fat globule, but there is very little formation of free fat via coalescence (*2*).

In protein-stabilized emulsions, the lipid−fluid phase interface is mostly stabilized by endogenous proteins and added stabilizers such as carrageenan (*3*, *4*). Carrageenans are sulfated linear polysaccharides that can react with water to increase the viscosity of the product or with casein and whey proteins to form stable complexes (*5*, *6*). Baduy (*7*) has concluded that, in infant formulas, carrageenan distributes itself according to the available fat globule area, probably through interactions with adsorbed surface proteins rather than with the free casein structure. This way, a weak gel network of colloidal particles is formed to prevent creaming and sedimentation (4). In rare instances, gelation may proceed to the extent that syneresis or wheying-off occurs. In such cases, a somewhat clear serum zone will form at the bottom of the product due to the aggregation of proteins and the entrapment of lipids in the upper gel (*1*).

However, the European Society for Paediatric Gastroenterology And Nutrition (ESPGAN) advises against the use of age thickening agents in infant formulas for infants <4 months of age (*8*). Recently, Capron et al. (*9*) have demonstrated that the degradation of κ-carrageenan in simulated gastric juice is very limited. In fact, only 10% of the carrageenan exposed to the gastric juice is reduced to a molecular weight of <100 kDa. However, this result is important considering the possible toxicological effect of degraded carrageenan. Consequently, there is a need to search for thickening agent substitutes to stabilize infant formulas.

Functional properties of whey protein hydrolysates have been extensively studied (*10*−*15*), and many studies have reported their potential use as emulsifiers in food formulations. Peptides produced by enzymatic hydrolysis are characterized by (1) lower molecular weights and fewer secondary structures; (2) higher numbers of ionizable groups; and (3) exposure of hydrophobic groups hitherto concealed (*16*), which may be expected to have increased solubility near the isoelectric point, decreased viscosity, and significant changes in foaming, gelling, and emulsifying properties compared to those of native proteins (*12*).

Many authors (*10*, *14*, *17*, *18*) have reported that solubility and emulsifying properties of tryptic whey proteins hydrolysates are higher than in native proteins. Jost and Monti (*10*) found that an oligopeptide preparation obtained by ultrafiltration of a whey tryptic hydrolysate showed better emulsifying and interfacial properties than chymotryptic peptides and significantly improved the stability of oil/water emulsions. In addition, Chobert et al. (*12*) showed that the emulsifying capacity of whey protein hydrolysates was higher than that of casein derivatives and much higher than that of native whey proteins. Moreover, a study by Turgeon et al. (*14*) demonstrated that the fractionation of a

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β-lactoglobulin tryptic hydrolysate resulted in oligopeptides, β-lg 21−40 and 41−60, with improved surface activity properties and emulsifying capacities. These low molecular weight peptides allow for a periodic distribution of polar and hydrophobic residues (*10*, *11*, *13*). Rahali et al. (*19*) found also that these peptides or shorter fragments (β-lg 21−32 and β-lg 41−57) generated from β-lactoglobulin by controlled trypsin hydrolysis are surface active peptides at the oil/water interface. Finally, a study by Huang et al. (*20*) suggested that the emulsifying properties of β-lg 41−100 and 149−162 were superior to those of β-lg 41−60 described by Turgeon et al. (*14*). Characterization of this 8.6 kDa oligopeptide suggested that native secondary and tertiary structures would correspond to a five-stranded antiparallel "β-barrel" fragment (*21*). Previous studies (*20*−*22*) have also suggested that the core β-barrel domain is the hydrophobic region that binds small hydrophobic molecules, such as fatty acids and retinol. Hence, because of its solubility, amphipolarity, secondary and tertiary structure, and structural flexibility, this domain fragment showed excellent surface activity (*20*).

The objective of the present study was to evaluate the effect of whey peptide fractions on the shelf life of a model infant formula, as a replacement for carrageenan. Both anionic and cationic ion-exchange chromatographies were used to generate different peptidic fractions from a tryptic hydrolysate of whey proteins. A stratification technique based on the determination of compositional changes in different strata of the product during its storage has been developed. This technique was used to evaluate the long-term physical stability of infant formula containing whey peptides by measuring early changes in the distribution of protein and fat throughout the product.

MATERIALS AND METHODS

Materials. PTN 6.0S trypsin from bovine pancreas was supplied from Novo Nordisk Co. (Bioindustrials Inc., Danbury, CT). Esterase activity was determined according to the method of Schwert and Takenaka (*23*) and was 3612 units of BAEE/mg of protein. Liquid whey protein concentrate (WPC) (4.68% w/v protein) prepared by ultrafiltration (UF) of mozzarella cheese whey was obtained from a local cheese plant (Groupe Lactel, Chambord, PQ). *O*-Phthaldialdehyde (OPA), L-leucine, bovine immunoglobulin G, β-lactoglobulin, α-lactalbumin, chain A insulin, kentsin, and *N*-formyl-Met-Ala were purchased from Sigma Chemical Co. (St. Louis, MO). Albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A were from Pharmacia Biotech Co. (Baie d'Urfé, PQ). All other reagents were of analytical grade. For chromatographic analysis, all samples were filtered through a 0.22 μm membrane (PVDF, Gelman Sciences, Ann Arbor, MI) before injection, and 18 MΩ water (Modulab, Fisher Scientific) was used for the preparation of mobile phases and filtered through Millipore 0.22 μm filters (Millipore, Bedford, MA).

