Pepsin-Induced Changes in the Size and Molecular Weight Distribution of Bovine Casein During Enzymatic Hydrolysis

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ABSTRACT

Bovine casein was digested with pepsin at pH 2.0 in a batch-stirred tank reactor. To investigate the effect of peptic digestion on the aggregate size and molecular weight distribution of bovine casein, the resulting hydrolysates were examined by size-exclusion chromatography coupled with multiangle laser light scattering and dynamic light scattering. Casein was resolved by size-exclusion chromatography into 2 major peaks corresponding to aggregates and monomers, both of which showed a continuous decrease as hydrolysis proceeded. However, the ratio of aggregates to monomers was maintained at almost 1 (2:2.5) during the initial 30-min hydrolysis, indicating that the caseins in solution were in a type of equilibrium between aggregates and monomers. Upon peptic hydrolysis, casein aggregates increased in size and molecular weight, and exhibited a decrease in intermolecular repulsion. This finding was confirmed by dynamic light scattering measurements, which traced the changes in the hydrodynamic radii and light scattering intensities of casein hydrolysates. In addition, the release kinetics of peptide fractions with different molecular weights was also examined. It was concluded that the increase in hydrophobic attraction and the reduction in intermicellar repulsion might promote the growth in aggregate size of bovine casein during the limited hydrolysis.

Key words: casein, pepsin, size-exclusion chromatography, light scattering

INTRODUCTION

Caseins, which represent approximately 80% of the total milk proteins, are among the most used functional proteins in food processing and formulation. In mammalian milk and aqueous solution, they exist in a micelle form aggregated by 4 phosphorylated proteins (i.e.,

α_{S1} -, α_{S2} -, β -, and κ -CN). To date, the mechanism of assembly remains unclear, although various models of CN structure have been proposed and reviewed (Dalglish, 1998; Farrell et al., 2006; Horne, 2006). Such models have often been supported or questioned by images from electron microscopy (McMahon and McManus, 1998; Dalglish et al., 2004). On the other hand, enzymatic hydrolysis has provided a new way to investigate the topography of proteins within the CN aggregates. It has been suggested that β -CN is present in the coat region, because it is very accessible to soluble trypsin (Diaz et al., 1996).

It is now accepted that changes in the size of CN aggregates can lead to chemical or physical instabilities, and thus affect the biological functions of CN. The effects of pH, temperature, ionic strength, concentration, and transglutaminase on the size and molecular weight of aggregates have been typically investigated (Vasbinder et al., 2003; Vetier et al., 2003; Smiddy et al., 2006). Recently, it has been shown that micellar aggregation of bovine CN could be promoted by added plasmin (Crudden et al., 2005). Aggregation and gelation of whey proteins, another important type of milk protein, have also been detected and assessed after enzymatic hydrolysis by immobilized trypsin (Chen et al., 1994), a protease from *Bacillus licheniformis* (Otte et al., 1997, 2000), and alcalase (Doucet et al., 2003a,b). In these studies, the intermolecular attractive forces, such as hydrophobic interactions, were regarded as the main reason for the formation of protein aggregates and gels.

Enzymatic hydrolysis of proteins is a complex phenomenon that involves significant changes in foaming and gelling abilities (van der Ven et al., 2002). At the same time, the original proteins are progressively cleaved into a number of peptides, some of which are biologically active. The structure, function, and application of such peptides derived from milk proteins have been extensively reviewed (Clare and Swaisgood, 2000; Silva and Malcata, 2005). One of the structure-activity relationships is that various functions of bioactive peptides depend strongly on their lengths. For example, to possess antimicrobial activity, the peptides should

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contain from 20 to 46 AA residues (Nicolas and Mor, 1995) so that they can cross the membrane barrier under certain circumstances. The angiotensin I-converting enzyme (ACE)-inhibitory peptides, also known as antihypertensive peptides, usually contain 2 to 12 AA residues, although some with up to 27 residues have been identified (Yamamoto et al., 1999; Saito et al., 2000; Robert et al., 2004). Very small peptides, especially di- and tripeptides, have a high intestinal absorption rate because they are subject to transmembrane transport (Daniel, 2004). Therefore, if protein hydrolysates are required to be selectively used, it would be important to control the extent of enzymatic hydrolysis and to characterize the resulting peptides based on their size and molecular weight.

