Effect of Casein Hydrolysates on Association Properties of Milk Proteins As Seen by Dynamic Light Scattering[†]

Zahur U. Haque,* Guillermo A. Casay, W. William Wilson,[‡] Pirkko Antila,[§] and Veijo Antila[‖]

Department of Food Science and Technology, Mississippi Agricultural and Forestry Experimental Station, Mississippi State University, Mississippi State, Mississippi 39762

Small amounts (nil, 1, 2, and 4% w/w) of tryptic (T-PEP) and chymotryptic (C-PEP) hydrolysates of casein were added to sodium caseinate (Na-CN) and β-lactoglobulin (A) (β-LA A) to observe their effect on association properties of the proteins. Association was studied by gel permeation chromatography (GPC) and dynamic light scattering (DLS). Structural alterations were monitored by determining the intrinsic fluorescence of the proteins with and without peptides. Model studies were conducted side by side with zwitterionic amphiphiles (sulfobetaines) of varying hydrophobicity (C:8, C:12, and C:16). The quantum yield of fluorescence was affected by the peptides indicating changes in structure. The zwitterionic amphiphiles also affected the fluorescence. Amphiphiles of low hydrophobicity decreased the quantum yield, whereas high hydrophobicity increased it. The observed changes in the quantum yield of fluorescence reflected a change in the associated states of the proteins. DLS showed that the peptides had a dissociating effect on Na-CN, whereas β-LA A showed a tendency to associate to a larger aggregate in their presence. C-PEP was more effective compared to T-PEP, which consisted of smaller peptides.

INTRODUCTION

There have been several reports of the influence of peptides on the functional properties of milk proteins (Adler-Nissen et al., 1983; Haque et al., 1990; Haque, 1991). Milk is a rich source of peptides (Brunner, 1977; Eigel et al., 1984). Since milk is an important source of protein-based food functional ingredients, the influence of these peptides on the film-forming properties of proteins is of considerable interest to the industry. The influence of amphipathic peptides in milk whey on the functionality of milk proteins has recently been reviewed (Haque, 1992).

The state of association of proteins in the interface is important for emulsion stability (Haque and Kinsella, 1988). In dispersions that contain both peptides and proteins, the peptides interact with proteins mostly via hydrophobic interactions and decrease surface hydrophobicity of proteins (Haque, 1990). This phenomenon is referred to as "protective colloid action" (William et al., 1978). Since protein–protein interactions are primarily driven by hydrophobicity (Catsimpoolas, 1978), a reduction in surface hydrophobicity is expected to reduce the aggregate size of proteins that aggregate spontaneously as well as affect protein functionality. This work investigates the effect of milk peptide preparations on the spontaneous aggregation of sodium caseinate (Na-CN) and β-lactoglobulin A (β-LA A). Evidence for molecular association was provided by dynamic light scattering (DLS), which gave estimates of the average molecular dimension of the associated protein molecules.

* Author to whom correspondence should be addressed [phone, (601) 325-3200; fax, (601) 325-2474/8766].

[†] Approved as Journal Article 7936 of the Mississippi Agricultural and Forestry Experimental Station.

[‡] Department of Chemistry, Mississippi State University.

[§] Department of Dairy Science, Helsinki University, Finland.

[‖] Food Research Institute, Agricultural Research Center, Jokioinen, Finland.

MATERIALS AND METHODS

Materials. (N,N-Dimethyl-3-ammonio)-1-propanesulfonate (Z 8), (N-decyl-N,N-dimethyl-3-ammonio)-1-propanesulfonate (Z 10), (N-dodecyl-N,N-dimethyl-3-ammonio)-1-propanesulfonate (Z 12), (N-tetradecyl-N,N-dimethyl-3-ammonio)-1-propanesulfonate (Z 14), and (N-hexadecyl-N,N-dimethyl-3-ammonio)-1-propanesulfonate (Z 16) were obtained from Calbiochem, San Diego, CA. β-Lactoglobulin A (β-LA A) was obtained from Sigma Chemical Co., St. Louis, MO. Fresh skim milk for Na-CN preparation was from the Mississippi State University dairy plant. All other chemicals were of analytical grade.

