Caseinomacropeptide Self-Association is Dependent on Whether the Peptide is Free or Restricted in κ -Casein

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ABSTRACT

There is a general agreement that the experimentally determined molecular weight (MW) of caseinomacropeptide (CMP) is greater than the theoretical MW. Some studies suggest that this is due to a pH-dependent aggregation of monomeric CMP. How this aggregation is influenced by pH is not understood. This study was carried out to study the nature of CMP aggregates and to clarify which conditions affect aggregation of CMP. The apparent MW of CMP at different pH values was determined using size-exclusion chromatography. Caseinomacropeptide was further characterized by immunochemical analysis, sodium dodecyl sulfate-PAGE, N-terminal sequencing, and mass spectrometry. The hydrophobicity of CMP was studied by means of 1-anilino-naphthalene-8-sulfonic acid binding experiments. Four CMP products prepared by different methods were studied: CMP produced by enzymatic (chymosin or pepsin) hydrolysis of κ -casein (CN), and 2 commercial CMP products. Both commercial products and CMP resulting from chymosin-hydrolysis of κ -CN (at pH 6.6) had elution volumes with a MW corresponding to 35 kDa at pH 8.0 and 3.4. Caseinomacropeptide prepared from pepsin-hydrolysis of κ -CN (at pH 2.5) eluted as multiple peaks with apparent MW of 35, 18, and 9 kDa, again independently of pH. Hydrolysis of κ -CN with chymosin or pepsin at different pH values (pH 2.5, 3.4, and 6.6) produced differently sized aggregates of CMP, largely depending on the pH of the hydrolysis. These results indicate that, whereas CMP molecules are irreversibly associated, CMP in κ -CN may associate reversibly in a pH-dependent manner. We suggest that interactions between para- κ -CN parts of the κ -CN molecules may be a requisite for the pH-dependent dissociation/association.

(**Key words:** caseinomacropeptide, molecular weight, aggregation, κ -casein)

Abbreviation key: ANS = 1-anilino-naphthalene-8-sulfonic acid, **CMP** = caseinomacropeptide, **MS** = mass spectrometry, **MW** = molecular weight, **SEC** = size-exclusion chromatography.

INTRODUCTION

Caseinomacropeptide (**CMP**) is released after specific cleavage of κ -CN by chymosin (Farrell et al., 2004) and pepsin (Delfour et al., 1965) at the Phe105-Met106 peptide bond. Caseinomacropeptide comprises the 64 amino acids in the hydrophilic C-terminal portion of κ -CN, and contains all the posttranslational modifications (glycosylation and phosphorylation) present in κ -CN that contribute to its marked heterogeneity. An increasing number of studies show that CMP may exert important biological activities (Abd El-Salam et al., 1996), and this explains the growing interest in CMP as an ingredient in dietetic food and pharmaceutical products. Much attention has been given to developing techniques for the isolation and purification of CMP, but a better understanding of the physicochemical properties of CMP is important to optimize isolation methods.

The sequence-derived molecular weight of CMP (genetic variant A) is 6.707 kDa (Jolles et al., 1972); however, most reports indicate much higher values for experimentally measured molecular weight (**MW**). About 40% of CMP is not glycosylated (Vreeman et al., 1986), and it is therefore not conceivable that the attached carbohydrate chains can account for the higher MW. Previous studies suggest that CMP forms aggregates, and that aggregation occurs at pH values above 4.5. Accordingly, methods for industrial scale preparation by ultrafiltration of CMP that rely on this phenomenon have been developed (Tanimoto et al., 1992; Holst and Chatterton, 2002). This apparent pH-dependent aggregation, however, is still debated (Brody, 2000), and in some studies, pH-dependent changes in the MW of CMP

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have not been observed (Minkiewicz et al., 1996; Nakano and Ozimek, 1998). In fact, some studies argue that the larger apparent MW is not caused by aggregation but can be attributed to a large hydrodynamic volume (Minkiewicz et al., 1996; Wang and Lucey, 2003).