Preparation of Tryptic Hydrolysate (TH). TH was prepared according to conditions proposed by Turgeon and Gauthier (*24*). Hydrolysis was performed in a 200 L stirred tank, equipped with a heat-jacket. A lot of 120 kg of liquid WPC (4.68% w/v protein) was adjusted to pH 8.0 with 4 N NaOH and heated to 40 ± 1 °C. The hydrolysis reaction was initiated by adding 600 mL of tryptic solution (9.34%, w/v, in 0.001 N HCl) corresponding to an E/S ratio of 1:100 (grams of enzyme/gram of protein). During hydrolysis, the pH of the reaction mixture was maintained to pH 8.0 by manual addition of 4 N NaOH according to the pH-Stat technique of Adler-Nissen (*25*). After the addition of 524 mL of 4 N NaOH, corresponding to a degree of hydrolysis (DH) of 5.6% measured by the pH-Stat technique, a sample of TH was taken and rapidly heated (78 °C, 3 min) to deactivate the enzyme. The rest of the reaction mixture was immediately ultrafiltered using a Koch ultrafiltration system (Koch Membrane Systems, Wilmington, MA) in order to stop the reaction by removing the enzyme. This system was equipped with two polysulfone spiral wound membranes (HFM 180, 5 m^2), layered to a cellulosic acetate support, and having a molecular weight cutoff of 18000 Da. The system was operating at a 50-psi inlet pressure and a 30-psi outlet pressure, and the hydrolysate was concentrated two times. The retentate, reaction mixture (RM), was heated (78 °C, 3 min) to deactivate the enzyme. The permeate, designated ultrafiltered tryptic hydrolysate (UF-TH), was evaporated to a solid content of 10% using an APV junior plate evaporator (APV Co. Ltd.). Liquid WPC and hydrolysate fractions (TH, RM, UF-TH) were freeze-dried and stored at −18 °C until further analysis.

Fractionation of UF-TH by Ion-Exchange Chromatography. UF-TH was fractionated in different peptidic fractions by anionic and cationic exchange chromatographies using an HPLC Beckman System Gold (Mississauga, ON, Canada) equipped with two pumps (model 126P), a variable UV−visible detector (model 166P) operating at 214 nm, and a Rheodyne injection valve (model 7725i, Cotati, CA) with a 100 μL injection loop. Data acquisition and chromatographic analysis were performed with Beckman System Gold software. Fractions were collected using an LKB 2211 Superak fraction collector (Pharmacia Biotech).

Anionic Exchange Chromatography (AE-HPLC). AE-HPLC was performed using a micropreparative column OPTIMA 5/10 Q-Hyper D 20 (4.6 mm i.d. × 10 cm) obtained from Biosepra S.A. Co. The column was equilibrated at 30 °C with a sodium phosphate buffer (20 mM, pH 8.0) at a flow rate of 2 mL/min. A sample of UF-TH (10 mg/mL 20 mM sodium phosphate buffer, pH 8.0) was applied onto the column, and elution was performed with a linear gradient of 0.5 M NaCl in the same buffer according to the following conditions: 0−2.5 min (0%); 2.5−4.65 min (0−28%); 4.65−7.5 min (28−65%); 7.5−9.75 min (65−90%); and 9.75−11.25 min (90−0%).

Cationic Exchange Chromatography (CE-HPLC). CE-HPLC was performed on a micropreparative column OPTIMA 5/10 S-Hyper D 20 column (4.6 mm i.d. × 10 cm) obtained from Biosepra S.A. Co. The column was equilibrated at 30 °C with a sodium acetate buffer (20 mM, pH 4.5) at a flow rate of 2 mL/min. A sample of UF-TH (10 mg/mL 50 mM sodium acetate buffer, pH 4.5) was applied onto the column, and elution was performed isocratically during 2.5 min in a sodium acetate buffer (20 mM, pH 4.5), then with a linear gradient of 1 M NaCl in a sodium acetate buffer (50 mM, pH 4.5) according to the following conditions: 2.5−7.5 min (0%); 7.55−11.35 min (0−35%); 11.35−16.35 min (35−100%); and 16.35−18.85 min (100−0%).

Desalting of Peptidic Fractions. The fractionation of UF-TH by ionic exchange chromatographies was obtained by applying a linear salt gradient, and nanofiltration was used to eliminate salt in peptidic fractions. Desalting was performed with an MX-07 membrane from Osmonics Co. (Minnetonka, MN) using a Sepa ST cell according to the method of Wijers et al. (*26*).

Evaluation of Emulsifying Properties in Model Infant Formulas. Model infant formulas were prepared using premixed proprietary ingredients provided by Wyeth-Ayerst Canada Co. (Ste-Claire, PQ). The preparation of 600 mL of sterilized concentrated infant formula started with the addition of nonfat dry milk (25.7 g) to 453 g of deionized water at 69 °C and under constant stirring. For TH, UF-TH, or peptidic fraction-based formulation, 30 mg of each fraction was dissolved in 50 g of deionized water and then added to the milk, without further addition of stabilizer (calcium carrageenan). Fat blend (41.9 g, mixture of coconut, soybean, safflower, and oleo oils and soy lecithin) prewarmed at 60 °C was added to the mixture, followed by the addition of 74.6 g of electrodialyzed whey powder (EDW), 10 g of lactose, 79.8 mg of calcium carrageenan as stabilizer, and some minerals and vitamins. The mixture was heated at 100 °C under constant stirring and then cooled to 70 °C using warm water. Formula was blended for 30 s using an Ultra-Turrax homogenizer (Janke & Kunkel,

IKA Labortechnik). Two-stage homogenization was performed at 4000 psi using a Microfluids model 110Y (Microfluidics Corp., Newton, MA); the first stage was carried out at 95 °C, and the second one was performed at 60 °C. Infant formula was refrigerated overnight at 4 °C; the next day, the remaining minerals and vitamins were respectively dissolved in a predetermined amount of deionized water, estimated from total solids analysis of the formula, in order to obtain 23.9% (w/w) total solids in the final product. Samples (110 g) of infant formula were poured into glass bottles, sealed, and sterilized in an autoclave at 121 °C for 8 min. The final composition of the product was 7.2% carbohydrate (mainly lactose), 3.6% fat, 1.5% protein (40% casein, 60% whey protein), and 0.25% minerals. Bottles were protected from light and stored at room temperature for 0, 2, 4, and 6 months. Samples at 0 months of storage were used to determined the physicochemical characteristics of model infant formula such as pH, viscosity, and fat and protein contents, whereas all other samples were stored to evaluate their shelf-life stability by the stratification method.