Methods. *Dynamic Light Scattering (DLS): Dynamic Light Scattering Theory.* Dynamic light scattering is a technique that measures intensity fluctuations due to particles passing through a beam of light. Since the particles act as independent scatterers, the net intensity of the scattered light will fluctuate with time due to the random motion of the particles. The intensity fluctuations can provide a measure of the particle's diffusion coefficient, which can subsequently be used to obtain an equivalent spherical hydrodynamic radius for the particle. Excellent reviews related to the technique are available (Chu, 1974; Ford, 1983).

For particles undergoing Brownian motion, DLS gives a correlation function, $g^{(2)}(\tau)$, of the form (Ford, 1983)

$$g^{(2)}(\tau) = A[1 + B \exp(-2DK^2\tau)] \qquad (1)$$

where A is the baseline, B is an instrument constant, D is the translational diffusion coefficient of the scattering particles, and K is the scattering vector with magnitude

$$K = \frac{4\pi n}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \qquad (2)$$

where n is the refractive index of the solvent, λ_0 is the incident wavelength in vacuum, and θ is the scattering angle.

Equation 1 shows that the DLS correlation function decays exponentially at a rate governed by the magnitude of the translational diffusion coefficient, D. The usual method for obtaining D from a system of monodispersed scatterers is to cast the DLS data as

$$\ln\left[\frac{g^{(2)}(\tau) - A}{A}\right] = \ln B - 2DK^2\tau \qquad (3)$$

A plot of the left-hand side (LHS) of eq 3 vs delay time, τ, is

linear, so that D can be determined from the slope. If the system is polydispersed, then $g^{(2)}(\tau)$ becomes a sum of exponentially decaying functions; i.e., there is a distribution of diffusion coefficients about a mean value, D. In this case, eq 3 has been modified as

$$\ln\left[\frac{g^{(2)}(\tau) - A}{A}\right] = \ln B - 2\bar{D}K^2\tau + \frac{1}{2}\mu_2\tau^2 - \frac{1}{6}\mu_3\tau^3 + \ldots \quad (4)$$

where the second cumulant, μ_2, and the third cumulant, μ_3, are moments of the distribution (Koppel, 1972). A plot of the LHS of eq 4 against τ gives D, and the value for μ_2 can be used to estimate the extent of polydispersity in the sample. For samples that are nearly monodispersed, the ratio $\mu_2/(DK^2)^2$ has values of less than about 0.05. Samples that are moderately polydispersed may give values for $\mu_2/(DK^2)^2$ of 0.5 or higher.

An estimate of the apparent hydrodynamic radius, $R_h(app)$, of the scattering particles can be made using the Stokes–Einstein relation (Einstein, 1908)

$$\bar{D} = k_B T/6\pi\eta_0 R_h(app) \quad (5)$$

where k_B is Boltzmann's constant, T is the absolute temperature, η_0 is the viscosity of the solvent, and R_h is the hydrodynamic radius.

The particle size distribution of a polydisperse system can be estimated from the correlation function using various algorithms. The technique used in this work is a Laplace inversion method developed by Ostrowsky et al. (1981).

It should be noted that results obtained according to this method of data analysis are intended to show changes in the particle size distribution rather than emphasize the absolute values for particle sizes.

Instrumentation. The laser source for the DLS experiment was a 50-mW Jodon He–Ne laser Model HN-50 with emission at 632.8 nm. The beam was focused using a 15-cm focal length lens into a cylindrical, water-jacketed (Hellma Model 165) scattering cell. The cell temperature was carefully regulated by using a circulating water bath. The cell was positioned on the center of a rotary mount that allowed the scattering angle to be varied from 0° to 130°.

Light scattered at an angle of 20° was collected and focused with a 10-cm focusing lens onto the surface of an RCA C31034 photomultiplier tube housed in a magnetically shielded and thermoelectrically cooled housing, Pacific Model 3457. A Fluke Model 415B high-voltage power supply provided power for the PMT. The signal from the PMT was amplified by a Pacific AD-6 amplifier–discriminator and routed to a Brookhaven BIC 2030AT digital correlator. The correlator was equipped with 72 real time data channels and 8 additional channels delayed by 1028τ for baseline determination. The autocorrelation functions were analyzed using both the method of cumulants, which gave a mean value for the molecular size, and the inverse Laplace transform method, which gave an estimate of the molecular size distribution.