The aim of the present study was to investigate the nature of CMP concerning MW as well as to identify the conditions that may affect aggregation. We investigated the effect of pH on the apparent MW of CMP, as well as the conditions under which CMP forms aggregates. A better knowledge of the physicochemical properties of CMP is essential to optimize production and isolation methods for CMP and provide a basis for understanding the biological activities of CMP.

Four CMP products prepared by different methods were studied: 2 commercial products (Sigma and Arla), and 2 CMP products produced in our laboratory by enzymatic hydrolysis of κ -CN with chymosin (at pH 6.6) or pepsin (at pH 2.5). According to the manufacturer, CMP from Sigma (Sigma Chemical Co., St. Louis, MO) was produced by precipitation of rennin-treated whole casein with 5% (wt/vol) TCA. The product commercialized by Arla (Arla Foods Amba, Viby, Denmark) was isolated from sweet whey by several ultrafiltration steps (Holst and Chatterton, 2002).

MATERIALS AND METHODS

Protein Material

Recombinant chymosin (CHY-MAX Plus, 205 international milk clotting units/mL, 0.92 mg/mL, EC 3.4.23.4) was a gift from Chr. Hansen, Hørsholm, Denmark; pepsin (EC 3.4.23.1), CMP, and κ -casein were from Sigma; and CMP (CCMP-10) was from Arla Foods Amba. The MW standards used for size-exclusion chromatography (SEC) were BSA (Sigma), ovalbumin (Sigma), carbonic anhydrase (Sigma), α -chymotrypsinogen A (Sigma), bovine pancreatic trypsin inhibitor (D-68298, Boehringer Mannheim, Mannheim, Germany). The set of MW markers used in SDS-PAGE was Mark 12 (LC-5677, Invitrogen, San Diego, CA). Water was drawn from a Milli-Q system equipped with an Organex cartridge (Millipore, Bedford, MA).

Chymosin Hydrolysis of κ -CN

A 10 mg/mL solution of κ -CN was prepared in 10 mM sodium phosphate buffer (pH 6.6) to which chymosin [0.02 international milk clotting units/mL; 1:1000 (wt/wt)] was added. A control containing no chymosin (designated chymosin-control) was prepared simultaneously. After 1 h at 37°C, the pH was adjusted to 9.0 with 1.0 M NaOH. After centrifugation (20 min at 12,000 $\times$ g, room temperature), the precipitate was discarded,

and the protein concentration in the supernatant was determined by amino acid analysis (Barkholt and Jensen, 1989). The supernatant was stored at -20°C until analysis. For chymosin hydrolysis at pH 2.5 and 3.4, the pH of a 10 mg/mL solution of κ -CN prepared in 10 mM NH_4HCO_3 was adjusted to the respective pH with 1 M HCl before hydrolysis, which was performed as described above.

Pepsin Hydrolysis of κ -CN

A 10 mg/mL solution of κ -CN was prepared in 10 mM NH_4HCO_3 . The pH was adjusted to pH 2.5 with 6 M HCl, and pepsin (1:1000, wt/wt) was added. A control containing no pepsin (designated pepsin-control) was prepared simultaneously. After 1 h at 37°C, the pH was adjusted to 7.4 with 1 M NH_4HCO_3 , and the sample was centrifuged (20 min at 12,000 $\times$ g, room temperature). The precipitate was discarded and the protein concentration in the supernatant was determined by amino acid analysis (Barkholt and Jensen, 1989). The supernatant was stored at -20°C until analysis. For pepsin-hydrolysis at pH 3.4 and 6.6, the pH of a 10 mg/mL solution of κ -CN prepared in 10 mM NH_4HCO_3 was adjusted to the respective pH with 1 M HCl before hydrolysis, which was performed as described above.

