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Implication of C-Terminal Deletion on the Structure and Stability of Bovine β-casein

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Bovine β-casein (β-CN) with its C-terminal truncated by chymosin digestion, β-CN-(*f*1-192), was examined and characterized using circular dichroism (CD) under various temperature conditions. CONTIN/LL analysis of the CD data revealed significant secondary structure disruption in β-CN-(*f*1-192) relative to its parent protein,β-CN, in the temperature range (5° to 70°C) studied. Near-UV CD spectra indicated significant temperature dependent structural changes. Analytical ultracentrifugation results showed significant reduction but not complete abolishment of self-association in β-CN-(*f*1-192) compared to whole β-casein at 2°–37°C. Furthermore, binding experiments with the common hydrophobic probe – 8-anilino-1-naphthalene sulfonic acid (ANS) illustrated that β-CN-(*f*1-192) is nearly incapable of binding to ANS relative to whole β-CN, suggesting a nearly complete open overall tertiary structure brought about by the C-terminal truncation. It has been demonstrated clearly that the tail peptide β-CN-(*f*193-209) is important in maintaining the hydrophobic core of β-CN but the residual association observed argues for a minor role for other sites as well.

KEY WORDS: β-casein; C-terminal deletion; chymosin cleavage; circular dichroism; protein structure; self-association; protein stability; conformational transition; analytical ultracentrifugation.

1. INTRODUCTION

The caseins of milk represent an unusual group of phosphoproteins that function to provide adequate supplies of bioavailable calcium, phosphate and magnesium to the neonate (Farrell, 1999; Patton, 2004). β-Casein comprises a major component of the casein complex and has been studied in some detail (Farrell, *et al.*, 2004). It has long been established (Schmidt and Payens, 1976; Andrews, *et al.*, 1979; Tai and Kegeles, 1984) that native bovine β-CN undergoes an endothermic self-association that reaches a limiting size depending on the ionic strength, and a critical micelle model (Waugh, *et al.*, 1970; Takase, *et al.*, 1980) has been applied to describe this highly concentration and temperature dependent process. It is these and other self-association reactions that lead to the formation of protein polymers, which in turn form the complex calcium and phosphate colloids necessary for casein secretion (Farrell, 1999).

In earlier studies (Herskovits, 1966; Noelken and Reibstein, 1968), β-CN was believed to exist as a complete random coil with little or no regular secondary structure under physiological conditions. The "rheomorphic" hypothesis proposed by Holt

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Abbreviations: ACS, American Chemical Society; ANS, 1-Anilino-8-Naphthalene Sulfonic Acid; CD, Circular Dichroism; ESI-MS, Electronspray Ionization Mass Spectrometry; FTIR, Fourier Transform Infrared Spectroscopy; MG, Molten Globule; MW, Molecular Weight; ORD, Optical Rotatory Dispersion; PIPES, Piperazine-N,N′-bis(2-Ethanesulfonic Acid); SDS, Sodium Dodecyl Sulfate; PPII, Left handed polyproline II helix; r.m.s.d., Root Mean Square Deviation; UV, Ultra Violet.

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and Sawyer (Holt and Sawyer, 1993) in the 90's suggested that all structures in caseins are completely flexible and form in response to flow. The latest development in understanding structure-function relationship of proteins, however, has categorized caseins as a member of the growing family of natively unfolded (Uversky, 2002b) or intrinsically unstructured proteins (Tompa, 2002). These proteins have been shown to exhibit little or no classical secondary structure, almost complete lack of globularity, low compactness, and high intramolecular flexibility under physiological conditions *in vitro*. The functional role of these proteins is particularly an evolving area of much research (Dyson and Wright, 2005). Our recent work (Qi, *et al.*, 2004; Farrell and Qi, 2005; Qi, 2005) appeared to implicate that caseins fall between the "pre-molten globule" and the "natively unfolded pre-molten globule" states described by Uversky (2002a).

Early work by Thompson *et al.* (1966) using sedimentation velocity ultracentrifugation suggested that removal of the three hydrophobic C-terminal amino acids of β-casein greatly diminished its ability to self-associate. Berry and Creamer (1975) used zonal GPC (gel permeation chromatography) to study a chymosin treated β-CN fragment 1-189. The center of the elution peak corresponded (within error) to a monomeric form of the protein, and this led them to suggest that self-association was completely abolished by this treatment. However, a recent report by Bu *et al.* (Bu, *et al.*, 2004) found that chymosin treatment of human β-casein did not completely abolish self-association and that colloidal calcium complexes were still formed. In this paper, we have reinvestigated the effect of C-terminal deletion obtained by chymosin digestion on the highly temperature dependent self-association behavior of bovine β-casein. In addition, Visser and Slangen (Visser and Slangen, 1977) suggested that Leu(192)-Tyr(193) is a more susceptible chymosin cleavage site. We used electrospray ionization mass spectrometry (ESI-MS) to establish quantitatively where this cleavage occurred.

2. MATERIALS AND METHODS

2.1. Materials

Anilino-8-naphthalenesulfonic acid (ANS) of high purity was purchased from Molecular Probes (Eugene, OR). All other reagents and chymosin used were of analytical grade or 'ACS certified' from Sigma (St. Louis, MO). Isolation and purification of β-casein were performed as described previously (Thompson, 1966).

β-CN-(*f*1-192) was prepared following initial pilot studies by HPLC using reverse phase C-18 column to optimize the digestion reaction of the parent protein (β-CN). Reaction conditions were as follows: 300 mg β-CN was dissolved in 300 ml 0.01*M* NH_4Ac. The pH of the solution was adjusted to 6.55 with acetic acid. The solution was placed into a 5°C water bath, and the temperature was monitored closely until equilibrated. 600 μl of 2.44 mg/ml chymosin (rennin) solution was then added, mixed and the sample digested for exactly one hour. 15 ml ethanol was added immediately to the solution, which was then transferred to a lyophilizing flask and freeze-dried. The freezing terminated the reaction and was done immediately after the sample was removed from the bath. The ethanol makes the final product less electrostatic and easier to work with. The genetic variant used in this work was β-casein A^1 and the larger fragment of chymosin cleaved products β-CN-(*f*1-192) as determined by electrospray ionization mass spectrometry (ESI-MS). The SDS-gel electrophoresis (Figure 1) shows purity of greater than 90%. Protein concentrations used are as follows: A & B are at 1.0 mg/ml; C, D & E are at 0.2 mg/ml.

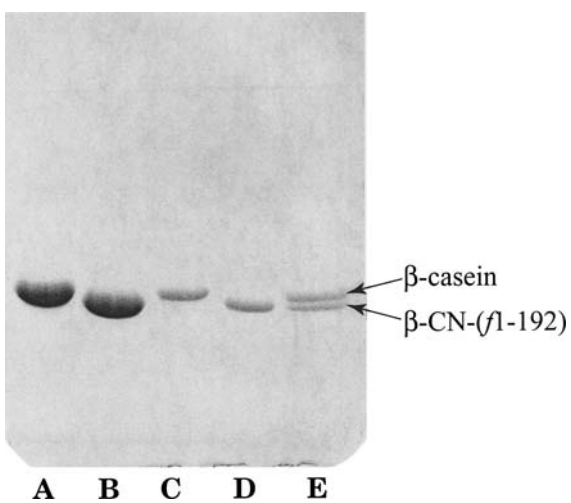


Fig. 1. SDS-PAGE gel electrophoresis of chymosin cleaved products of bovine β-CN. Lanes A & C represent native β-casein, lanes B & D represent fragment β-CN-(*f*1-192), and lane E is the digestion mixture.