Viscosity Measurements. After sterilization, viscosity (centipoise) was determined at 22 °C using a Brookfield viscometer model LVD II + (Stoughton, MA) fitted with an LV spindle no. 1.

Evaluation of Emulsion Stability by the Stratification Method. This method is based on the changes observed in lipid and protein distributions in infant formula during storage. Two 12-mL aliquots (top and bottom) of infant formula were taken out without agitation in the bottle to avoid dispersion of the blend and to ensure representative sampling. The first aliquot was taken at 4.5 cm from the bottom of the bottle. Then, the second 12-mL sample was taken at 1 cm from the bottom of the bottle. Both aliquots, identified as the bottom and top strata, were analyzed for protein and fat contents using the Kjeldhal and Mojonnier methods, respectively.

Characterization of Peptidic Fractions by Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis. Peptidic fractions obtained from anionic (Q-Hyper D) and cationic (S-Hyper D) chromatographies were analyzed by RP-HPLC using a system from Waters Co. (Millipore, Milford, MA), consisting of a pump (model 600E), a variable UV−visible detector (model 484) operating at 220 nm, and a Rheodyne injection valve (model 7725i) with a 20 μL injection loop. Data acquisition and chromatographic analysis were performed with Waters Millennium 2010 software (Millipore). RP-HPLC analyses were performed on a Nova-Pak C_{18} column (4 μm, 3.9 i.d. × 150 mm) from Waters Co. (Millipore) connected to a guard column (4 μm, 3.9 i.d. × 20 mm) filled with the same matrix and with the following conditions: flow rate of 1 mL/min; column temperature of 39 °C; solvent A, 0.11% TFA in water; solvent B, 60% (v/v) acetonitrile plus 40% (v/v) water and 0.10% TFA. An aliquot (13 μL) of peptidic solutions (10 μg/μL of 18 MΩ water) was injected onto the column and elution was obtained with a linear gradient from 0 to 60% of solvent B in 30 min, from 60 to 100% B in 5 min, and from 100 to 0% B in 3 min. The purity of peptidic peaks was further evaluated with the same column but using different conditions. Peptidic peaks in higher concentration (>5% of total peptides) were collected, dried in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY), and analyzed with the following conditions: solvent A, 25 mM ammonium acetate (pH 6.0); solvent B, 50 mM ammonium acetate (pH 6.0)/60% acetonitrile; elution obtained with a linear gradient from 100% solvent A to 60% solvent B in 30 min, from 60 to 100% B from 30 to 35 min, and 100% B from 35 to 37 min.

Characterization of Peptidic Fractions by Pico-Tag Analysis. For peptide identification, peptidic peaks were collected from RP-HPLC and dried in a Speed-Vac concentrator (Savant Instruments). After acid hydrolysis under vacuum in the presence of 6 N HCl for 24 h at 110 °C in a Pico-Tag station (Waters), amino acids were derivatized with phenylisothiocyanate (PITC) according to the method of Bidlingmeyer et al. (*27*) and quantified by RP-HPLC using a Pico-Tag C_{18} column (3.9 mm i.d. × 15 cm; Waters). Dried samples were dissolved in 95% Na_2HPO_4 (2 mM, pH 7.4)/5% acetonitrile. The column was equilibrated at 38 °C in solvent A (94% 0.14 M CH_3-COONa plus 0.5 mL of TFA/L, pH 6.4/6% acetonitrile), and elution was performed according to the method of Bidlingmeyer et al. (*27*) at a flow rate of 1 mL/min. Absorbance was recorded at 269 nm.

Chemical Analysis. Protein content (N × 6.38) of fractions obtained from enzymatic hydrolysis (WPC, TH, MR, UF-TH) and strata from model infant formula was determined in duplicate by the Kjeldahl method (*28*) performed on a Buchï block digester 430 equipped with distillation unit 323 (Brikman Instruments, Montreal, PQ). The degree of hydrolysis (DH) was evaluated in triplicate by the OPA spectrophotometric method of Church et al. (*29*), using L-leucine as a standard. Lactose content in the samples was obtained in triplicate by spectrophotometry using the lactose/D-glucose enzymatic kit (*30*) provided by Boehringer Mannheim Co. (Laval, PQ). Fat was determined in duplicate according to the Mojonnier method (*31*). Minerals were determined by atomic absorption (Ca^{2+}) and emission (Na^+, Mg^+, and K^+) using an Instrumentation Laboratory spectrophotometer (model IL751, Wilmington, MA). The total phosphorus was measured in triplicate by the colorimetric method of Allen (*32*). Moisture content was measured in duplicate by an AOAC method (*33*). Ash was obtained by charring the samples during 12−18 h in a muffle furnace at 550 °C. After cooling, 2 mL of H_2O_2 (*34*) was added and the samples were heated until a white ash was obtained.

Molecular Weight Distribution Profiles. Molecular weight distribution profiles of the water-soluble proteins of WPC and of its hydrolysate fractions (TH, RM, UF-TH) were determined by high-performance size exclusion chromatography (HPSEC) using the Waters HPLC system previously described for RP-HPLC analysis. HPSEC analysis was performed with a Biosep-SECS2000 column (300 mm × 7.8 mm i.d.) from Phenomenex Co. (Torrance, CA), connected to a guard column (75 mm × 7.8 mm i.d) filled with the same matrix. Protein (WPC) and hydrolysate fraction (0.8% protein base in 100 mM sodium phosphate buffer, pH 6.8) samples were injected onto the column, and elution was performed isocratically in a sodium phosphate buffer (100 mM, pH 6.8) at a flow rate of 1 mL/min. Detection was performed at 220 nm. Protein standards used for the calibration were immunoglobulin G (150000 Da), albumin (67000 Da), ovalbumin (43000 Da), dimeric β-lactoglobulin (36000 Da), chymotrypsinogen A (25000 Da), α-lactalbumin (14000 Da), ribonuclease A (13700 Da), chain A insulin (2531.6 Da), kentsin (500.6 Da), and *N*-formyl-Met-Ala (248.3 Da). The total areas of the chromatograms were integrated and separated into three molecular weight ranges (>5000, 2000−5000, and <2 000 Da), expressed as percentage of the total surface.