After milk is fed to adults or suckling offspring, pepsin and pancreatin play the most important roles in the hydrolysis of mammalian CN during the process of gastrointestinal digestion. Pepsin digests the proteins at an optimal acidic pH of 1.5 to 2 in gastric juice and exhibits a preference for peptide bonds between hydrophobic AA residues (Tang, 1963). To date, the molecular weight distribution (MWD) and peptide composition of the resulting hydrolysates from milk proteins by the site-specific pancreatic proteases, especially trypsin, have been typically examined by bulk experimental methods such as liquid chromatography and mass spectrometry (Qi et al., 2003; Su et al., 2007). Although the reaction of CN hydrolysis with pepsin had been investigated in several previous studies (Dalgalarondo et al., 1995; Awad et al., 1998), the changes in the aggregate size and MWD of peptic hydrolysates are still not well understood.

The objective of this study, therefore, was to 1) characterize the aggregate size and MWD of bovine CN by high-performance size-exclusion chromatography coupled with multiangle laser light scattering (HPSEC-MALLS); 2) monitor the pepsin-induced changes in the sizes of CN aggregates by HPSEC-MALLS and dynamic light scattering (DLS); and 3) follow the MWD of the resulting peptides released from peptic hydrolysis of bovine CN.

MATERIALS AND METHODS

Materials

Bovine CN (C7078) of technical grade and porcine pepsin (EC 3.4.23.1; 800 to 2,500 units/mg) were purchased from Sigma Co. (St. Louis, MO). Acetonitrile (ACN) of HPLC grade was obtained from Merck Co. (Darmstadt, Germany). Decahydronaphthalene (decalin) was purchased from Fisher Scientific (Pittsburgh, PA). Ultrapure water was obtained from an ELGA water purification unit (ELGA Ltd., Bucks, UK). All other

common reagents and solvents were of analytical grade from commercial sources.

Peptic Hydrolysis of Bovine CN

Bovine CN at 10 mg/mL was digested with pepsin (0.5 mg/mL) at 37°C in a batch-stirred tank reactor. The pH was kept stable at 2.0 by adding 0.2 M HCl with a pH-stat method. During the reaction, aliquots of CN and its hydrolysates were taken out at 0 (control), 2.5, 5, 10, 30, 60, 120, and 180 min. Enzymatic hydrolysis was stopped by addition of ammonium hydroxide up to pH 8.0. The samples were lyophilized and then stored at -20°C.

The degree of hydrolysis (DH); that is, the percentage of peptide bonds cleaved during the enzymatic reaction, was measured by pH-stat method as previously described (Qi et al., 2007) using the equation given by Zhao et al. (1996).

HPSEC-MALLS

The high-performance size-exclusion chromatographic system consisted of an Agilent 1100 isocratic pump (Agilent Technologies, Waldbronn, Germany), a Rheodyne 7725i injector loop (Rheodyne, Cotati, CA) of 100 μ L, and 3 columns in series including PL aquagel-OH Guard, PL aquagel-OH MIX and PL aquagel-OH 83 (Polymer Laboratories, Shropshire, UK). The columns were kept at a constant temperature of 35°C. The buffer used was water containing 30% ACN, pH 7.0; the flow rate was 0.5 mL/min. The elution profiles were monitored by a UV detector, a BI-MwA MALLS detector equipped with a 30-mW He-Ne linearly polarized laser at a wavelength of 660 nm, and a BI-DNDC differential refractometer [refractive index (RI) detector; Brookhaven Instruments, Holtsville, NY].