2.2. Circular Dichroism (CD) Measurements

Far-UV CD experiments were carried out with 0.17 ± 0.02 mg/ml (~7.20 μ*M*) β-CN-(*f*1-192) in a 2 m*M* PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)), 4 m*M* KCl buffer (I = 7.5 m*M*) at pH 6.75 or a 33 m*M* potassium phosphate buffer at pH 6.75 (50 m*M* ionic strength). Successive measurements in the far UV (190–250 nm) were made with overlapping samples at 4°, 10°, 15°, 20°, 25°, 37°, 50°, 60° and 70°C. All solvents for CD measurements were first filtered through a Millipore 0.22 μm pore filter. Dissolved protein and peptide samples were filtered through a 0.45 μm pore regenerated cellulose filter. The extinction coefficient of β-CN-(*f*1-192) was calculated to be $\varepsilon_{280} = 0.43$ l $g^{-1}cm^{-1}$ based on Wetlaufer (Wetlaufer, 1962) from absorbance spectra obtained on a Beckman DU-7 UV-Vis spectrophotometer (Palo Alto, CA), using an extinction coefficient of $\varepsilon_{280} = 0.45$ l $g^{-1}cm^{-1}$ for native β-CN. Circular dichroism spectra were recorded on an Aviv model 215 spectropolarimeter (Aviv Associates, Lakewood, NJ) using cells of appropriate path lengths and a scan time of 4.0 s/nm. The jacketed cells were attached to a circulating constant temperature bath; the equilibration time of the sample was calculated to be 30 min for a 30° change in the bath temperature. All spectra are corrected for solvent contributions and are expressed in units of mean residue ellipticity (degrees·cm^2·$dmole^{-1}$) relative to wavelength. Analysis of protein secondary structure from CD spectra was accomplished using the CONTIN/LL procedure of CDPro software package (Sreerama, *et al*., 2000; Sreerama and Woody, 2000) with 48 proteins in the reference set.

Near-UV CD spectra were recorded over the wavelength range of 250 to 310 nm, a 2-cm path length cylindrical cell was used for protein concentrations of ~1.0 mg/ml and each spectrum represents the average of five scans. 1-cm and 2-mm quartz cuvettes were used for protein concentrations of ~2.0 mg/ml and ~10 mg/ml.

2.3. Non-linear Regression Analysis

We have previously (Farrell, *et al*., 1988; Farrell, *et al*., 1990) used a series of equations derived from the thermodynamic linkage theory (Wyman, 1964) to describe qualitatively the concentration, temperature dependent self-association behavior of β-casein. The derivation of these equations has been presented in detail elsewhere (Rusling and Kumosinski, 1996). By their nature, they are a series of saturation binding isotherms with exponential functions to allow cooperativity. These equations have been applied (Qi, *et al*., 2004) to correlate potential changes in conformation as a function of temperature via changes in an experimental parameter, such as absorbance, ellipticity (θ) and fluorescence (F) with temperature. These temperature induced transitions may in turn be related to the well-described (Andrews, *et al*., 1979; Schmidt, 1979) self-association behavior of β-CN.

Since the total protein concentration remains constant throughout all experiments and in many instances, the protein is nearly monomer based on calculated k_a values, the most important changes in θ (or F) occur with temperature and may reflect conformational changes rather than self-association. The temperature dependent transitions were fitted with these equations. Our primary interest here lies in comparing transition mid-points, so the following relations hold true for bifunctional transitions:

$$\theta_{app} = \frac{\theta_0}{1 + k_1^n T^n} + \frac{\theta_1 k_1^n T^n}{1 + k_1^n T^n} + \frac{(\theta_2 - \theta_1) k_2^m T^m}{1 + k_2^m T^m} \quad (1)$$

It should be noted that the above expression is valid for sequential binding only, *i.e*. assuming $k_1^n \gg k_2^m$ and $k_1^n \gg 1$.

Equation 1 is closely related to the linkage between temperature and conformational changes as derived by Wyman (1964). The application of this equation was not apriori but occurred after a series of curve fitting attempts. The empirical meaning of the terms is as follows $\theta_i = \theta_{max}$ or θ_{min}, the maximum or minimum extrapolated θ at infinite T, and k_i is the half point of the transition. The term n may reflect a delay in onset, cooperativity, or could just be a fitting function. All fitting routines were accomplished by fixing the values of n and m and calculating the best least square fit for the optimally evaluated k_1 and k_2 values. The n and m values were then fixed to new integer values and the whole procedure repeated. The n and m values which yielded the minimum root-mean-square and lowest error values for k_1 and k_2 were then reported following the procedures previously reported for ligand binding (Farrell, *et al*., 1988) and enzyme kinetics (Farrell, *et al*., 1990).

2.4. Analytical Ultracentrifugation

For analytical ultracentrifugation, the protein samples (β-CN and β-CN-(*f*1-192)) were dissolved in 25 m*M* PIPES buffer and 8 nm KCl (ionic strength 0.05 *M*), pH 6.75 at concentrations ranging from 1.0 to 3.0 mg/ml. Samples and solvents were filtered through a Millipore (Milford, MA) HVLP 0.45 um membrane filter. Phast gel electrophoresis in SDS showed a nearly identical pattern of protein components before and after filtration; less than 1% of the material was retained on the filter as ascertained by UV spectroscopy. Sedimentation equilibrium experiments were performed using a Beckman Optima XL-A (Palo Alto, CA) analytical ultracentrifuge at speeds ranging from 6,000 to 12,000 rpm at various temperatures. A 12 mm charcoal-epon 6 channel centerpiece was used with quartz windows in a wide aperture window holder. The solvent density used in these experiments was 1.0044 at 25°C. This value was calculated from the data as previously described using 0.564 cc/g as the partial specific volume of PIPES. The partial specific volumes of β-CN and β-CN-(*f*1-192) were taken as 0.728 and 0.739 respectively. Data were collected at 280 nm using the standard XL-A procedure. The plots of absorption relative to radius were analyzed directly for weight average molecular weight using the program IDEAL1; a part of the Optima XL-A data analysis software. As the absorbance offsets were not allowed to float in these analyses, weight average molecular weights were obtained. Increased molecular weight with increased ionic strength was anticipated by the data of Schmidt (Schmidt, 1982) who clearly demonstrated self-association for native β-casein. Schmidt and coworkers used the following model to analyze their data:

$$i\beta \overset{k}{\rightleftharpoons} \beta i \tag{2}$$

where iβ refers to the unbound protein and $i \gg 1$ ranging from 20 to 50. Other models such as the formation of polymer through a critical micelle concentration have also been used. Analysis of the current data was accomplished using ASSOC4 which is for a system of up to four species. This model represents a parallel association scheme where monomer is simultaneously in equilibrium with dimer, trimer and tetramer when n_2, n_3 and n_4 are the integers 2, 3 and 4 in Eq. 3 below. In our experiments with β-CN and β-CN-(*f*1-192) the best fits were obtained by fixing the sequence molecular weight at 24,600 Da and 22,600 Da for the monomer and floating K values at the increasing integer values of n noted above; only one constant was required to fit the data. Analysis of all the data was accomplished using the following equation:

$$\begin{aligned} A_r = {} & EXP[\ln(A_0) + H \times M \times (X^2 - X_0^2)] \\ & + EXP[n_2 \times \ln(A_0) + \ln(K_{a2}) + n_2 \\ & \times H \times M \times (X^2 - X_0^2)] + E \end{aligned} \tag{3}$$

where A_r is total absorbance of all species at radius X; EXP is exponent; ln is natural log; A_o is absorbance of the monomer species at reference radius X_o; H is constant $[(1-\bar{v}\rho)\omega^2]/2RT$; M is apparent monomer molecular weight; X_o is reference radius; n_i is stoichiometry for the polymer (number of monomers); K_{ai} is association constant for the monomer-nmer equilibrium; and E is baseline offset.