RESULTS AND DISCUSSION

The chemical composition, degree of hydrolysis (DH), and molecular weight distribution of protein components from WPC, TH, and its fractions obtained by ultrafiltration (RM, reaction mixture; UF-TH, ultrafiltered tryptic hydrolysate) are given in Table 1. Liquid WPC showed a chemical composition corresponding to that of a commercial 35-WPC (35% proteins) (*35*). TH showed a lower protein content (37.0% dry basis) than WPC (38.9%), which resulted from the increase in ash content (8.3%) caused by pH adjustments with NaOH during the hydrolysis reaction. This result was confirmed by the large amounts of sodium (1898 mg/100 g) measured in the TH. UF fractionation of the TH was performed at the end of the reaction to remove the enzyme as well as the nonhydrolyzed proteins and resulted in the concentration of proteins and fat in the retentate fraction (RM), whereas lactose concentrated in the permeate (UF-TH). Furthermore, the passage of salts through the UF membrane varied according to the

Table 1. Chemical Composition (Percent Dry Matter), Degree of Hydrolysis (DH), and Molecular Weight Distribution of WPC, TH, and Fractions Obtained by Ultrafiltration of TH (RM, UF-TH)

	WPC	TH	RM	UF-TH
protein (%)	38.9	37.0	47.4	28.2
lactose (%)	49.7	53.1	33.5	66.0
lipid (%)	4.2	4.6	8.3	0.1
ash (%)	6.4	8.3	8.7	8.3
Ca (mg/100 g)	707	638.3	1393.4	211.4
Na (mg/100 g)	769.4	1898.5	1496.6	2472.0
Mg (mg/100 g)	100.9	103.2	113.0	75.9
K (mg/100 g)	1400.5	1383.2	1268.3	1633.1
P (mg/100 g)	680.7	679.1	1054.5	610.1
DH[a] (%)	-	5.2	3.9	9.9
MW distribution[b] (%)				
>5000 Da	92.1	49.6	66.4	26.6
2000−5000 Da	1.8	19.0	13.6	24.1
<2000 Da	6.1	31.4	20.0	49.3

[a] DH was determined by OPA method. [b] Molecular weight distribution was calculated from the integration of the total area of the chromatogram obtained from HPSEC; the chromatogram was separated into three ranges of molecular weight and expressed in percent total area.

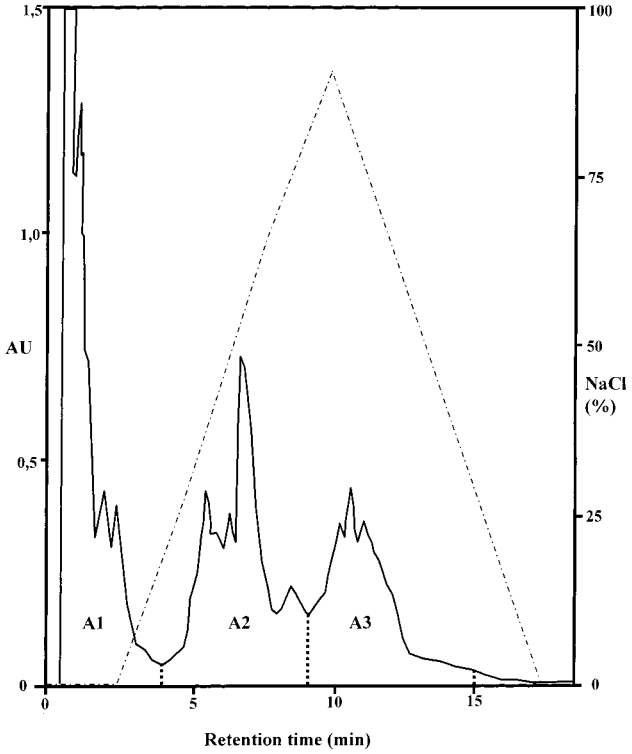

Figure 1. AE-HPLC profile of UF-TH fractionated into three peptidic fractions (A1−A3). Conditions: column, OPTIMA 5/10 Q-Hyper D 20; 20 mM sodium phosphate buffer, pH 8.0; gradient 0.5 M NaCl (-·-·-); flow rate, 2 mL/min; detection, 214 nm.

nature of ions with larger amounts of calcium, magnesium, and phosphorus in the retentate fraction (RM), whereas sodium and potassium were concentrated in the filtrate fraction (UF-TH). A mass balance calculation indicates that 60% of nitrogen was found in the filtrate fraction (UF-TH) and the remaining 40% in the retentate fraction (RM) following ultrafiltration of the TH.