Determination of Light Scattering. The freshly hydrated protein dispersion (see below) was injected into a sample loop that consisted of an inert tubing that formed a closed circuit through a 0.45-μm filter, a peristaltic pump, and the scattering cell. The total capacity of this sample loop was 1 mL. The peristaltic pump was then run for 30 s to exclude particles, if any, that were bigger than the cutoff point of the on-line filter. This effectively removed foreign matter that could give artifactural data. The light scattering data were then recorded.

Preparation of Proteins. Sodium caseinate (Na-CN) was obtained by isoelectric precipitation of casein from skim milk followed by readjustment of pH to neutral with NaOH. The process of precipitation and subsequent redispersion with NaOH was repeated. Na-CN thus produced and β-LA A that had been purchased from Sigma were dialyzed exhaustively (10 000-fold) against distilled water. The proteins were then freeze-dried and stored in a desiccator at 4 °C until needed.

Protein Dispersions. All proteins were dispersed (1 mM) in 5 mM sodium phosphate buffer (pH 6.8) and allowed to hydrate for 1 h at 22 °C prior to use.

Gel Permeation Chromatography (GPC). The "native" associated state of the proteins used was determined by GPC using a Waters Protein-Pak 300SW column (8 mm/300 mm). A Waters 510 pump was routed through a universal (U6K) injector to a differential refractometer (Waters R401). The elution buffer used was 50 mM sodium phosphate (pH 7) containing 0.02% sodium azide. The flow rate was 1 mL/min, and the amount of protein loaded was 5 mg. Experiments were conducted at 25 °C.

Intrinsic Fluorescence. An Amino scanning fluorescence spectrophotometer coupled to a computerized data acquisition unit was used for the study. The protein dispersion was pipetted in a 1-cm cell path quartz cuvette, and the scans were recorded at 120 nm/min at 22 °C. The excitation wavelength was fixed at 295 nm (10 μ) to assure tryptophan excitation. The emission was varied from 320 to 440 nm (8 μ). All readings were taken at 25 °C.

Enzyme Hydrolysates. Chymotryptic hydrolysate (C-PEP) was obtained by mild hydrolysis of casein with α-chymotrypsin. Tryptic hydrolysate (T-PEP) was obtained by extensive digestion of casein with trypsin for 1 h. In both cases, the protein to enzyme ratio was 1000:1, pH was 8, and the incubation was at 37 °C. The reaction was stopped by boiling for 5 min.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the modified caseins was carried out according to a modified version of the method of Schagger et al. 1988) in the presence of sodium dodecyl sulfate (SDS). Reservoir buffer (0.192 M glycine, 0.025 M Tris, pH 8.3) containing 0.1% (w/v) SDS and 0.05% Coomassie Brilliant Blue G-250 was used for the separation and visualization of proteins (Schagger et al., 1988). During the passage through the stacking gel, the current was regulated at 15 mA, following which it was increased to 25 mA.

RESULTS AND DISCUSSION

Casein associated much more than β-LA A as observed by GPC (Figure 1). The caseins in sodium caseinate self-associated and eluted at the void volume (as determined by Blue Dextran) at a retention volume of 5 mL. A broad component eluted between 6 and 11 mL, indicating a wide range of aggregate sizes. The arrows A and B in Figure 1A indicate the elution points of Blue Dextran and lysozyme (MW 14 000). The peak maxima of the second (smaller aggregates) peak was around a retention volume of 7 mL, which was the retention volume of bovine serum albumin (Figure 1B, arrow B) (MW 67 000).

The GPC profile of β-LA A indicated much less association (Figure 1B). A single peak was obtained under the conditions of our experiments and appeared at a retention volume of about 9.5 mL compared to 12 mL for lysozyme (Figure 1B, arrow C). This is consistent with the expected retention time for a β-LA A dimer.

Intrinsic Fluorescence. The quantum yield of the fluorescence of casein dispersion was affected by the type of amphiphile used (Figure 2). Whereas ZW 8 decreased the relative intensity markedly, ZW 16 increased it. The shape of the fluorescence profile was, however, not affected. Thus, it is unlikely that the environment of the chromophores was changed. In other words, the protein structure was not noticeably changed. Fluorescence quenching has been associated with energy transfer to/ from small molecules bound to proteins (Udenfriend, 1969). Thus, it appears that the larger zwitterionic amphiphile (ZW 16) bound less to the proteins as indicated by the increased fluorescence.