As determined above in Eq. 3, K_{ai} may be converted to molar units using:

$$K_{conc} = K_{abs}\left[\frac{el}{n}\right]^{n-1} \tag{4}$$

where K_{abs} is K_{ai} of the software, *e* is the molar extinction coefficient, 1 is the path length in cm and n is the fitted integer of the software.

In turn, the molar constant, K_{conc} can be converted to the weight constant k_n by:

$$k_n = \frac{k_{conc}}{\left[\frac{M_{monomer}}{n}\right]^{n-1}} \tag{5}$$

$M_{monomer}$ is the monomer molecular weight and n is defined as above. The k_n values can thus be determined.

2.5. Fluorescence Measurements

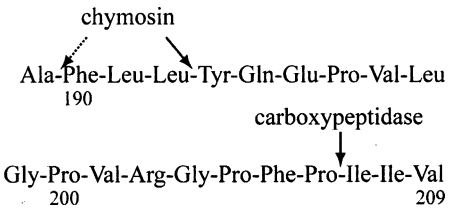
Fluorescence spectra were recorded with a Florolog-3 spectrofluorimeter (Jobin Yvon, Inc., Edison, NJ) equipped with a Peltier sample cooler. A 1 cm path-length quartz cell at room temperature (20°C–24°C) was used in this work. ANS binding studies were carried out by titrating increasing ANS concentration into a fixed protein concentration (5.0 μ*M*) in PIPES buffer, I = 0.05 *M*, pH 6.75. An excitation wavelength of 380 nm was used, and fluorescence emission was recorded from 400 to 600 nm. All the spectra were corrected for solvent and protein contributions to ANS fluorescence.

3. RESULTS AND DISCUSSION

3.1. Characterization of β-CN-(*f*1-192)

A brief report (Thompson, *et al*., 1967) on the treatment of β-casein by carboxypeptidase at pH 7.0 indicated that the removal of the last three residues greatly decreased the self association process. Creamer *et al*. (Creamer, *et al*., 1971) studied the action of milk-clotting enzyme chymosin (rennin) on β-CN by zonal column chromatography at 36°C. They found that chymosin preferably splits off a C-terminal fragment from β-CN with a molecular weight of approximately 2000 Da. In further studies, they thought (Berry and Creamer, 1975) that the chymosin cleavage site is at Ala(189)-Phe(190) (see below) and suggested that the C-terminus is totally responsible for the self-association behavior of bovine β-CN. However, it should be pointed out that zonal column chromatography could only qualitatively describe an associated system, Visser and Slangen (1977) reexamined the specificity of chymosin on β-casein at 13°C and pH 5.4, and reported that Leu(192)-Tyr(193) is a much more susceptible site for chymosin cleavage. The following scheme summarizes the cleavage sites by chymosin and carboxypeptidase in the C-terminal region of β-casein.

We have carried out chymosin digestion on β-casein according to the method described in the Materials and Methods section. The purified larger fragment from the reaction mixture was subjected to carboxypeptidase A analysis, and showed a rapid liberation of Leu followed by Phe and Ala. The final molar ratios were 1.0:0.9:1.9 for Ala:Phe:Leu, which



is indicative of the sequence of Ala-Phe-Leu-Leu. Further quantitative characterization of the larger fragment was achieved by electrospray ionization mass spectrometry (ESI-MS). The obtained experimental molecular weight unambiguously identified that the larger digestion fragment was β-CN-(*f*1-192) and the parent protein was genetic variant A^1 as shown in Table 1. Sedimentation equilibrium analysis of the fragment showed a weight average molecular weight in accord with the ESI-MS analysis at 2°C (Table 2), but as the temperature was increased the molecular weight increased indicating some residual self-association.

Table 1. ESI-MS Analysis of β-CN and β-CN-(*f*1-192)

	Expected MW(Da)[1]	Experimental Value (Da)
β-casein A^1	24023	24019 ± 1
β-casein A^2	23983	–
β-casein A^3	23974	–
β-CN-(*f*1-192)A^1	22160	22156 ± 1
β-CN-(*f*1-189)A^1	21786	–

[1] Calculated based on molecular formula.

3.2. Reduction of Self-association in β-CN-(*f*1-192)

The critical micelle model (Takase, *et al*., 1980, Tai and Kegeles, 1984; Thum, *et al*., 1987) has long been established to describe the predominant physical chemistry of temperature and concentration mediated self association of β-casein. It has also been shown that the association process is highly salt-dependent (Schmidt and Payens, 1976). The shape of the monomer, and the contributions of conformational change in the early stages of the association, however, remained controversial. Perhaps one reason for the controversy over the nature of the β-casein monomer is that much of the physical-chemical data has been collected at protein concentrations (1 to 3% w/v) or ionic strengths (0.2 to 0.4 *M*) which generally favor self-association. In contrast, spectroscopic data are often collected on more dilute samples. Accordingly, we have reexamined the β-CN-(*f*1-192) self-association process and compared it to that of its parent protein, β-CN, at much reduced concentrations (1 to 2 mg/ml) and lower ionic strength (0.05 *M*) at neutral pH (6.75).

Sedimentation equilibrium analysis was carried out from 2 to 37°C at various rotor speeds, as described in the experimental section. A significant degree of self-association is clearly present as signified by the upward curvature of the data for β-CN at 25°C as shown in Figure 2a while Figure 2b represents analytical ultracentrifugation behavior of β-CN-(*f*1-192) where it becomes apparent that some association is still present. To further quantify this data, both curves were fitted with a two species system (Eq. 3). The temperature dependence of the self-association behavior for β-CN-(*f*1-192) in comparison with that of β-casein was summarized in Table 2. To overcome the temperature limitation of