The impact of tryptic hydrolysis of WPC was observed by the increase of the DH value (5.2%) for the TH. From the UF fractionation of the TH, peptides were concentrated in the filtrate fraction (UF-TH) as indicated by its higher DH value (9.9%) compared to that of the retentate fraction (RM, 3.9%). The molecular weight

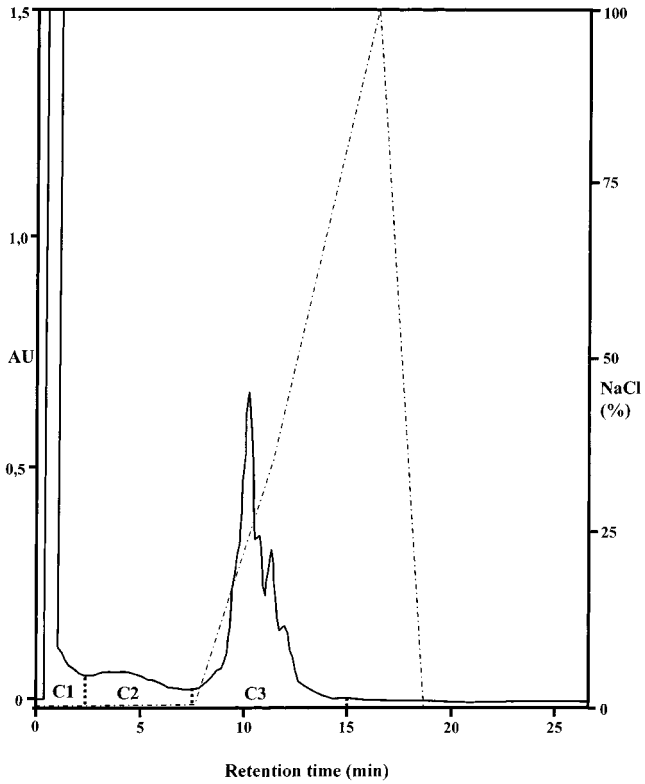

Figure 2. CE-HPLC profile of UF-TH fractionated into three peptidic fractions (C1−C3). Conditions: column, OPTIMA 5/10 S-Hyper D 20; isocratic mode during 2.5 min using 20 mM sodium acetate buffer, pH 4.5; 50 mM sodium acetate buffer with gradient 1 M NaCl (-·-·-); flow rate, 2 mL/min; detection, 214 nm.

Table 2. Physicochemical Characteristics of Model Infant Formula for Standard, Reference, Hydrolysate (TH, UF-TH), or Peptidic Fraction-Based Formulations at 0 Months of Storage

formulation	pH	viscosity (cP)	protein (%)	fat (%)
standard[a]	6.5	9.2	3.03	6.87
reference[b]	6.5	8.1	3.05	6.76
TH	6.6	8.4	3.25	6.90
UF-TH	6.6	9.2	3.15	6.74
anionic 1	6.4	10.2	3.19	6.87
anionic 2	6.4	8.3	2.95	6.76
anionic 3	6.4	7.4	2.94	6.63
cationic 1	6.4	7.8	2.94	6.57
cationic 2	6.4	8.6	2.99	6.65
cationic 3	6.4	9.8	3.06	6.84

[a] With carrageenan. [b] Without carrageenan.

distribution of the protein components for the different fractions is in agreement with the DH values. Liquid WPC is mainly composed of protein molecules (>5000 Da) with low amounts of peptidic material (7.9% of <5000 Da). From tryptic hydrolysis, about half of the proteins were hydrolyzed in smaller components (50.4% of < 5000 Da) that were concentrated (73.4% of < 5000 Da) in the UF-TH fraction following ultrafiltration of the TH. High amounts (66.4%) of protein material (>5000 Da) in the RM fraction resulted from enzyme and nonhydrolyzed proteins, which were concentrated in this fraction.

Ultrafiltered TH (UF-TH) was fractionated using anion- (Figure 1) and cation- (Figure 2) exchange chromatographies to obtain different peptidic fractions for their evaluation in the model infant formula as a carrageenan replacer. From anion-exchange chroma-

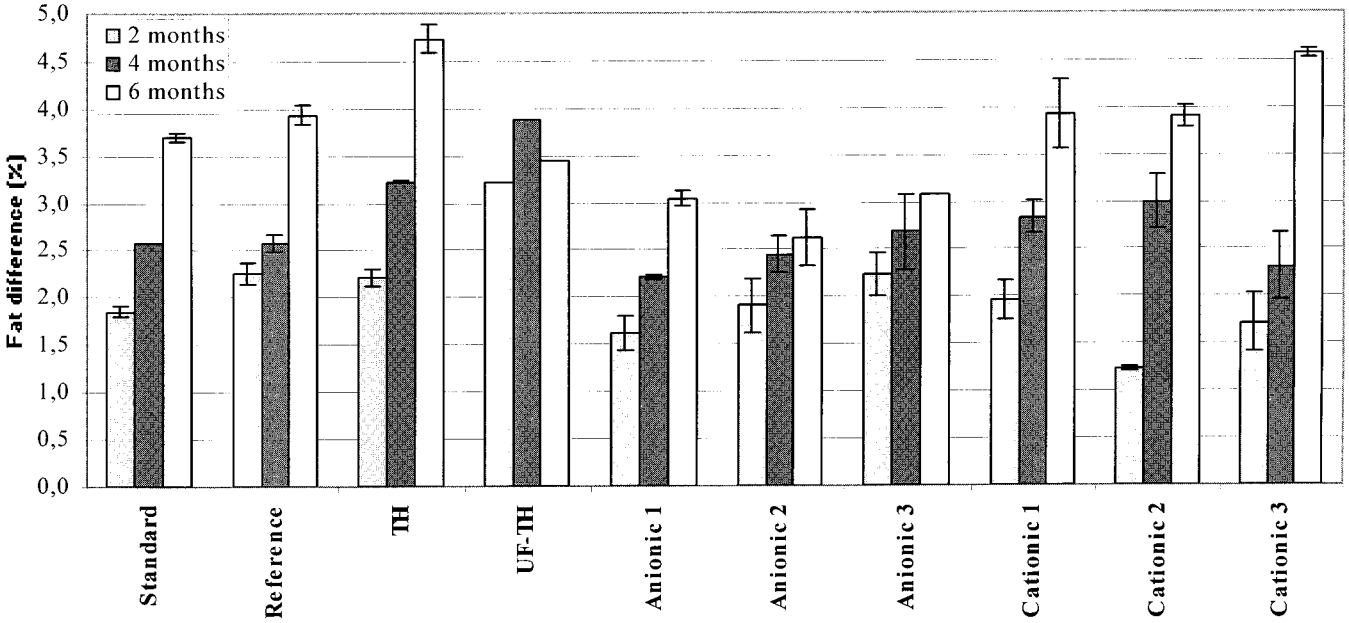

Figure 3. Fat differences (percent) between top and bottom strata after 2-, 4-, and 6-month storage periods (20 °C) for standard, reference, hydrolysate (TH, UF-TH), or peptidic fractions obtained from AE-HPLC (A1−A3) or CE-HPLC (C1−C3).