The casein hydrolysates were then used to observe the effect of zwitterionic peptides. On the basis of the electrophoretic data, the C-PEP consisted of a heterogeneous mixture of peptides, whereas T-PEP almost totally consisted of much smaller peptides that traveled close to the electrophoretic front (Figure 3). When a small amount (4% by weight of protein) of the T-PEP was used (4%
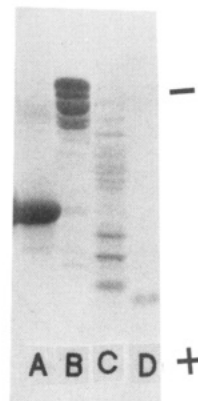
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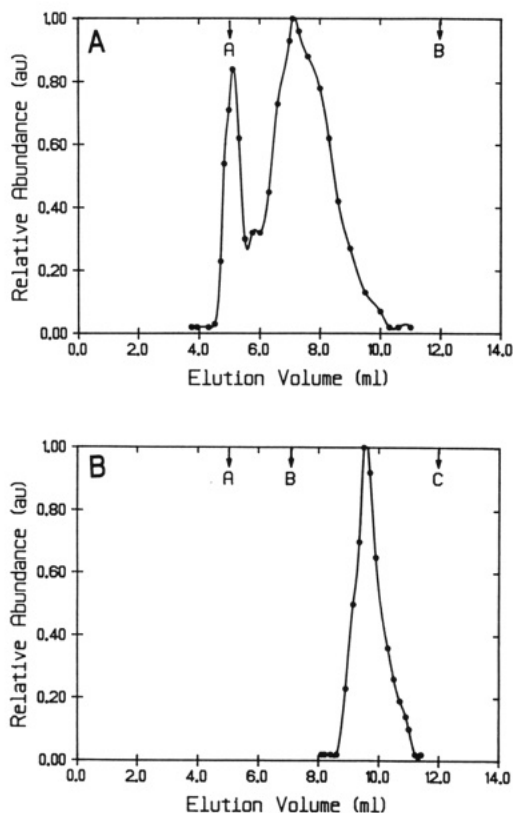
Figure 1. (A) Gel permeation chromatography of sodium caseinate. The pH was 7 (50 mM phosphate) at 25 °C, and the flow rate was 1 mL/min. The X and Y axes represent the retention volume and detector intensity, respectively. The arrows A and B represent the void volume (Blue Dextran) and elution point of lysozyme (MW 14 300), respectively. (B) Gel permeation chromatography of β-lactoglobulin (A). The conditions were the same as in (A). The arrows A, B, and C represent the void volume (Blue Dextran) and the elution points of bovine serum albumin (MW 67 000) and lysozyme (MW 14 300), respectively.
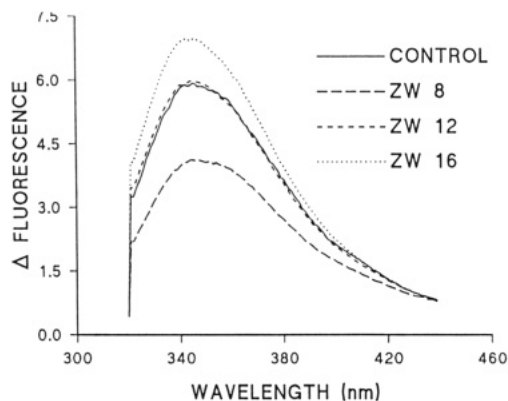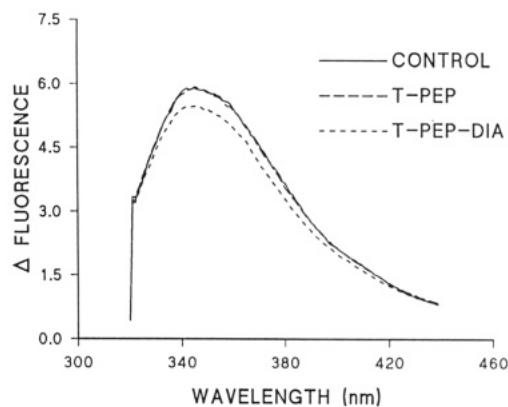

Figure 2. Fluorescence spectra of casein in the presence or absence of zwitterionic amphiphiles. The buffer used was 10 mM sodium phosphate, pH 6.8 (25 °C). Excitation was at 295 nm. ZW 8, ZW 12, and ZW 16 represent zwitterionic amphiphiles of increasing hydrophobicity as represented by the number (see Materials).