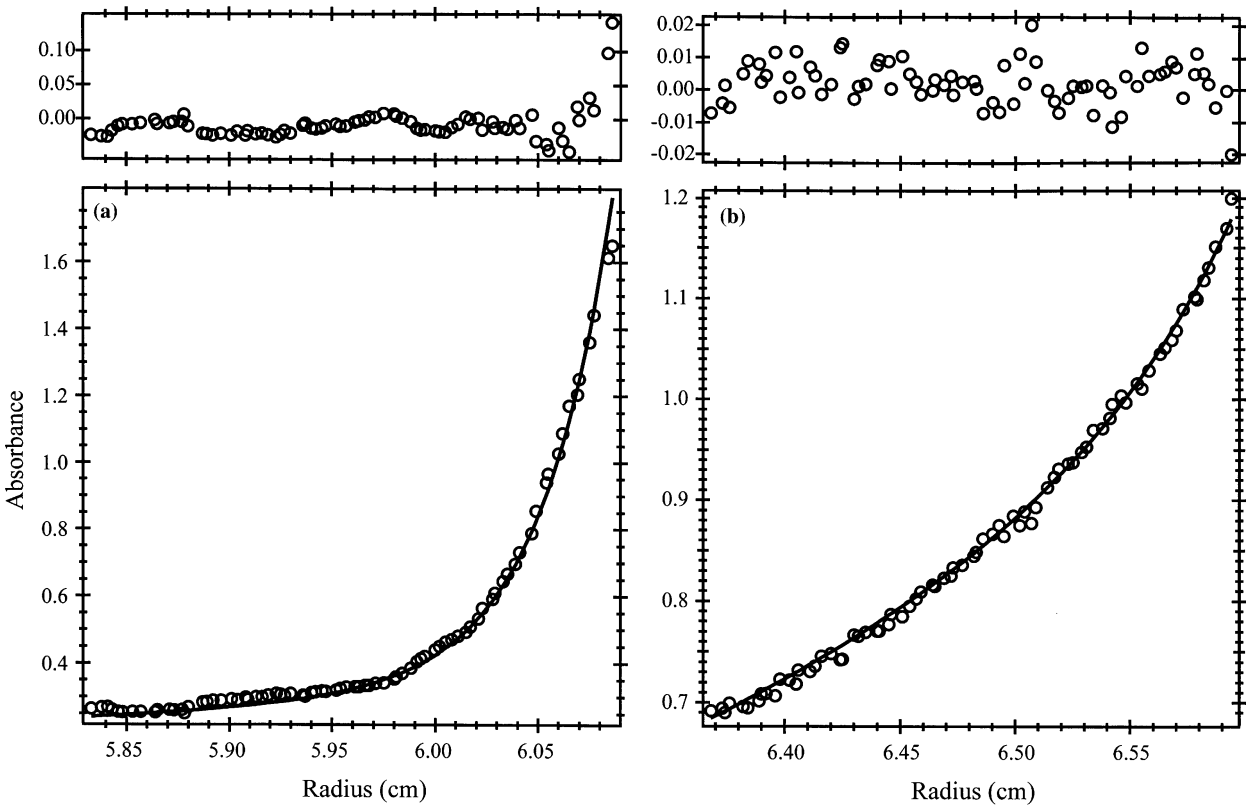


Fig. 2. Analytical ultracentrifugation analysis of native β-CN (a) and β-CN-(*f*1-192) (b) at 25°C in PIPES buffer, at I = 50 m*M* and pH 6.75. The fits to the absorbance vs. radius plot for Eq. 3 at 10,000 rpm for β-CN-(*f*1-192) and 9,000 rpm for β-CN, and the residual for the fits were also shown.

Table 2. Temperature Dependence of the Association Constants and ΔG° of β-CN and β-CN-(*f*1-192) Derived from Equilibrium Sedimentation

	Temperature (°C)	Rotor Speed (rpm $\times 10^{-3}$)	Weight Average MW (Da)	k_n(l/g)	n	k_{conc}(l/mol)	ΔG°(kcal/mol)
β-CN	70[1]	–	–	2.14×10^{31}	30	6.02×10^{115}	−182
	50[1]	–	–	3.69×10^{24}	29	3.29×10^{106}	−157
	37	6	495,000 ± 7084	4.38×10^{14}	29	3.90×10^{96}	−137
	25	6,9	235,000 ± 9800	2.00×10^{5}	23	3.04×10^{75}	−102
	20	9	128,000 ± 3167	1.83×10^{1}	20	8.67×10^{59}	−80.3
	15	9,12	60,760 ± 2828	6.36×10^{-2}	19	2.22×10^{57}	−75.3
	10	12	36,900 ± 1501	1.35×10^{-2}	14	1.95×10^{40}	−52.0
	5	12	29,880 ± 1260	3.00×10^{-2}	10	1.02×10^{29}	−36.9
	2	12	28,070 ± 790	1.23×10^{-2}	10	3.93×10^{28}	−36.0
β-CN-(*f*1-192)	70[1]	–	–	5.56×10^{19}	34	4.69×10^{112}	−176.8
	50[1]	–	–	1.60×10^{3}	31	7.70×10^{88}	−131.4
	37	10	90,222 ± 4954	4.68×10^{-3}	18	1.73×10^{50}	−71.3
	30	10	38,494 ± 1909	5.77×10^{-4}	11	6.63×10^{29}	−41.3
	25	10	34,343 ± 1975	8.28×10^{-4}	10	1.11×10^{27}	−36.9
	20	10	30,943 ± 2233	7.59×10^{-4}	9	1.06×10^{24}	−32.2
	15	10	29,121 ± 1335	3.07×10^{-4}	9	4.29×10^{23}	−31.1
	10	10	26,461 ± 782	5.68×10^{-4}	8	7.33×10^{20}	−27.0
	5	10	24,259 ± 1219	4.76×10^{-4}	7	4.91×10^{17}	−22.5
	2	10	23,531 ± 1130	6.23×10^{-3}	3	3.43×10^{5}	−7.0

[1] Estimated from fit Eq. 1 to Figure 3a and 3b.

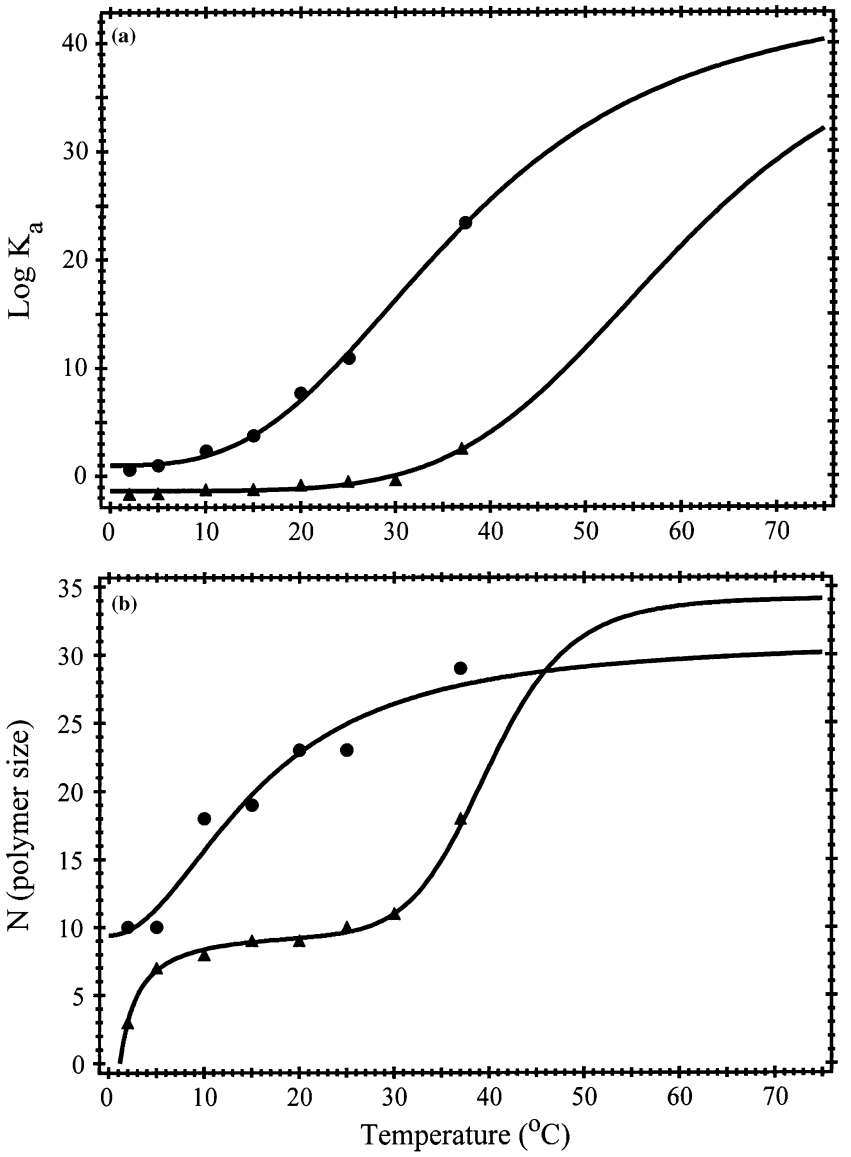


Fig. 3. (a) Temperature dependence of Log K_a for V-CN-(*f*1-192) (triangles) and β-CN (circles) at pH 6.75. (b) Temperature dependence of n (polymer size) for β-CN-(*f*1-192) and β-CN at pH 6.75. K_a and n were obtained by fitting the analytical ultracentrifugation data to a multi-component equilibrium (Eq. 3). The temperature dependence of Log Ka and n were fitted with Eq. 1.