tography (AE-HPLC) (Figure 1), three peptidic fractions were collected, corresponding to retention times of 0−3.85 min (A1), 3.86−9.10 min (A2), and 9.11−13.00 min (A3) and representing 42, 34, and 24% of the protein content of UF-TH, respectively. Three peptidic fractions were also collected from cation-exchange chromatography (CE-HPLC) (Figure 2) at retention times of 0−2.21 min (C1), 2.22−6.72 min (C2), and 6.73−15.00 min (C3). In contrast to AE-HPLC, peptidic material recovery was disproportionate in the three fractions collected from CE-HPLC, representing 51% (C1), 11% (C2), and 38% (C3) of the protein content of UF-TH. Because ion-exchange chromatography used a salt gradient for the elution of the peptidic material, all fractions were desalted by nanofiltration to avoid salt effects for the study of storage stability of model infant formulas.

Model infant formulas were prepared with TH, UF-TH, and peptidic fractions obtained from AE-HPLC (A1−A3) and CE-HPLC (C1−C3) and then compared to the standard formulation containing carrageenan and reference sample without carrageenan. Table 2 presents the physicochemical characteristics of the different formulations at 0 months of storage. All infant formulas were similar to the standard formulation in terms of pH (6.4−6.6), viscosity (7.4−10.2 cP), protein (2.94−3.25%), and fat (6.57−6.90%) contents. Slight changes in pH, viscosity, and overall composition of the formulas containing hydrolysate or peptidic fractions were observed and indicated that there are no major problems associated with the incorporation of these fractions in model infant formulas.

Figure 3 shows the results obtained from the evaluation of storage stability for the different formulations using the stratification method. Results are expressed as the percent difference between the top and bottom strata in terms of fat content. During storage (2−6 months), fat difference values increased for all samples except for the UF-TH fraction-based formulation, for which the fat difference value at 6 months was lower than that at 4 months. Also, the creaming rate of the fat in the product varied according to the nature of the added ingredient (carrageenan, hydrolysate, or peptidic fractions). These results showed that the stratification method represents a suitable technique for evaluating the impact of ingredients added to the formulation by assessing early changes occurring in fat movement throughout the product.

To identify carrageenan replacers from hydrolysate or peptidic fractions, all formulations were compared to the standard product in terms of fat difference during storage (2−6 months). In fact, a higher fat difference value than that of the standard indicates lower product stability. Without carrageenan in the product (reference), the fat creaming rate in the product was slightly faster than in the standard (with carrageenan), which indicates lower stability of the reference sample during storage. With hydrolysate-based formulations (TH and UF-TH), this phenomenon was even more pronounced. In these products, carrageenan was replaced by the hydrolysate fractions and the fat difference values at the 2-, 4-, and 6-month storage periods were higher than in the standard or reference samples. Thus, the addition of TH or a polypeptides mixture obtained from UF fractionation of TH (UF-TH) to the model infant formula seemed to accelerate the creaming process of the product during storage. Agboola et al. (*36*, *37*) also reported the destabilization of oil-in-water emulsions containing whey protein hydrolysate as the sole emulsifier/stabilizer.

The storage stability of peptidic fraction-based formulations varied according to the chromatographic method used for the UF-TH fractionation. For peptidic fractions obtained from CE-HPLC, the stability of the product after a 2-month storage period was similar to (C1) or better than (C2, C3) that of the standard. However, the creaming rate of these samples increased more rapidly during storage, resulting in higher fat difference values for all cationic fraction-based formulations than in the standard after 6 months. Thus, it seems that peptidic fractions obtained from CE-HPLC are unable to or cannot stabilize the model infant formula. For peptidic fractions obtained from AE-HPLC, very promising results were obtained concerning the stability of the product during storage and their use as