Size-Exclusion Chromatography

Size-exclusion chromatography was performed on a Superdex 75 PC 3.2/30 column mounted on a SMART system (Pharmacia Biotech AB, Uppsala, Sweden). Chromatography was carried out at room temperature at pH 8.0 (150 mM NH_4HCO_3) or at pH 3.4 (25 mM CH_3COOH , 125 mM NaCl), at a flow rate of 50 $\mu\text{L}/\text{min}$. The run time for each experiment was 75 min. Elution profiles were monitored at 280 and 220 nm. Peaks containing CMP (that does not contain tryptophan or tyrosine) generally showed very little if any absorbance at 280 nm. Standards used for column calibration were BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), α -chymotrypsinogen A (23 kDa), and bovine pancreatic trypsin inhibitor (6.5 kDa). Fifteen microliters of the sample (1 mg of protein/mL) was applied to the column. For runs performed at pH 3.4, all samples were adjusted to pH 3.4 with 2.0 M acetic acid and incubated for 2 h at room temperature before loading on the column. No prior incubation was carried out for runs at pH 8.0. Insoluble material in all samples was removed by quick centrifugation (1 min at 12,000 $\times$ g) before loading on the column.

To collect larger amounts of protein eluate for further analysis, preparative SEC was carried out at pH 3.4 (5 mM CH_3COOH , 125 mM NaCl) on a Superdex 75 col-

umn (Hiload 26/60, AP Biotech, Uppsala, Sweden) operating at a flow rate of 2 mL/min. Six milliliters of sample (3 mg/mL) was applied to the column, and 2-mL fractions were collected.

ELISA

Microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.5 µg/mL CMP (Sigma) in carbonate buffer (50 mM, pH 9.6). After washing, the CMP samples were added (50 µg/mL, 50 µL) together with monoclonal anti-CMP antibody (50 µg/mL, 50 µL) that was produced in our laboratory and demonstrated to bind specifically to κ -CN and CMP. Fractions collected from SEC were serially diluted in PBS containing 0.1% Triton X-100, pH 7.4. The plates were incubated for 1 h at room temperature. After washing, plates were incubated for 1 h with horseradish peroxidase-conjugated rabbit antimouse antibody (DAKO A/S, Denmark; 1:1000 in PBS with 0.1% Triton X-100). Plates were developed by adding 100 µL/well of substrate solution containing 3,3',5,5'-tetramethylbenzidine (Merck, Darmstadt, Germany) in hydrogen peroxide. The reaction was stopped after 10 min by adding 100 µL/well of 2 M phosphoric acid. Optical density was measured at 450 nm with 630 nm as reference. Caseinomacropeptide from Arla was used as a standard for assessing the CMP concentration in each fraction.

SDS-PAGE

Sodium dodecyl sulfate-PAGE of CMP products was carried out using precast Tricine porosity gradient gels (10 to 20% monomer, EC6625; Novex, Invitrogen, Groningen, The Netherlands). Samples were mixed 1:1 with sample buffer (0.1 M Tris-base, 8% (wt/vol) SDS, 24% (vol/vol) glycerol, 0.025% (wt/vol) Coomassie, 0.04 M dithiothreitol; adjusted to pH 6.8), and boiled for 5 min (Schagger and von Jagow, 1987). Sample lanes were loaded with 10 to 20 µg of protein, or with 2 µL of a MW standard solution. Electrophoresis was carried out for 80 min at constant voltage (125 V). Gels were fixed and silver stained according to published procedures (Blum et al., 1987).

Protein Sequencing

Protein sequencing was carried out by automated α -amino terminal Edman degradation in a Procise 494 sequenator according to the recommendations of the manufacturer (Applied Biosystems/Perkin Elmer, Foster City, CA). At least 3 residues were sequenced for each determination.

Mass Spectrometry

Before mass spectrometry (MS), samples previously lyophilized and redissolved in 0.1% (vol/vol) aqueous trifluoroacetic acid were purified from nonprotein components using Zip-Tip C18 prepacked microcolumns (Millipore) from which proteins were eluted in 40% (vol/vol) aqueous acetonitrile containing 0.1% trifluoroacetic acid. Mass spectrometry analyses were performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (PerSeptive Biosystem, Framingham, MA) equipped with an N₂-laser (337 nm, 3 ns pulse width, 20 Hz repetition rate). The instrument operated in positive linear ion mode with an accelerating voltage of 25 kV and for each mass spectrum, 250 laser pulses were acquired. As matrix, both sinapinic acid (3,5 dimethoxy-4-hydroxy-cinnamic acid) and α -cyano-4-hydroxy-cinnamic acid, prepared by dissolving 10 mg of crystalline powder in 1 mL of aqueous 50% acetonitrile containing 0.1% trifluoroacetic acid were used. External mass calibration was performed by a separated analysis of a mixture of standard proteins. Mass spectra were elaborated using Data Explorer software furnished with the MS equipment.