Figure 3. Polyacrylamide gel electrophoresis of casein hydrolysates. A, B, C, and D represent β-lactoglobulin, casein, chymotryptic digest (C-PEP), and tryptic digest (T-PEP), respectively.

Figure 4. Fluorescence spectra of casein in the presence or absence of tryptic hydrolysate (4% w/w) of casein. Conditions were as in Figure 2. T-PEP and T-PEP-DIA represent tryptic digest of casein and digest that had been dialyzed, respectively.
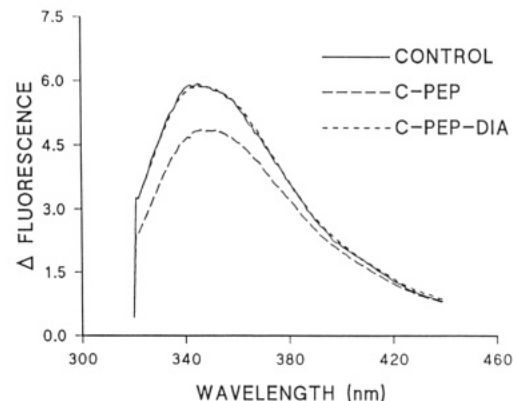
Figure 5. Fluorescence spectra of casein in the presence or absence of chymotryptic hydrolysate (4% w/w) of casein. Conditions were as in Figure 2. C-PEP and C-PEP-DIA represent chymotryptic digest of casein and digest that had been dialyzed, respectively.

w/w), there was little or no effect on the fluorescence, even when the hydrolysate used had been previously dialyzed to remove smaller peptides (T-PEP DIA) (Figure 4). On the other hand, the observations with the C-PEP (larger peptides) (Figure 5) were different. The undialyzed hydrolysate depressed the quantum yield compared to that of the dialyzed hydrolysate.

To understand the above observations, we look at the electrophoresis data again (Figure 3). Electrophoresis showed that C-PEP was a heterogeneous mixture of

peptides. Dialysis would reduce the content of smaller peptides, thus increasing the relative proportion of the larger peptides (>12 000 MW). The extensively hydrolyzed peptide preparation (T-PEP), on the other hand, consisted primarily of small peptides; dialysis did not markedly alter the relative size distribution of the peptides.

Effect of Amphiphiles on Association Tendency of Casein. Dynamic light scattering data corroborated the data obtained from the GPC experiments where two associated components were observed for casein. DLS was able to detect changes in the association tendency of
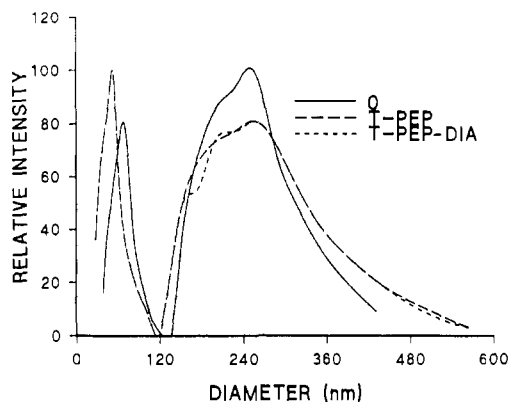
Figure 6. Size distribution of the sodium caseinate aggregates in the presence and absence of tryptic hydrolysate (4% w/w) determined by dynamic light scattering. Buffer used was 10 mM phosphate buffer, pH 7 at 25 °C. 0, T-PEP, and T-PEP-DIA represent the control, chymotryptic digest of casein, and digest that had been dialyzed, respectively.