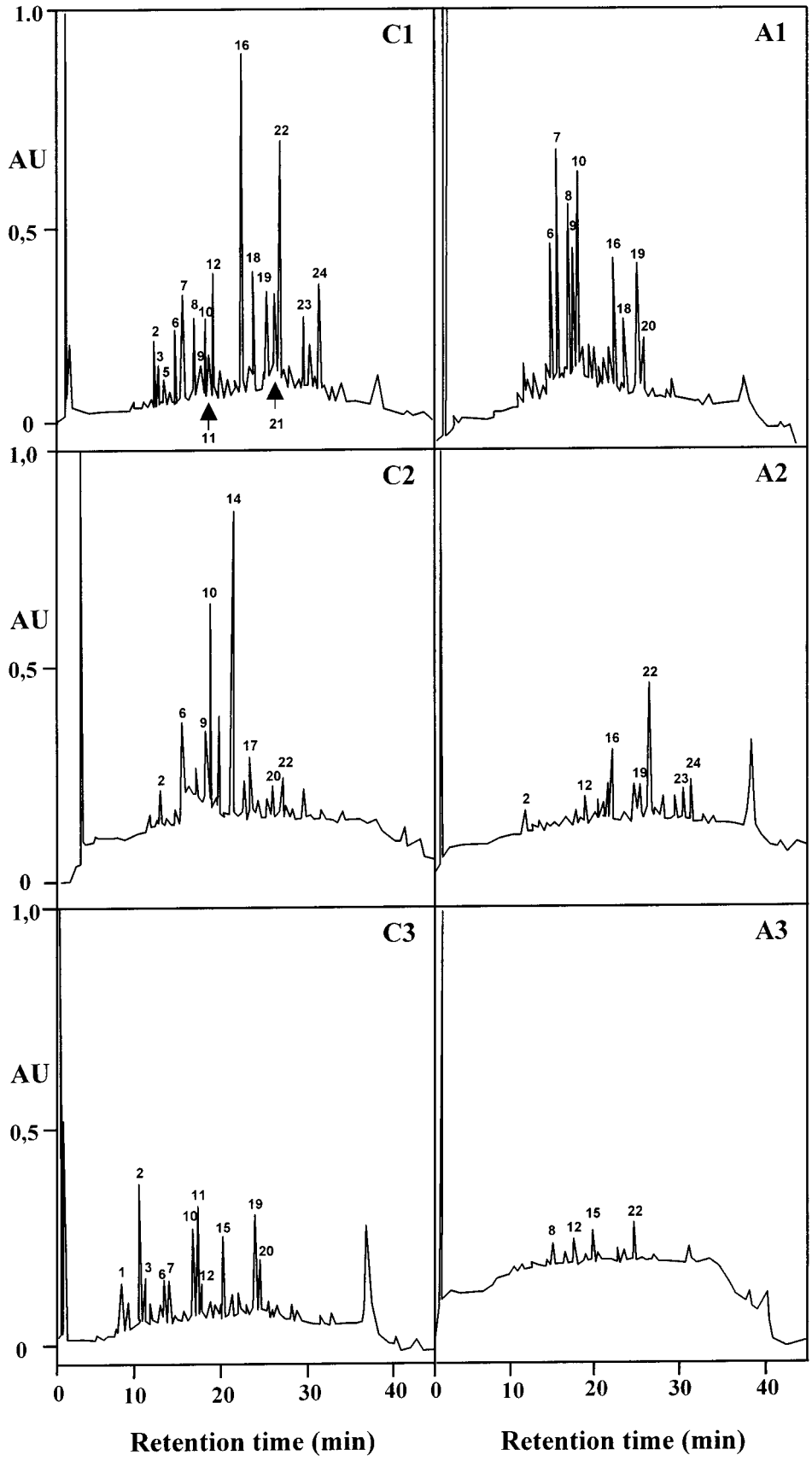

Figure 4. RP-HPLC profiles of peptidic fractions obtained from AE-HPLC (A1−A3) or CE-HPLC (C1−C3) of UF-TH. Conditions: Nova-Pak C_{18} column; column temperature, 39 °C; solvent A, 0.11% (v/v) TFA in water; solvent B, 60% acetonitrile/40% water in 0.10% (v/v) TFA; gradient 0−60% solvent B in 30 min, 60−100% solvent B in 5 min; flow rate, 1 mL/min; detection, 220 nm.

a carrageenan replacer. In fact, all anionic fraction-based formulations resulted in a better storage stability than that of the standard after 6 months. From the three fractions (A1−A3) isolated from AE-HPLC, the A2

Table 3. Content (Percent)[a] and Physicochemical Characteristics of Peptides Identified by HPLC (RP-C_{18} and Pico-Tag) in Peptidic Fractions Isolated from AE-HPLC (A1−A3) or CE-HPLC (C1−C3) of UF-TH

peptidic sequence	peak[b]	MW[c] (Da)	pI[d]	charge pH 7.0	$H_{\phi av}$[e]	UF-TH	A1	A2	A3	C1	C2	C3
β-lg 1−8	14	933	9.36	+1	1.34						13.74	
β-lg 9−14	10a	673	6.23	0	1.14		10.29					6.53
α-la 105−108	10b	468	9.36	+1	1.16						10.70	
β-lg 15−20	16b	696	5.77	0	1.61	7.88	7.57			9.7		
β-lg 33−40	7	857	6.23	0	0.91		16.08					
β-lg 41−60	24	2314	5.40	−3	1.37	6.61		7.12				
β-lg 61−69 + 149−162	22	2703	5.75	−3	0.94	8.69		23.62	22.36	10.23		
β-lg 61−70 + 149−162	21	2851	4.00	−2	0.96					6.42		
β-lg 71−75	6	573	9.36	0	1.63		13.12				9.30	
β-lg 76−82	20	775	9.36	+1	1.80							5.26
β-lg 78−82	19	546	5.77	0	2.19		8.53	7.39		5.21		7.10
β-lg 83−91	9	1044	6.24	0	1.01		9.77				6.82	
β-lg 84−91	8	916	4.54	−1	0.95		9.24		16.02			
β-lg 125−135	12	1245	4.52	−4	0.85			5.29	17.64			
β-lg 139−141	1a	330	9.36	+1	1.55							5.30
not identified	11											11.04
β-lg 142−148	15	837	10.22	+1	1.54							5.07
β-lg 146−148	2	425	10.22	+1	1.23							10.24

[a] Only the peptides present in higher concentration (>5% of total peptides) are reported in this table. [b] As reported in Figure 4. [c] Theoretical mass was calculated from ExPaSy molecular biology server. [d] Calculated from dissociation constants of ionic groups according to the method of Tanford (*41*). [e] Average hydrophobicity was calculated according to the method of Bigelow (*42*).

fraction appeared to be the best sample in terms of storage stability, with a fat difference value of 2.62% after a 6-month storage period, which corresponds to the value (2.58%) obtained after 4 months of storage in the standard formulation. Thus, it seems that the presence of specific peptidic fractions in infant formulas may have contributed to the retardation of the separation of fat in the product and improve their storage stability and that the chromatographic method used for the fractionation of peptidic components from UF-TH has an important impact on the functionality of the resulting fractions.

The results obtained for the different formulations in terms of the difference in protein content between the top and bottom strata are not presented because, for all samples, the differences in protein content remained very low (<0.5%) after a 4-month storage period.