1-Anilinonaphthalene-8-Sulfonic Acid Binding Experiments

Protein surface hydrophobicity in the various samples was assessed by spectrofluorimetric titration with 1-anilinonaphthalene-8-sulfonic acid (ANS), as reported elsewhere (Iametti et al., 1996, 1998). In short, emission fluorescence spectra were recorded from 450 to 550 nm with excitation at 390 nm (2.5 nm band width) on mixtures containing 80 µg of sample protein in 1 mL of the appropriate buffer and increasing concentrations of ANS, added as very small volumes from 2 or 20 mM stock solutions in water. Binding curves were analyzed according to standard procedures (Bonomi et al., 1988; Pagliarini et al., 1990).

RESULTS

MW of CMP is Independent of pH

The effect of pH on the apparent MW of 4 different CMP products was analyzed by SEC at pH 8.0 and pH 3.4 (Figure 1). Caseinomacropeptide from chymosin-hydrolyzed κ -CN as well as commercial CMP products (Sigma and Arla; Figure 1, panels A to C) eluted at both pH values, as a single, broad peak with a mean MW of approximately 35 kDa.

The elution profile of pepsin-hydrolyzed κ -CN (Figure 1D) however, differed from that of the other 3 products. At pH 8.0, this preparation resolved into 4 peaks with

Figure 1. Elution profiles of caseinomacropeptide (CMP) after size-exclusion chromatography at pH 8.0 and 3.4. A) CMP from Sigma, B) CMP from Arla, C) chymosin-hydrolyzed (pH 6.6) κ -casein, D) pepsin-hydrolyzed (pH 2.5) κ -casein. Sample size = 25 μ L (3 mg/mL); AU = absorbance units; vertical dashed line represents MW = 35 kDa.

MW >70, 35, 18, and 9 kDa. The largest fraction was absent when the separation was carried out at pH 3.4. The peak with MW >70 kDa had only little absorbance at 280 nm; hence, it may represent a high-MW aggregate of CMP. At both pH values, a peak with MW <6

kDa was observed, which might be related to para κ -CN-derived peptides.

Given that CMP has a sequence-calculated mass of 6.707 kDa, our SEC data clearly illustrate that both at pH 8.0 and 3.4, CMP migrates as a mixture of polymers

Figure 2. Inhibition curves from competitive ELISA. Plates were coated with 0.5 µg/mL CMP (Sigma) and incubated with anti-CMP antibody and CMP or κ -casein as competitor. ■ = pepsin hydrolyzed (pH 2.5) κ -casein, ● = chymosin hydrolyzed (pH 6.6) κ -casein, ▼ = CMP from Arla, ◆ = CMP from Sigma, □ = pepsin-control, ○ = chymosin-control, ▽ = κ -casein. AU = Absorbance units.

and that the apparent aggregation of CMP is not solely influenced by the actual pH. In fact, incubation of CMP (Arla) in 0.1 M HCl at 80°C (1 h) or at pH 3.4 (4°C at 20 h) did not result in dissociation of CMP into lower MW forms (results not shown).

As the mean theoretical MW of CMP is approximately 7 kDa, the peaks observed in the SEC corresponding to 35, 18, and 9 kDa may represent tetrameric, dimeric, and monomeric forms of CMP, respectively.

Immunochemical Analyses of Enzymatic Hydrolysis Products

The immunochemical reactivity of the 4 CMP products was compared in a competitive ELISA, based on inhibition of binding of monoclonal anti-CMP antibodies to immobilized CMP. The resulting inhibition curves (Figure 2), which are representative of curves obtained using 3 different monoclonal antibodies toward CMP, showed that hydrolyzed κ -CN (from either chymosin or pepsin hydrolysis), as well as both commercial CMP products, were equally efficient at inhibiting antibody binding, indicating immunochemically identical structures of all the CMP products.