The freeze-dried CN (control) and its hydrolysates were redissolved in the eluant buffer at 5.0 mg/mL. The apparent pH values of samples were approximately 7.0 to 7.5, and were finally adjusted to 7.0 by using 0.2 M HCl. All samples were filtered through 0.45- μ m filters (Pall Corp., East Hills, NY) before injection. A dn/dc (the RI increment as a function of concentration) value of 0.186 mL/g was applied for the molecular weight calculations (Wen et al., 1996).

The WINGPC 7.2 software (Polymer Standard Service, Mainz, Germany) was applied to collect and process the elution data. Bovine serum albumin was used for normalizing the signals of UV and RI detectors relative to those of MALLS detector. The number-averaged (M_n) and weight-averaged (M_w) molecular weights were calculated by the Debye method; that is, regression on the light scattering parameter: $R(\theta)/K^*C$ vs. $\sin^2(\theta/2)$.

To calculate the molecular weight of peptides, a molecular weight calibration curve was developed from the average elution volumes of the following standard proteins: BSA (monomer 66.5 kDa, dimer 133 kDa), cytochrome C (12.3 kDa), insulin (5.73 kDa), and vitamin B₁₂ (1.36 kDa).

DLS

Dynamic light scattering measurements at a 90° scattering angle were carried out by a package containing a MGL-III model 100-mW He-Ne laser (Changchun New Industries Optoelectronics Tech Co., Changchun, China) tuned to a wavelength of 532 nm, a computer-controlled BI-200SM goniometer, and a BI-9000AT digital correlator and signal processor (Brookhaven Instruments). Decalin, as an index-matching liquid to reduce flare, was recirculated through a 47-mm outside diameter, 0.1- μ m pore-size hydrophobic membrane filter (Millipore, Bedford, MA) until visual inspection showed no dust. The freeze-dried CN and its hydrolysates were redissolved in 0.1 M PBS (pH 7.0) at a concentration of 1.0 mg/mL, and carefully filtered with 0.45- μ m filters (PALL Corp.) directly into borosilicate glass tubes (Fisher Scientific). The test tubes were placed in the sample holder and kept at a constant temperature of 37°C for 10 min. The average hydrodynamic radius (R_h) was calculated from the diffusion coefficient by using the method of cumulants (Brown et al., 1975).

RESULTS AND DISCUSSION

Analysis of Bovine CN

The HPSEC elution profiles of bovine CN obtained from the laser scatter detector at an angle of 90° (MALLS), the RI signal, and the absorbance at 214 nm (UV) are shown in Figure 1. The RI signal was similar to the UV response but different from the light-scattering response. This is the nature of heterogeneous protein mixtures, because the RI and UV signals were related to the concentration, whereas the light-scattering signal was in proportion to both the concentration and the molecular weight (Wyatt, 1993). Both RI and UV chromatograms for bovine CN displayed 2 distinct peaks at elution volumes of 5.0 and 6.6 mL by using the eluant buffer of water containing 30% ACN. In front of these 2 peaks, the MALLS detector also displayed a shoulder near the elution volume of 4.7 mL, which was almost invisible to the UV and RI detectors because its concentration was very low.

Separation methods including liquid chromatography for CN analysis are not effective on physiological media (Bramanti et al., 2001). Lucey et al. (2000) reported that SEC of sodium caseinate using a Superose

Figure 1. High-performance size-exclusion chromatography-UV-multiangle laser light scattering-refractive index (HPSEC-UV-MALLS-RI) elution profile of bovine casein and its calculated molecular weight distribution (MWD). The UV detector was monitored at 214 nm; the MALLS response was followed at 90°; and the column temperature was 35°C. Peak (P) 1 = CN aggregates; P2 = individual CN.

6HR 10/30 column resolved the caseins into a very small peak near the void volume and 2 large peaks by using an eluant buffer of 20 mM imidazole and 50 mM NaCl at pH 7.0. The similar SEC profiles for sodium caseinate were also observed by adding SDS (Lynch et al., 1997) or urea (Lorenzen et al., 1998) to the eluant buffers.