Table I. Mean Apparent Hydrodynamic Radius of Sodium Caseinate and β-Lactoglobulin in the Presence and Absence of Peptides[a]

	control	T-PEP	T-PEP-DIA	C-PEP	C-PEP-DIA
Sodium Caseinate					
R_h(app),[b] nm	90	89.5	88	72.5	85.5
poly[c]	0.166	0.31	0.26	0.25	0.32
β-Lactoglobulin					
R_h(app),[b] nm	3.4	3.75	32.6	36.5	41.5
poly[c]	0.38	0.53	0.49	0.54	0.49

[a] T-PEP and C-PEP are tryptic and chymotryptic digests (see Materials and Methods). DIA represents dialysis. [b] R_h(app), Hydrodynamic radius as determined by DLS. [c] Poly, a measure of the width of the distribution.

the proteins. The particle size distribution of casein in the presence of T-PEP and T-PEP-DIA showed no marked change (Figure 6; Table I). The hydrodynamic radii presented in Table I are mean values and are not to be considered as absolute values. The slightly greater poly values for T-PEP compared to those for T-PEP-DIA are an indication of the broadening of the size distribution. This was comparable to the fluorescence data. On the other hand, in the undialyzed C-PEP, the peak of the particle size distribution of the larger aggregates was shifted to a lower aggregate size at about 130 nm; however, the impact of dialysis in the casein distribution was clearly observed, and the peak distribution was shifted to a higher aggregate size at about 300 nm (Figure 7). The fluorescence data showed a decrease in quantum yield caused by the presence of C-PEP, and this effect disappeared when the hydrolysate was dialyzed. We have hypothesized above that decreased quantum yield was due to the attachment of small peptides. Since the casein–casein association is primarily driven by hydrophobicity (Wong et al., 1988), masking of hydrophobic sites by peptides is expected to decrease association tendency.

On the basis of small-angle X-ray diffraction, crystallography, and hydrodynamic properties, the native molecular diameter of β-LA A was found to be approximately 2 nm (Bell et al., 1970; Zimmerman et al., 1970; Gilbert and Gilbert, 1973). As observed before, β-LA A gave a single homogeneous peak by GPC (Figure 1B). The DLS data gave some interesting data related to β-LA A (Table I). In the presence of T-PEP, the mean hydrodynamic radius of β-LA A did not change, but when T-PEP-DIA was used, there was a dramatic increase in the mean hydrodynamic radius of the β-LA A aggregate from 3.4 to 32.6 nm. When the larger peptide containing C-PEP was
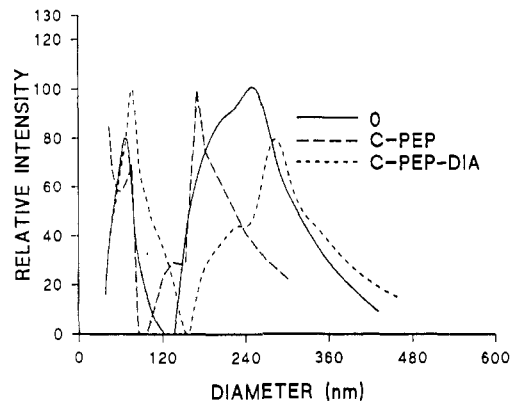
Figure 7. Size distribution of the casein aggregates in the presence and absence of chymotryptic hydrolysate (4% w/w) determined by dynamic light scatter. 0, C-PEP, and C-PEP-DIA represent the control, chymotryptic digest of casein, and digest that had been dialyzed, respectively. Conditions were the same as in Figure 6.

used, the aggregate size was similarly increased whether the hydrolysate was dialyzed or not.

Thus, β-LA A associated markedly in the presence of both large and small peptides, whereas caseinate was much less affected. In fact, the peptides appeared to dissociate the casein–casein aggregate. The increased tendency for association of β-LA A in the presence of peptides may be attributed to the following: (i) a subtle alteration in the structure as a result of the change in free energy due to protein–peptide binding; (ii) the formation of mixed micellar structures that may be better able to decrease hydrocarbon–aqueous interface, a thermodynamically favorable phenomenon (Tanford, 1980).

These data emphasize the marked effect of amphipathic peptides on the physicochemical properties of milk proteins. The impact varied depending on the type of protein and was also affected by the size of peptides. In an extension of this study, the peptides in the hydrolysates have been characterized. Further study is in progress.

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