The data in Figure 2 also provide evidence of a large difference in the inhibitory capacities of intact κ -CN vs. CMP, with a more than 10-fold higher inhibitory concentration for CMP compared with intact κ -CN. In other words, CMP-specific antibodies recognize CMP-specific epitopes in intact κ -CN much better than in isolated (but heteropolymeric) CMP. These data also

confirm that κ -CN was completely hydrolyzed by both enzymes as shown by the SEC tracings in Figure 1, panels C and D. Intact κ -CN eluted in the void volume in separate SEC runs (results not shown).

CMP is Present in SEC Peaks with MW Corresponding to 35, 18, and 9 kDa

Sodium dodecyl sulfate-PAGE, competitive ELISA, protein sequencing, and MS were used to confirm that CMP was present in the SEC fractions corresponding to 35, 18, and 9 kDa as collected from chymosin- and pepsin-hydrolyzed κ -CN. Elution profiles from a preparative column (Superdex 75 column; Hiload 26/60, AP Biotech) were similar to those obtained from the analytical column (Superdex 75 PC 3.2/30) presented in Figure 1.

Analysis of pepsin-hydrolyzed κ -CN (pH 2.5) by SDS-PAGE, followed by silver-staining of the gels, showed protein bands of approximately 18 kDa (lanes 1 to 4, Figure 3B) in fractions corresponding to a MW of both 35 kDa (fractions 1 to 3, Figure 3A) and 18 kDa (fraction 4, Figure 3A). The 9-kDa peak (fraction 5) contained protein bands with MW of 7,000 and lower (lane 5, Figure 3B). Caseinomacropeptide from Arla and Sigma, as well as chymosin-hydrolyzed κ -CN, were visible as a broad smear with somewhat resolved bands having MW around 35, 18, and 10 kDa (lanes C_A, C_S, and C, Figure 3B). A faint band at 7 kDa is also visible in the Arla product (lane C_A, Figure 3B).

Caseinomacropeptide could be detected by competitive ELISA in the 35, 18, and 9 kDa fractions (Figure 3A). It was not possible to detect any CMP in peaks with a MW lower than 9 kDa (fractions 6 and 7) that are therefore likely to exclusively represent peptides derived from further degradation of either para κ -CN or CMP. Protein sequence analysis demonstrated that the main sequence in the 35, 18, and 9 kDa fractions corresponded to the N-terminal sequence of CMP (Table 1). Note that peptides originating from the N-terminal region of κ -CN would not be detected by Edman degradation, as the pyroglutamic residue is impervious to this reaction. Up to 22 residues of the samples corresponding to the 18- and 9-kDa peaks were sequenced to confirm the CMP sequence. In the 9-kDa fraction, other peptides were also detected, with N-terminals representing Ala138 and Ser80 of κ -CN, respectively.

Mass spectrometry analysis demonstrated that the main component in the CMP product from Arla was the monophosphorylated monomeric form of CMP (both genetic variants A and B; data not shown). The monomeric form was also detected in the 35 kDa SEC peaks of chymosin- and pepsin-hydrolyzed κ -CN (Table 1, fraction 3). Moreover, MS detected a species with a

Figure 3. A) Elution profiles from size-exclusion chromatography (SEC) at pH 3.4 of pepsin-hydrolyzed κ -casein. The numbers indicate collected fractions. Gray bars indicate CMP content in fractions ($\mu\text{g/mL}$) determined by ELISA. B) Silver-stained SDS-PAGE gels of fractions collected from SEC after pepsin-hydrolysis of κ -casein. St = Molecular weight standard, C_A = CMP from Arla; C_S = CMP from Sigma; C = chymosin-hydrolyzed κ -casein; lanes 1 to 7 = fractions 1 to 7 (as indicated by arrows in Figure 3A).

mass corresponding to that of the monomeric CMP in the 9-kDa SEC peak of pepsin-hydrolyzed κ -CN (Table 1, fraction 5). Consistent with the results of SDS-PAGE, matrix-assisted laser desorption ionization time-of-

flight MS analysis indicated the absence of covalently linked tetrameric CMP in the various SEC fractions.