As shown in Figure 1, the absolute molecular weights obtained from the HPSEC-MALLS-RI analysis appeared to be a typical negative plot with the elution volume. The peak at 6.6 mL (28 to 42 kDa) could be assigned to individual CN, whereas the peak at 5.0 mL (>500 kDa) could be assigned to CN aggregates. The molecular weights at the 2 peaks were approximately 30 and 850 kDa, respectively. It should be noted that the HPSEC-MALLS-RI analysis was carried out in the eluant buffer containing 30% ACN, which might cause more aggregation than the aqueous medium during CN hydrolysis. In fact, the weight-average molecular weight found here for the control CN was 1.35 million compared with 110,000 reported by Farrell et al. (2006). Therefore, the HPSEC-MALLS-RI result indicated that there might be a dynamic system between monomers and aggregates, and thus supported the viewpoint of Walstra (1999) that CNs were a type of equilibrium system.

Pepsin-Induced Changes in the Size and Molecular Weight of CN Aggregates

Peptic hydrolysis of bovine CN induced growth in aggregate size (Figure 2). Figure 2A depicts the

Figure 2. High-performance size-exclusion chromatography-multiple angle laser light scattering (MALLS) profiles of bovine casein undergoing hydrolysis with pepsin for a) 0 (control), b) 5, c) 10, d) 30, and e) 120 min at pH 2 and 37°C. The UV detector was monitored at 214 nm (panel A) and the MALLS response was followed at 90° (panel B). The column temperature was 35°C; the UV and MALLS responses are in arbitrary units (AU). The resulting peptides in peptic hydrolysates could be separated into 5 major peaks with different molecular weights: 1 = 20.0 to 7.0 kDa; 2 = 7.0 to 3.0 kDa; 3 = 3.0 to 1.0 kDa; 4 = 1.0 to 0.4 kDa; 5 = <0.4 kDa.

HPSEC-UV elution profiles of bovine CN peptic hydrolysates. Bovine CN was fragmented into different peptides during hydrolysis. As the reaction proceeded, CN aggregates underwent a reduction in their peak areas, together with a decrease in elution volumes. In other words, the longer the hydrolysis time, the lower the concentrations (C) of CN aggregates, but the larger their sizes and molecular weights (M). Therefore, the value of $M_w \cdot C$ might display a fluctuating trend upon peptic hydrolysis. As shown in Figure 2B, the strength of the MALLS signal was fluctuating during the course

of hydrolysis, because it was proportional to $M_w \cdot C$ (Wyatt, 1993). A small number of large micelles in 30-min hydrolysates gave the largest response by the MALLS detector. After 2 h of hydrolysis, the MALLS system still detected large aggregates that eluted close to the void volume. But their amount was almost invisible, as measured by the UV detector, indicating that very few CN aggregates survived. Because the UV detector was more sensitive to small molecules than the MALLS detector, the resulting peptides were detected only in the UV chromatograms.

By using HPSEC-MALLS-RI, the MWD of CN aggregates upon hydrolysis was calculated, as displayed in Figure 3. The size and molecular weight of CN aggregates increased gradually with hydrolysis time. After 30 min of hydrolysis, the elution volume of the aggregates (4.76 mL) was much lower than that of the original aggregates (5.03 mL). A summary of the M_n , M_w , second virial coefficient (A_2) and the z-average radius of gyration ($\langle R_g \rangle_z$) values of the aggregates after different hydrolysis times is shown in Table 1. The aggregates formed after 5 min of hydrolysis displayed a M_w of 8.05×10^6 Da, whereas those formed at 30 min of hydrolysis showed a larger M_w (4.63×10^7 Da). Upon peptic hydrolysis, a dramatic increase in the $\langle R_g \rangle_z$ of CN aggregates was also detected, from 31.4 to 165.5 nm, which indicated pepsin-induced aggregation. During the course of hydrolysis, the second virial coefficients (A_2) of CN aggregates were all positive, which suggested that the protein-protein interactions were repulsive (Liu et al., 2005). After peptic hydrolysis of bovine CN, however, A_2 became less positive, indicating that the electrostatic repulsion was gradually reduced. Such screened repulsion might be correlated to the simultaneous increased size and molecular weight of the aggregates. Similar behavior has been reported for CN micelles hydrolyzed by plasmin (Crudden et al., 2005). The decrease in intermicellar repulsion was thought to be responsible for the formation of aggregates.