the sedimentation experiments, a plot of log K_a (absorbance data) versus temperatures was made (Figure 3a). It should be pointed out that the curve is non-linear, and the transformation to 1/T does not overcome this feature. The plot of log K_a versus T does follow the type of analysis used in Eq. 1. When log K_a is substituted for θ, the data may be fitted with Eq. 1, and $K_{a\ max}$ and $T_{1/2}$ calculated. K_a values at 50 and 70°C could thus be obtained from extrapolation. In a similar fashion, n versus T yielded values for the size of self-association at elevated temperatures (Figure 3b). This resulted in n = 29 and n = 30 for β-casein and n = 31 and 34 for β-CN-(*f*1-192) for 50° and 70°C (see Table 2). The n values for β-casein derived in this way are consistent with the calculations of Thurn *et al.* (1987) from light scattering data at 40°C. Conversion to k_n and k_{conc} are therefore possible and results are

given in Table 2. From k_{conc}, $\Delta G°$ can be calculated at each temperature. The values at 20°C for β-casein are somewhat lower than those obtained by Takase *et al.* (1980) at 0.4 *M* ionic strength. This suggests that charge screening in the formation of β-casein polymers of high n (>20) might be important. However, at 10°C where polymer formation is just beginning (n = 10), the value of $\Delta G°$ is in reasonable agreement with Takase's work (−58.6 kcal/mol) and with the Payens and van Markwijk's (Payens and van Markwijk, 1963) value of −48 kcal/mol at 8.5°C for β-casein. Table 2 and Figure 3 clearly show a much lower K_a value, higher free energy (ΔG) and a smaller sized self-associated polymer for β-CN-(*f*1-192) relative to β-casein at each given temperature, suggesting the ability to self-associate in β-CN-(*f*1-192) has significantly diminished but is not completely abolished as suggested by Berry and Creamer on β-CN-(*f*1-189) (Berry and Creamer, 1975). As shown in Figure 3b, the size of the self-association of β-casein assumed a smooth monotonic function (n = 2 in Eq. 1) in response to increasing temperature. β-CN-(*f*1-192), on the other hand, showed two distinctive sharp transitions with n > 5 (Eq. 1). Moreover, within the experimental temperature 5–25°C, K_a showed a small gradual increase (Fig. 3a) while n displayed a plateau region (Fig. 3b). In contrast, the self-association of native β-casein proceeds rather rapidly in this region. Extreme temperatures (≥ 30°C) resulted in the increase of the amount as well as the size of self-associated polymers in β-CN-(*f*1-192). The limiting size of the self-associated state is 20 and 30 for β-CN-(*f*1-192) and β-casein respectively. This would suggest that sites other than the C-terminal 20 residues are also important in the self-association of bovine β-CN, Similar observations were reported by Bu *et al.* (Bu, *et al.*, 2004) for human β-CN.

3.3. Secondary Structure of β-CN-(*f*1-192) in Comparison to β-CN

The CONTIN/LL, a variant of the CONTIN procedure developed by Provencher and Glockner (Provencher and Glöckner, 1981) has been widely used to analyze the CD data and estimate secondary structure content of proteins and peptides. The database of 48 proteins (Sreerama and Woody, 2000) was chosen in this work because it contains both denatured proteins as well as left-handed polyproline II (PPII) structural elements even though the output of the program does not explicitly index the content of PPII. In addition, the calculated CD spectra seemed to be generally in good agreement with our experimental data. Analysis of far-UV CD data of β-casein at room temperature (25°C), low ionic strength (I = 0.05 *M*) and pH 6.75 indicates approximately 17% α-helix, 2.5% β-strand, 16% turns, and 65% unordered. The relative percentage falls to 5%, 2%, 7%, and 87% for β-CN-(*f*1-192) under the same experimental conditions. The calculated percentages of sheet and turns for native β-casein from FTIR and a different CD database (from the one used herein) (Farrell, *et al.*, 2001) differ somewhat from those derived from far-UV CD, but both techniques support the assumption that extended strand and turns comprise a significant portion of the protein structure (∼20–50%). Even though residues 1 to 6 of β-casein were strongly predicted to adopt α-helix conformation in the secondary structure prediction, the lack of α-helix in the N-terminal portion of β-casein was clearly demonstrated in the NMR studies of the 1–25 peptide (Wahlgren, *et al.*, 1993) and 1–42 peptide (Wahlgren, *et al.*, 1994) as well. Our recent work (Farrell, *et al.*, 2002) on native β-casein 1–25 peptide and its dephosphorylated form revealed possible existence of polyproline II and turns in this region. Previous studies by Raman Optical Activity (Syme, *et al.*, 2002) also estimated nearly 25% PPII in β-casein, Figure 4 compares the CD spectra of β-CN-(*f*1-192) at various temperatures, β-CN, and β-CN-(*f*1-25) at pH 6.75 and 25°C. Clearly, there is a stronger similarity between β-CN-(*f*1-192) and β-CN-(*f*1-25) than between β-CN-(*f*1-192) and native β-CN. As suggested previously in the case of β-casein (Qi, *et al.*, 2004), the presence of polyproline II short fragments in β-CN-(*f*1-192) is also apparent, perhaps in reduced amount. The strong negative CD band in the region of 190–200 nm signifying an unordered structural element in proteins (Saxena and Wetlaufer, 1971) not only blue shifted for ∼5 nm for β-CN-(*f*1-192) relative to β-CN, an increase of ∼2,500 (degree cm^2 $dmol^{-1}$) in intensity at 25°C was also observed. This could likely be attributable to the increase of about 22% in unordered structure and a simultaneous reduction in both α-helical and turn contents compared to β-CN as indicated by the CONTIN/LL analysis. Overall, the CD spectra of β-CN-(*f*1-192) and β-CN-(*f*1-25) resemble that of systemin which was found (Toumadje and Johnson, 1995) to contain poly(L-proline) II type helix. This perhaps is

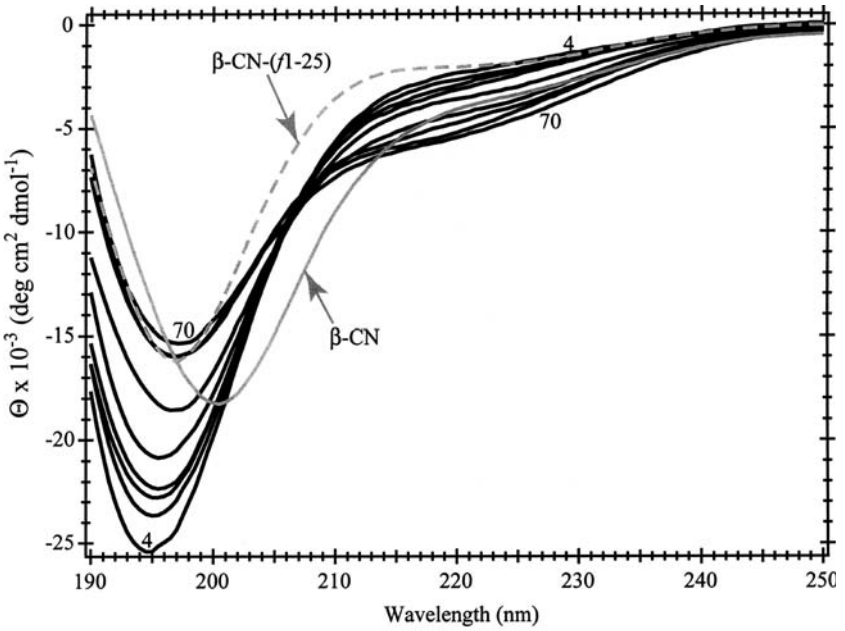


Fig. 4. Far-UV CD spectra of β-CN-(*f*1-192) at pH 6.75 and various temperatures (dark solid lines). Numbers on the lines represent temperatures (°C). The CD spectra of native β-CN (solid gray line) and β-CN-(*f*1-25) peptide (dashed gray line) in PIPES buffer (pH 6.75) at 25°C were also plotted as a comparison.

expected because of the high number of proline residues, 34, which constitutes over 11% of the total residues in β-CN A[1]. Consequently, β-CN may possess a strong tendency to form left-handed PPII, which has been increasingly recognized as an important structural element in unfolded proteins (Rucker and Creamer, 2002; Shi, *et al*., 2002).