The results described above demonstrate that CMP is present in the 35, 18, and 9 kDa SEC peaks, thus

Table 1. Characterization of fractions from size-exclusion chromatography.

Fraction no. ¹	Peptide sequence ²	Position in κ -casein	MW, ³ kDa	MW, ⁴ Da
3	MAI	Met106	35	6756, 6788, 6985, 7151
4	MAI	Met106	18	ND ⁵
5	MAI, AVE, SNT	Met106, Ala138, Ser80	9	3943, 6783, 9787
7	VQV	Val162	<7	ND

¹Size-exclusion chromatography fractions as shown in Figure 3A.

²Major species only.

³Mean apparent molecular weight (MW) determined by size-exclusion chromatography.

⁴Determined by mass spectrometry, major species only.

⁵ND = Not determined.

indicating that CMP can exist as a tetramer, dimer, and monomer. The dimeric and monomeric forms of CMP were seen when κ -CN was cleaved by pepsin, but not with chymosin. A major difference in these enzymatic cleavages is the pH at which they were carried out: pH 2.5 for pepsin-hydrolysis compared with pH 6.6 for chymosin-hydrolysis.

pH Influences Interactions Between CMP Units in κ -CN Molecules Before and During Hydrolysis

A series of experiments were performed where the aim was to study the effect of pH on the association of CMP in the κ -CN molecule, focusing on the peptide—peptide interactions at the very moment in which CMP is released by enzymatic hydrolysis. For this purpose, the aggregation state of CMP products resulting from chymosin- or pepsin-hydrolysis of κ -CN at pH 2.5, 3.4, and 6.6 was investigated by SEC. In all experiments, κ -CN was incubated at pH 2.5 before hydrolysis at different pH values.

Hydrolysis of κ -CN at pH 6.6 with either pepsin or chymosin resulted in a CMP product that eluted as a single peak with a MW of 35 kDa (Figure 4, panels A and B). However, when chymosin- or pepsin-hydrolysis was carried out at either pH 3.4 or 2.5, the resulting products also contained lower MW components (Figure 4, panels A and B), including peaks corresponding to dimeric and monomeric CMP. Front peaks containing nonhydrolyzed κ -CN were seen for pepsin-hydrolysis of κ -CN at pH 3.4 and 6.6. Long-term (up to 24 h) incubation of κ -CN at pH 2.5 before hydrolysis did not affect the distribution of CMP product obtained by subsequent enzymatic hydrolysis (result not shown).

These results demonstrate that the pH at which proteolysis of κ -CN took place influenced the form and consequently the MW of the resulting CMP product. Thus, the CMP parts of κ -CN molecules appear to associate and dissociate depending on pH, and the degree of association of the CMP molecules will depend on the

state of aggregation of the κ -CN molecule at the time of hydrolysis.

The Tetrameric Form of CMP is More Hydrophobic than the Dimeric and Monomeric Forms

Given the absence of covalent bonds in CMP aggregates, and their insensitivity to pH in SEC separations, strong interaction through hydrophobic contacts can be hypothesized to hold together polymeric forms of CMP. The surface hydrophobicity of various CMP aggregates isolated by SEC were studied by measuring the binding of ANS, a hydrophobic fluorescent probe that preferably binds to hydrophobic surfaces of proteins (Matulis and Lovrien, 1998). Titration studies with ANS demonstrated that tetrameric forms of CMP from SEC fractions were significantly more hydrophobic than the dimeric and monomeric forms of CMP isolated by SEC (Figure 5).

Size-exclusion chromatography fractions containing monomeric CMP showed a limited number of surface hydrophobic sites (inferred from fluorescence values at saturating ANS (Iametti et al., 1996, 1998), and this parameter only increased slightly upon dimerization. However, tetrameric CMP forms isolated by SEC showed a 3-fold increase in the number of ANS-accessible surface hydrophobic sites relative to dimeric CMP, a behavior that has been associated with the establishment of new hydrophobic sites upon contact between specific protein regions (Iametti et al., 1998). Figure 5 also makes it clear that the establishment of these interprotein connections does not involve major structural rearrangements, as it does not modify the apparent affinity of the various forms of CMP for the probe, as indicated by the slopes of lines in the Scatchard plots (Figure 5C), reflecting the apparent K_d for dissociation of the ANS-protein complex (Pagliarini et al., 1990; Iametti et al., 1996).