DLS Analysis of Pepsin-Induced Changes in the Size of CN Aggregates

Changes in the aggregate size of bovine CN upon peptic hydrolysis were further studied by DLS. Figure 4 shows the reaction-time dependence of R_h and the light scattering intensity (I_s). The latter is approximately proportional to both the concentration and the weight-average molecular weight. During the initial stage of the reaction (0 to 30 min), I_s increased gradually, accompanied by a significant growth in R_h from about 67 to 79 nm, suggesting the aggregation of CN upon peptic hydrolysis. In the latter stage (30 to 120 min), I_s dramatically decreased, which indicated a sig-

Figure 3. Molecular weight distributions (■) and refractive index (—; arbitrary units) of casein aggregates after hydrolysis with pepsin for (panel A) 0 (control), (panel B) 5, (panel C) 10, and (panel D) 30 min.

nificant reduction in the content of aggregates; however, values of R_h continued to increase gradually. Finally, the aggregate size and the scattering intensity became constant in the period from 120 to 180 min.

The increase in aggregate size might be elucidated in terms of changes in molecular structure of bovine CN upon peptic digestion. In the submicelle and dual-binding models for CN aggregate structures (Horne, 2006), hydrophobic interactions are believed to play an important role in the formation of micelles by individual

Figure 4. Average hydrodynamic radius (R_h , ●) and light scattering intensity (I_s , □ in kilo-counts per second) of bovine casein peptic hydrolysates as a function of reaction time. Bovine casein was digested with pepsin at 37°C; the DLS measurements were performed at 90°. The lines are to guide the eye; the arrows denote the corresponding coordinates for $\langle R_h \rangle$ and I_s .

CN. It is thought that CN aggregates have hydrophobic cores and hydrophilic surfaces, and that the outer surface of the micelle is quite diffuse (Chu et al., 1995; Kumosinski et al., 1996). As is well known, pepsin is an enzyme that preferentially hydrolyzes hydrophobic peptide bonds. During the process of hydrolysis, pepsin might cleave the peptide bonds inside the aggregates, inducing exposure of the hydrophobic cores. By assembling the exposed aggregates, large particles could be formed due to hydrophobic attractions, together with the screened intermolecular repulsion.

MWD of Bovine CN Peptic Hydrolysates

As displayed in Figure 2B, the MALLS intensity of the resulting peptides was too low to calculate their molecular weight with sufficient accuracy. Therefore, the HPSEC system should first be calibrated if the molecular weight characterization of peptic hydrolysates is required. We selected the columns in this study to separate the standard proteins and CN aggregates according to their hydrodynamic size. The relationship between elution volume (V_e) and $\lg(M)$ (the logarithm

Table 1. Number- (M_n) and weight-average (M_w) molecular weights, second virial coefficient (A_2), and z-average radius of gyration ($\langle R_g \rangle_z$) for casein aggregates after different hydrolysis times (mean $\pm$ SD)

Time (min)	M_n (g/mol $\times 10^6$)	M_w (g/mol $\times 10^6$)	M_w/M_n	A_2 (mL·mol/g ² $\times 10^{-2}$)	$\langle R_g \rangle_z$ (nm)
0	0.95 $\pm$ 0.04	1.35 $\pm$ 0.05	1.42	2.04 $\pm$ 0.06	31.4 $\pm$ 0.6
5	6.53 $\pm$ 0.23	8.05 $\pm$ 0.26	1.23	0.56 $\pm$ 0.03	73.7 $\pm$ 2.4
10	8.84 $\pm$ 0.22	10.7 $\pm$ 0.25	1.21	0.32 $\pm$ 0.02	81.2 $\pm$ 2.9
30	29.2 $\pm$ 0.45	46.3 $\pm$ 0.59	1.59	0.28 $\pm$ 0.02	165.5 $\pm$ 4.5