3.4. Effect of Temperature on the Secondary Structure of β-CN-(*f*1-192)

The temperature dependence of CD spectra of β-CN-(*f*1-192) (see Figure 4) appears to follow that of β-CN closely. The movement of polyproline II model peptide from aqueous to a more non-polar (hydrophobic) environment (Tiffany and Krimm, 1968) seems to mirror the changes seen for both proteins. Similar results (data not shown for reason of clarity) were also obtained in analyzing the temperature dependence of CD of a polyglutamate (MW 750–1500) peptide at neutral pH which has been regarded as containing polyproline II conformations. Systemin has been demonstrated (Toumadje and Johnson, 1995) to contain PPII elements whose CD spectra were also found to display a similar temperature dependence behavior.

Unlike β-casein, where the only meaningful change is an apparent increase in α-helical content going from 2° to 70°C at pH 6.75 (Farrell, *et al*., 2001; Qi, *et al*., 2004), the amount of α-helix in β-CN-(*f*1-192) does not show noticeable temperature dependence based on the analysis from CONTIN/LL, indicating the calculated values for these elements are significantly lower (2%–5%), they are thus difficult to detect. One other explanation could be that there is little thermal unfolding in this class structure (however low it may be) as the protein is heated at pH 6.75. The amount of β-strand was found to follow a similar trend. The amount of turns, however, experienced noticeable reduction, to 7% at 25°C, and then returned to the value of 14% (comparable to that at 4°C) as temperature continues to increase. It is interesting to note that the amount of turns in its parent protein, β-CN, is also about 15% at higher temperature. The difference in self-association behavior between the two proteins could be attributable to the observed difference at elevated temperature, even at low concentration. The extended (unordered) structure remains relatively unchanged as a function of temperature. A similar conservation of extended turns at high temperature had been previously observed for intact

Table 3. Analysis of Change in Θ_{224}, Θ_{min} and λ_{min} Based on Eq. 1.

	β-CN	β-CN-(*f*1-192)
$T_{\Theta\ 224}$(°C)	33	37
$\Delta_{\Theta\ 224}$(deg·cm^{2}dmole^{-1})	−3740	−3447
n	2	2
$T_{\Theta\ min}$ (°C)	78	34
$\Delta\Theta_{min}$ (deg·cm^{2}dmole^{-1})	21,500	10,010
N	1	2
$T_{\lambda\ min}$(°C)	10,41	12,36
$\Delta\lambda_{min}$(nm)	3.4	2.0
n	20	24
m	3	32

β-casein (A^1 variant) by Farrell *et al.* (2001) and Graham *et al.*, (Graham, *et al.*, 1984).

The temperature dependence of the CD spectra of β-CN-(*f*1-192) was compared to that of β-casein in Figure 5. The data can be quantitated when fitted with Eq. 1, the predicted maximum change at temperatures greater than 70°C, the minimum ellipticity value (Θ) at 0°C and the midpoint for the transitions ($T_{\Theta\ 224}$ and $T_{\theta min}$ can be obtained for both β-CN-(*f*l-192) and β-CN. Table 3 compares changes in ellipticity at 224 nm (Θ_{224}, Fig. 5a), the minimum ellipticity (Θ_{min}, Fig. 5b), and the wavelength change occurred at the minimum ellipticity (λ_{min} Fig. 5c). It appears that temperature has a comparable effect on the ellipticity at 224 nm for both β-casein and β-CN-(*f*1-192), indicated by a similar maximum ellipticity change ($\Delta\Theta_{224}$) and a slightly higher midpoint transition temperature ($T_{\theta\ 224}$) for β-CN-(*f*1-192) than for β-casein. A hint of cooperativity was found in both cases, *i.e.* n = 2. Conversely, the maximum changes at the minimum ellipcity ($\Delta\Theta_{min}$) were estimated to be ~10,010 and ~21,500 degrees· cm^{2}dmole^{-1} for β-CN-(*f*l-192) and β-casein respectively. A much higher midpoint transition temperature, 78°C, was found for β-casein when only 41% of β-CN exists as monomer, than for β-CN-(*f*1-192) (34°C) when it remains nearly 100% monomer according to calculations from K_n (Table 2). Moreover, cooperativity of the transition is apparently less prominent for β-casein (n = 1) than for β-CN-(*f*l-192) (n = 2). It is important to point out that both transitions observed in Fig. 5a & b are highly reversible and showed little or no hysteresis.

Graham *et al.* (1984) calculated that the far-UV CD spectrum of β-casein at ~200 nm arises from larger Cotton effects in its ORD curves in the near vacuum UV area. If this hypothesis holds true,

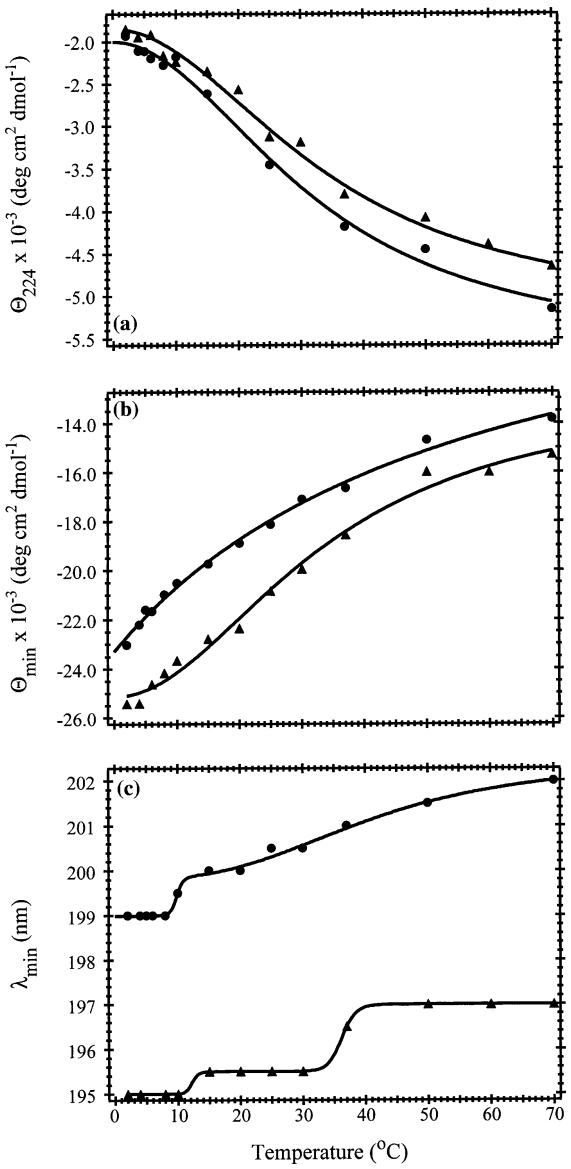


Fig. 5. Temperature dependence of far-UV CD spectra of β-CN-(*f*1-192) (triangles) in comparison with native β-CN (circles) at pH 6.75 and I = 50 m*M*. (a) Ellipticity at 224 nm (θ_{224}), (*b*) Minimum ellipticity (θ_{min}) in the far-UV region, and (c) Wavelength at minimum ellipticity (λ_{min}) were plotted as a function of temperature. Data were fitted with Eq. 1.