Figure 4. Effect of A) chymosin- and B) pepsin-hydrolysis of κ -casein at pH 6.6, 3.4, and 2.5. Elution profiles after size-exclusion chromatography at pH 3.4 are illustrated [sample size = 25 mL (3 mg/mL)].

DISCUSSION

The greater than theoretical MW often reported in the literature for CMP is thought to be caused by a large hydrodynamic volume or by association of monomers to form aggregates. The results presented in this study support the latter hypothesis. These aggregates do not associate and dissociate in a pH-dependent manner. Moreover, pH-dependent interactions between CMP molecules seem to take place within the κ -CN micelles, and we suggest that these interactions will influence the resulting CMP product arising from enzymatic cleavage. Overall, the results indicate that CMP units in κ -CN appear to associate reversibly depending on pH, whereas free CMP monomers do not tend to associate once they have been formed.

In the present study, we investigated the effect of pH (pH 3.4 and 8.0) on the MW of 4 CMP products by SEC. We found that independently of pH, CMP had a greater than expected MW although a lower MW form of CMP, corresponding to a monomeric form, was produced together with a dimeric form by pepsin- and chymosin-hydrolysis of κ -CN at pH 2.5. Competitive ELISA, protein sequencing, and MS confirmed that CMP was present in the peaks corresponding to dimeric and monomeric CMP forms. Secondary hydrolysis products, which have also been reported by Shammiet et al. (1992), were identified in the monomeric peak. Although secondary hydrolysis products add to the complexity of the multippeak elution profiles, the results presented for pepsin hydrolysis at pH 2.5 are in accordance with the presence of nonhydrolyzed monomeric CMP.

The formation of aggregates has been reported in a study similar to the current study (Nakano and Ozimek, 1998), in which it was concluded that CMP could aggregate to form trimers having a MW of 36 kDa. Different sized forms of CMP have also been reported in studies where ultrafiltration was used to isolate CMP. Aggregates of different sizes of CMP were found, the majority having a MW ranging from 30 to 50 kDa, but also some having a MW >50 kDa at pH 4.6 (Kawasaki et al., 1993; Chu et al., 1996).

The lack of pH-dependent change in MW of free CMP seen in the present study is in agreement with previous studies (Minkiewicz et al., 1996; Nakano and Ozimek, 1998) although Kawasaki et al. (1993) reported that a decrease in pH resulted in a decrease in MW. This discrepancy in results could be caused by different production methods of CMP or pH-dependent interaction with SEC column material.

Sodium dodecyl sulfate-PAGE analysis revealed that CMP in both the tetrameric and dimeric fractions appeared as an 18-kDa protein band. Thus, although the tetrameric CMP product appears to be resistant to changes in pH (as described above) it can be reduced to 18 kDa by SDS-PAGE. As the dimeric form appears to be resistant to SDS-PAGE, we suggest that dimeric CMP is composed of 2 tightly associated CMP monomers. In the monomeric fraction (Figure 3B, lane 5), there is a protein band with a MW of approximately 7 kDa, which may represent monomeric CMP. This protein band is not present in fractions 6 and 7, which elute at volumes corresponding to a MW lower than 9

Figure 5. 1-Anilinonaphthalene-8-sulfonic acid (ANS) binding curves for A) tetrameric, and B) dimeric and monomeric fractions of caseinomacropепptide (CMP) from chymosin- and pepsin-hydrolyzed κ -casein; C) Scatchard plot of ANS binding to fractions of CMP: ■ = chymosin-hydrolyzed κ -casein (tetrameric fraction); ● = pepsin-hydrolyzed κ -casein (tetrameric fraction); ▼ = CMP from Arla; ◆ = pepsin-hydrolyzed κ -casein (dimeric fraction); □ = pepsin-hydrolyzed κ -casein (monomeric fraction).

kDa. Our studies showed that silver-stained gels revealed more lower MW proteins than did Coomassie-stained gels (results not shown). In relation to this, most previous studies report only the aggregated form of CMP (Coolbear et al., 1996; Chow and Harper, 2001; Nakano et al., 2002), which may be due to the use of Coomassie staining, which requires higher protein concentrations than silver staining.