Figure 5. The calibration curves of molecular weight (M) and elution volume (V_e) by using the standard proteins ($\square$) and the casein aggregates ($\bullet$) after different hydrolysis times.

of molecular weight) is shown in Figure 5. At $V_e \geq 5.5$ mL, the calibration curve was $\lg(M) = 8.318 - 0.5826 V_e$ (V_e in mL, M in g/mol). At $V_e < 5.5$ mL, the SEC columns were calibrated with the V_e and M of CN aggregates, as determined by HPSEC-MALLS-RI at their peak positions. The value of $\lg(M)$ decreased exponentially with the increasing elution volume at $V_e < 5.5$ mL, because the large molecules would elute near the void volume of the columns.

By applying the V_e - $\lg(M)$ relationship, we could estimate the molecular weight distribution of bovine CN peptic hydrolysates. Due to the relative specificity of pepsin (C-terminal cleavage at F, L, and E), the peptide chains of individual CN were cleaved into 5 major fragments including macropeptides (20.0 to 7.0 kDa, peak 1; 7.0 to 3.0 kDa, peak 2), polypeptides (3.0 to 1.0 kDa, peak 3; 1.0 to 0.4 kDa, peak 4) and oligopeptides (<0.4 kDa, peak 5), as shown in Figure 2A. These fractions all appeared in the initial stage of hydrolysis and their concentrations increased as the reaction proceeded.

It should be noted that the HPSEC-UV elution profiles in Figure 2A were recorded at 214 nm, a wavelength generally used for the detection of proteins and peptides. Absorption at this wavelength was mainly caused by peptide bonds, which implied that the amount of small peptides is underestimated. However, it was difficult to use a correction factor, because the exact composition of each molecular weight fraction was not known. Therefore, the peak area of UV chromatograms at 214 nm was applied to examine the changes in the protein/peptide concentration in spite of its imperfection.

Figure 6 shows the kinetic appearance of 5 molecular weight fractions during peptic hydrolysis of bovine CN.

Figure 6. Changes in the mass percentages of different protein and peptide fractions, based on molecular weight intervals of the high-performance size-exclusion chromatography profiles, during peptic hydrolysis of bovine casein. The lines are to guide the eye.

It can be seen from Figure 6 that 1) the reductions in both CN micelles and monomers were rapid in the initial stage of hydrolysis ($DH < 5\%$), but slower subsequently. The original substrates were almost disappeared at the DH value of 9.5%; 2) the macropeptides (20.0 to 3.0 kDa, peaks 1 and 2 in Figure 2A), which might include various antimicrobial peptides, showed rapid formation at $DH < 2\%$ and then a slower increase. Above 2%, the intermediate fraction would be still generated and converted to the smaller peptides; 3) the polypeptides (3.0 to 0.4 kDa, peaks 3 and 4 in Figure 2A) might be a rich source of antihypertensive peptides, and they displayed a similar trend in the contents as the macropeptides. However, the mass difference between polypeptides and macropeptides was gradually reduced, which suggested that the latter might be more susceptible to pepsin; 4) the oligopeptides (<0.4 kDa, peak 5 in Figure 2A) should be mainly composed of di- and tripeptides, which possess a high intestinal absorption rate. The concentration of this fraction, which might be underestimated due to its lower absorbance at 214 nm, increased continuously during the course of hydrolysis. A similar trend for the HPSEC profiles of the hydrolysates was previously observed in the case of bovine CN digested with trypsin (Qi et al., 2003).

The most remarkable phenomenon, as depicted by HPSEC-UV chromatograms in Figure 2A, was that the concentration ratio of CN aggregates to individual CN was almost constant at approximately 1 (2:2.5) during the initial 30-min hydrolysis. This distribution of aggregates and monomers again suggested that the CN in

the solutions exist as a dynamic system of CN aggregates and monomers.

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