To relate improvements in the storage stability of infant formulas to the functionality of whey peptides and to some of their physicochemical characteristics, the main (>5%) peptides of each peptidic fractions were isolated by RP-HPLC and further identified by their amino acids analysis (Pico-Tag). Figure 4 shows the RP-HPLC chromatographic profiles obtained for each peptidic fraction (A1−A3 and C1−C3). Each peak of these chromatograms was individually collected, repurified by RP-HPLC when necessary by using different solvents, and followed by their Pico-Tag amino acid analysis to identify their peptidic sequence in the major whey proteins (β-lactoglobulin and α-lactalbumin).

Table 3 reports the results of the peptide analysis with a list of the most abundant (>5% of total content) peptidic sequences identified, their physicochemical properties (molecular weight, isoelectric point, charge at pH 7.0, and average hydrophobicity), and their concentration in UF-TH fractions and peptidic fractions obtained from AE-HPLC (A1−A3) and CE-HPLC (C1−C3). A first examination of the overall peptidic sequences reveals that the majority of peptides originated from β-lactoglobulin (β-lg), with only the sequence f105−108 from α-lactalbumin (α-la). According to the specificity of trypsin for basic amino acids (Lys and Arg), five nonspecific cleavages (Leu, Trp, Phe, Met, and Asp) were observed for β-lg or α-la leading to peptides f33−40, f76−82, f78−82, f83−91, f130−135, and f146−148 from β-lg and to the sequence f105−108 from α-la. This result may be related to the presence of chymotrypsin in the trypsin preparation (PTN-6S), the specificity of which is mainly toward aromatic and hydrophobic amino acids. Also, many peptidic sequences previously identified in TH from whey proteins and reported by Dalgalarondo et al. (*38*) and Turgeon et al. (*14*) do not appear in Table 3 for the UF-TH or peptidic fractions obtained from ion-exchange chromatography. For the UF-TH fraction, a complex mixture of 24 peptides (result not shown) was identified, but only those in sufficient concentration (>5%) are listed in Table 3. This explanation is also valid for the peptidic fractions obtained from ion-exchange chromatography (A1−A3 and C1−C3). It is also possible that some peptidic material may have been lost during the desalting of the peptidic fractions by nanofiltration.

Peptidic sequences showed various physicochemical characteristics (Table 3). The molecular weight range of the peptides expands from 330 to 2851 Da, isoelectric points range from 4.00 to 10.22, and average hydrophobicity values range from 0.78 to 2.19 kcal/residue. This apparent heterogeneity in the physicochemical properties of peptides may explain why some peptidic fractions performed differently from others in the model infant formula. For example, the peptidic fractions providing the best improvement in storage stability of model infant formula (Figure 3) and which were obtained from AE-HPLC (A1−A3) are composed of neutral or highly negatively charged peptides. In these fractions, no positively charged peptides are found in amounts >5%. Furthermore, the A2 fraction, which represents the best sample in terms of storage stability (Figure 3), is composed of four peptides (>5% concentration), accounting for 50% of its peptidic content and including the three most negatively charged peptides (β-lg 41−60, 61−69 + 149−162, 125−135) identified in the UF-TH. From this group of negatively charged peptides, we noticed the presence of the β-lg 41−60 peptide, previously identified by Turgeon et al. (*14*), and a fragment of the β-barrel domain of β-lactoglobulin (β-lg 61−69 + 149−162) already identified by Huang et al. (*20*); both peptidic sequences are reported to have high emulsifying properties. Compared to the A2 fraction, the lower performance of the A1 and A3 fractions in terms of

storage stability could be related to the absence of the peptide β-lg 41−60 in these fractions. As a consequence of the prevalence of negatively charged peptides in anionic fractions, the isoelectric points of the peptides composing these fractions are in the acidic range (4.5−6.3), with the only exception being the peptide β-lg 71−75, which has an isoelectric point of 9.36 and was found in the A1 fraction. However, no other specific relationship was found for the molecular weight or the hydrophobicity of the anionic fractions. In fact, the molecular weights of the peptides composing these fractions varied from 546 to 2703 and their average hydrophobicity from 0.78 to 2.19.

The destabilizing effect of the cationic fractions in the model infant formula may be related to their relatively high content of positively charged peptides and the absence or low amounts of emulsifying peptides. In fact, peptides with positive charges account for >20% of the C2 and C3 fractions, which may interfere with the stabilizing effect of the negatively charged peptides. These fractions are composed of peptides with higher isoelectric points (5.8−10.22) and lower molecular weights (<1000 Da) than peptides found in anionic fractions. Also, the peptidic sequences identified as potential emulsifiers (β-lg 41−60 and β-lg 61−69 + 149−162) are absent in the C2 and C3 fractions. In contrast, no positively charged peptide appeared in the C1 fraction, but its total content in negatively charged peptides is low (16.5%) compared to the A2 fraction (36%). Also, this fraction contains low amounts (10%) of the peptide β-lg 61−69 + 149−162 compared to the A2 fraction (23%) and the β-lg 41−60 peptide is absent. Finally, even if only a few peptides appear in Table 3 for the C1 fraction, its peptidic composition is more complex (see Figure 4). Thus, the stabilizing effect of the emulsifying or negatively charged peptides may be reduced by their interactions with other peptides present in low amounts in the C1 fraction.

The stabilizing role of peptides in a complex system such as infant formulas is difficult to understand, but in dispersions that contain both peptides and proteins, the peptides may interact with proteins adsorbed at the fat globule interface. The protein and peptides that make up the fat globule membranes may be close enough to create a semicontinuous network, thereby stabilizing the whole system, as in a salad dressing (*39*). Portions of the peptide chains can be responsible for creating a weak gel state similar to the carrageenan network and may reduce creaming. The peptides can then distribute themselves in infant formulas according to the available fat globule area as does the carrageenan as studied by Baduy (*7*) and Samant et al. (*40*) in processed milk. In addition, the larger amounts of water that the peptide can hold may lead to an increased stability and viscosity and keep particles from interacting. Finally, the peptide regions that are directly associated with the fat globule may withstand higher gravitational forces and may provide enough steric repulsion or residual charges to prevent fat globule−fat globule interaction as seen in casein micelles (*4*).

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