The present studies on the effect of pH at the moment of κ -CN hydrolysis, demonstrated that the MW of the resulting CMP product was influenced by pH. Hydrolysis of κ -CN by either chymosin or pepsin at pH 6.6 (whether κ -CN had been preincubated at pH 2.5 or not) resulted in only the tetrameric form of CMP. Hydrolysis of κ -CN by both enzymes at pH 2.5 or 3.4, however, produced elution profiles consisting of peaks with MW corresponding to dimeric and monomeric forms of CMP as well as the tetrameric form. We propose that pH may induce a change in conformation of κ -CN that affects association of CMP units between adjacent κ -CN molecules and that this change is reversible as long as CMP is still a part of κ -CN.

The overall structure of κ -CN has been shown to be flexible (Griffin and Roberts, 1985), and a decrease in pH can cause slight changes in conformation such as increased α -helical content (Plowman et al., 1997). κ -Casein molecules form micelles (Farrell et al., 1996), in which κ -CN molecules are orientated with their hydrophobic regions (para- κ -CN segment) forming the core, and the hydrophilic CMP segment projecting outwards (Vreeman et al., 1981). Based on our findings, we suggest that the pH-induced change in the conformation of κ -CN in turn affects the association or interaction of CMP units between adjacent κ -CN molecules. A lower pH may result in a structure where CMP molecules are less closely associated, perhaps due to conformation changes in the para- κ -CN part, whereas at pH 6.6, κ -CN exists as a more tightly bound aggregate, resulting in a closer association of CMP units.

In support of the above, a recent study by Kim et al. (2005) demonstrated that in SDS-PAGE, recombinant human CMP had mobility corresponding to a MW of 7 kDa. In contrast, the mobility of natural bovine CMP corresponded to a MW of 20 kDa.

In this study, analysis of surface properties of CMP by ANS-binding experiments revealed that CMP (from Arla) and the tetrameric fraction from SEC had greater hydrophobicity than did the monomeric and dimeric fractions. These results indicate that aggregation of monomers having a relatively small hydrophobic surface form a tetrameric structure with a hydrophobic patch. As increased hydrophobicity will decrease the binding of water molecules to the protein aggregate, these results imply that hydration is not the cause of

the increased MW of CMP as previously suggested by Minkiewicz et al. (1996).

Regarding previous proposals that carbohydrate chains are involved in the greater than expected MW, we found that incubation under acidic conditions, which would cause removal of sialic acid (Schauer, 1982), did not influence the elution pattern of CMP in SEC (results not shown). Similar results have been published (Nakano and Ozimek, 1998, 2000). Furthermore, as about 40% of CMP is glycosylated (Vreeman et al., 1986), it is unlikely that the increased MW is caused by carbohydrate attachments alone.

The results presented in this study support the hypothesis that the increased MW determined for CMP is caused by aggregation of monomeric CMP forms, and is not due to attached carbohydrate chains. It should be noted however, that although the MW observed in SEC correspond well with the suggested presence of monomeric, dimeric, and tetrameric forms of CMP, it is possible that changes in the apparent MW are caused by binding of hydrophobic peptides, derived from para- κ -CN under acidic conditions, to CMP. Such peptides bound to CMP would ultimately cause a decrease in apparent MW and decrease the ANS reaction. Further studies need to be done to clarify this hypothesis.

CONCLUSIONS

This study demonstrated that the greater than theoretical apparent MW of CMP is due to aggregation of monomeric CMP. We propose that CMP aggregates consist of 4 CMP molecules and that these aggregates, once formed, are resistant to changes in pH. We suggest that the formation and conformation of the CMP aggregates are influenced by properties of the CMP units in κ -CN, and that CMP units attached to κ -CN are reversibly influenced by pH. These findings are important for gaining a better understanding of the properties of CMP, which is crucial for optimizing methods of isolation of this peptide in such a way that the bioactive properties are preserved.

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