the red shift in λ_{min} observed here (Fig. 5c) could suggest meaningful structural changes in the backbone. A close examination of λ_{min} (Table 3) found that two distinctive transitions could be fitted with Eq. 1 for β-casein, $T_{\lambda 1} = 10$°C and $T_{\lambda 2} = 41$°C, and $T_{\lambda 1} = 12$°C and $T_{\lambda 2} = 36$°C for β-CN-(*f*l-192). It is apparent that these midpoints of transition are comparable for each protein. A much diffused tran-

sition behavior (m = 3) was implicated for β-casein contrary to a sharp two-state transition for β-CN-(*f*1-192) in the higher temperature range (> 30°C), This apparent distinction in the two proteins is most likely related to the self-association properties of β-casein as temperature is increased. The temperature dependence behavior of λ_{min} of β-CN-(*f*1-192) in Fig. 5c grossly parallels a "typical" protein denaturation curve with a two-state transition. CONTIN/LL analysis of the CD spectra for the two proteins suggested a slight but visible loss of α-helical content and perhaps an equal amount of increase in unordered structures. Using Eq. 1, a detailed analysis of Garnier's ORD data of β-casein (Garnier, 1966) showed a transition at 5 to 10°C for the change in A_{436} as a function of temperature. This, in all likelihood, approximates that of both β-casein and β-CN-(*f*1-192) in the low temperature range found in this work. The ORD data would, however, represent about 40% monomer at the experimental concentration using our association constants (Table 2).

Because β-casein is known to self-associate even at room temperature, the initial explanation for the mid-ranged (33°C) transition in β-casein might be cooperative self-association into the more hydrophobic environment of the polymer, *i.e.* the rheomorphic hypothesis. Analytical ultracentrifugation studies revealed, however, the monomer content remains greater than 98% for β-casein at the concentrations (0.17 ± 0.02 mg/ml) used in CD experiments even at 37°C. The extrapolation of the data to 50° and 70°C (Fig. 3a & 3b) showed 71% and 46% monomer in β-casein at these respective temperatures and concentrations used in this work, and nearly 100% monomer in β-CN-(*f*1-192) throughout. It is therefore reasonable to assume that the mid-ranged transitions observed in Fig. 5 and Table 3, at ~33°C, reflect "true" structural changes for β-casein and β-CN-(*f*1-192) monomers, rather than structural accommodation to the self-associated state. If the changes observed in the CD spectra indeed reflect movement to a more hydrophobic environment, then the monomer may be becoming more compact with increasing temperature. This model appears to reconcile with what was described as the apparent thread-like structures at 2 to 4°C that become more compact monomers in the presence of polymer at 8.5°C in β-casein (Payens and van Markwijk, 1963). At extreme temperatures, greater than 50°C, increasing degree of self-association in β-CN can arguably be attributed to the mid-point transition seen at 78°C, consistent with our previous findings (Qi, *et al.*, 2004). In contrast, this transition is apparently absent for monomeric β-CN-(*f*1-192). Based on these observations, we hypothesized that β-CN-(*f*1-192) may possess less secondary structure (α-helix) relative to its parent protein, β-casein monomer, in the temperature range studied in this work, but may contain more thermal-stable structural elements, such as extended structures.

3.5. Near-UV CD Spectra: Effects of Temperature and Protein Concentration

Near-UV CD spectra of β-CN-(*f*1-192) and β-casein at pH 6.75, I = 50 m*M* and various temperatures are shown in Fig. 6. The major contribution to the near-UV CD comes from the aromatic side chains. β-CN contains one Trp (W143), four Tyr (Y60,114,180,193), and nine Phe (F33, 52, 62, 87, 111, 119, 157, 190, 205) residues. The near-UV CD spectra at pH 6.75 and 5°, 25° and 60°C showed the absence of a negative peak at 295 nm that corresponds to the $^1L\beta$ transition (Valeur and Weber, 1977) of Trp 143 in both proteins, indicative of its considerably solvent exposed environment. The near-UV CD spectra of β-CN-(*f*1-192) are similar to that of its parent protein, β-CN, in many aspects, suggesting similar Trp and Tyr packing in these proteins. At 5°C when both proteins, β-CN, exist as monomers, greater than 98% for β-CN and nearly 100% for β-CN-(*f*1-192) at 1.0 mg/m1, the removal of one Phe and one Tyr that are located in the deleted 193–209 segment is likely to cause the observed difference in the spectrum of β-CN-(*f*1-192) relative to that of β-CN, especially, in intensities at the positive peak, ~258 nm, and the negative peaks at 275 nm and 282 nm. The peak in the 280 nm region may contain contributions from the $^1L_\alpha$ of Trp 143 and $^1L_\beta$ transition of Tyr residues based on assignments made by Valeur and Weber (1977). At 25°C, the fast reduction in relative intensity at 258 nm, 275 nm and 282 nm compared to that at 5°C for β-CN-(*f*1-192) is likely brought about by temperature as it remains mostly (~100%) monomeric under the experimental conditions employed. The peak at 258 nm, in particular, appears to be more sensitive to temperature for the monomeric β-CN-(*f*1-192) and is likely attributable to the Phe residues. Combined with the results obtained from near-UV CD experiments of β-CN at high pH (10.5) where the protein is much denatured (Qi,

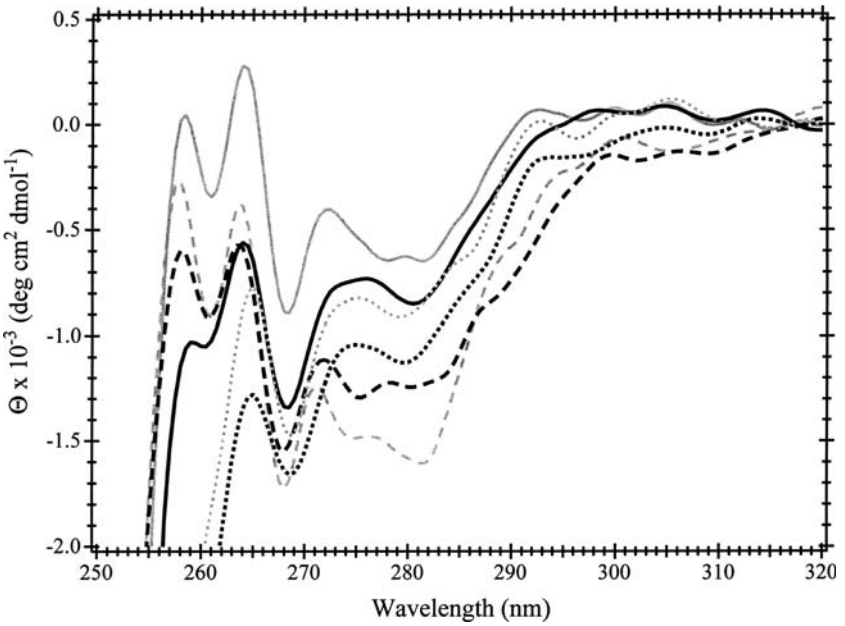


Fig. 6. Near-UV CD spectra of β-CN-(*f*1-192) (dark lines) and β-CN (gray lines) in PIPES buffer at pH 6.75 and 5° (dashed lines), 25° (solid lines), and 60°C (dotted lines). The protein concentrations were 1.0 mg/ml, *i.e.* 40.6 μ*M* for β-CN and 44.4 μ*M* β-CN-(*f*1-192).

et al., 2004), it may be reasonable to suggest this peak can be indicative of denaturation caused by heating in a less associated system, such as β-CN-(*f*1-192) and β-CN at pH 10.5. The presence of self-association in the case of β-CN where 57% remains monomeric at 25°C may somewhat slow down the disappearance of the peak at 258 nm. At 60°C, when there is less than 10% monomer for β-CN and about 46% for β-CN-(*f*1-192) the near-UV CD spectra are in close resemblance to one another, suggesting the coupled effect of temperature denaturation and self-association.

The CD spectra recorded for β-CN at 10 mg/ml (0.4 m*M*) (data not shown for reason of clarity) where it is mostly self-associated even at 5°C (~18% monomer) closely overlap with that at 1.0 mg/ml where it remains predominantly monomeric (~98%). The exception is that the intensity of all peaks is greatly reduced, presumably caused by an increasing degree of self-association as discussed above. The spectrum for β-CN-(*f*1-192) at higher concentration, 7.9 mg/ml, showed an apparent increase in relative intensity at 258 nm at 5°C when there is only ~46% monomer compared to that at much lower concentration, 1.0 mg/ml. As temperature is increased, self-associated species become predominate for both proteins, changes observed in the near-UV CD spectra manifested the highly temperature and concentration dependent self-association behavior.

3.6. ANS Fluorescence

ANS fluorescence has been widely used as a diagnostic indicator of exposed hydrophobic sites in proteins, especially in the detection of ''molten globule'' states (Semisotnov, *et al.*, 1991). Although ANS binds to proteins mainly through electrostatic interactions (Matulis and Lovrien, 1998), the fluorescence of ANS is enhanced by the presence of hydrophobic regions. There is a 2.5 fold enhancement of ANS fluorescence in the presence of β-CN at 480 nm, which is also blue-shifted relative to ANS alone. In contrast, fluorescence of ANS was almost unaffected by β-CN-(*f*1-192) (Fig. 7). The K_a of the binding complex between ANS and β-CN-(*f*1-192) can not be determined accurately because of the ineffective binding while the K_a of β-casein-ANS complex was determined to be $\sim 5.26 \times 10^4$ M^{-1} as reported previously (Qi, *et al.*, 2004). The near elimination of binding of β-CN to ANS brought about by the C-terminal truncation, consistent with the findings for β-CN-(*f*1-189)

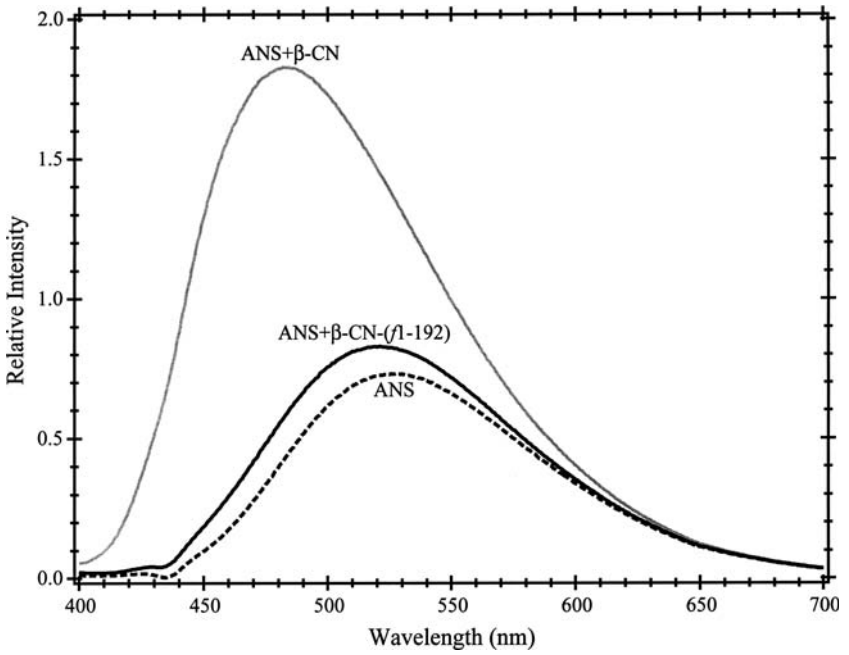


Fig. 7. Fluorescence emission spectra of ANS only, in the presence of native β-CN and β-CN(*f*1-192) at pH 6.75, I = 0.12 *M* and 20°C. λ_{ex} = 380 nm. Protein concentrations were 5.0 μ*M* and ANS was 40.0 μ*M*.

(Berry and Creamer, 1975), suggests that C-terminal peptide plays an important role in the formation of the complex between β-casein and ANS despite an additional aromatic (F190) and two hydrophobic (L191 & L192) residues in β-CN-(*f*1-192). Combining the results obtained from the far-UV CD studies, we propose that β-CN-(*f*1-192) resembles a "fully" unfolded globular protein in many respects although it is evident that a considerable amount of secondary structural elements such as turns and PPII still persists.

3.7. Significance of the C-terminal Deletion in Protein Function, Protein-Protein and Protein-Lipid Interactions

β-Casein has long been known to be an important protein in food formulation and stabilization because of its superior emulsification properties (Damodaran and Rammovsky, 2003). It thus posses surface active function and may well be involved in protein-protein as well as protein-lipid interactions. The critical role of C-terminal peptide in protein-protein and protein-lipid interactions has been identified for several proteins (Pagani, *et al*., 2001; Fimland, *et al*., 2002; Rodriguez-Zavala and Weiner, 2002) where the C-terminus anchors the protein into a membrane or initiates penetration into a cell. An excellent example of the latter is tailspike protein of phage P22. The tailspike recognizes the lipo-polysaccharide on the surface of *Salmonella* bacteria, which the Phage infects. The mature native tailspike protein is a timer containing three copies of the same protein chain, which is very stable when exposed to heat, detergent and protease. It has been established that the C-terminus plays an integral role in the function and the formation of the stable trimer of tailspike protein. It is interesting to note that the C-terminus of tailspike protein bears 64% sequence identity to that of β-casein (Figure 8). DeLano *et al*. (2000) have suggested that many protein-protein and protein-receptor complexes at membranes may have found "convergent solutions" to binding at

```
tailspike protein     FLPYWENNSTSLKALVKKPNGELV
bovine β-casein       FLLYQE----PVLGPVRGPFPIIV
sequence identity     ** * *     .: . *: *   :*
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Fig. 8. Pair-wise sequence alignment of tailspike protein C-terminus and bovine β-CN C-terminus by using T-COFFEE. Sequence identity score is 64%.

interfaces. Therefore, an intriguing question arises: Is this simply just a coincidence or could it be meaningful in a fundamental evolutionary sense?

4. CONCLUSIONS

We have shown clearly that C-terminal deletion of bovine β-casein by chymosin cleavage can significantly affect its structure and stability in the monomeric state. The secondary structure of β-casein, α-helix in particular, has been considerably disrupted due to the truncation in the C-terminal region. The degree of self-association has greatly been decreased, but not completely abolished. We further suggest that the C-terminal 17 amino acid peptide plays a less critical (than previously believed) role in the formation and stabilization of self-association of β-CN.

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