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Secondary structure and colloidal stability of beta-casein in microheterogeneous water-ethanol solutions

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ABSTRACT

Dynamic light-scattering (DLS), fluorescence spectroscopy (FS) and circular dichroism (CD) techniques were applied to study the influence of alcohol on beta-casein (b-CN) self-association and the secondary structure in a wide range of temperatures and ethanol concentrations. Temperature induced micellization and demicellization transitions of b-CN in water-ethanol solutions are revealed on the basis of the DLS data. The obtained results indicate that the association of b-CN at low and high alcohol concentrations proceeds through different mechanisms. It is suggested that the solvent microheterogeneity independently modulates both the secondary structure and the colloid properties of b-CN.

1. Introduction

Beta-casein (b-CN) is one of the members of casein family (Wong, Camirand, & Pavlath, 1996) and the most abundant milk protein. The amphiphilic character and high surface activity of b-CN are responsible for its good foaming and emulsifying properties (Dalgleish, 1997; Dalgleish, Spagnuolo, & Goff, 2004; Halling, 1981). b-CN molecules have a strong tendency to self-associate in aqueous environment forming globe-shaped surfactant-like micelles with hydrodynamic radius 7–14 nm (Dickinson, 1999; Faizullin, Konnova, Haertlé, & Zuev, 2013; Holt, 1998; Horne, 1998; Payens & van Markwijk, 1963; Portnaya et al., 2006; Rollema, 1992; Stroylova et al., 2013; Swaisgood, 1992). As was established by a variety of methods (Evans, Phillips, & Jones, 1979; Kajiwara et al., 1988; Niki, Takase, & Arima, 1977; Payens & Vreeman, 1982; Pearce, 1975; Portnaya et al., 2006; Schmidt & Payens, 1972; Swaisgood, 2003; Thurn, Burchard, & Niki, 1987) the critical micelle concentration of protein ranges from 0.3 to 0.7 mg/ml depending on temperature, pH and ionic strength of solution. The balance of two main driving forces – the attraction of hydrophobic domains and the electrostatic repulsion of the charged hydrophilic N-terminal regions forms the basis of the b-CN micellization process (De Kruif & Grinberg, 2002; Horne, 1998, 2002; Kumosinski, Brown, & Farell, 1993; Mikheeva, Grinberg, Grinberg, Khokhlov, & de Kruif, 2003; O'Connell, Grinberg, & de Kruif, 2003; Portnaya et al., 2008). The key point of this model is the ability of water molecules to form a continuous three-dimensional network of hydrogen bonds with enthalpy depending on temperature and configuration of bonds (Cinelli, Onori, & Santucci, 1997; Privalov, 1987; Ruckenstein & Shulgin, 2001). The presence of the third amphiphilic component in solution (ethanol in our case) which competes with water for the formation of hydrogen bonds affects spatial network of hydrogen bonds and weakens hydrophobic interactions. Consequently, all these effects can modify the colloidal state of b-CN.

Recently, b-CN has been classified as an "intrinsically unstructured protein" (IUP) (Tompa, 2002). Holt has argued (Holt & Sawyer, 1993) that caseins have the rheomorphic nature meaning that their secondary structure has rather large conformation space. Therefore, the secondary (probably, also tertiary) structure of b-CN

Abbreviations: b-CN, beta-casein; CD, circular dichroism; DLS, dynamic light-scattering; IUP, intrinsically unstructured proteins; PPII, polyproline II helix.

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and specific properties of its colloidal micelles are strongly dependent on the properties of protein environment such as solvent structure or interactions with low- and high-molecular ligands. The study of b-CN in water and water-ethanol mixtures presents an interest both in practical aspects (b-CN is used as a mild emulsifier and ingredient in formulation of many alcoholic beverages and for development of advanced materials) and in understanding of molecular mechanisms crucial for b-CN specific structural and functional properties. It is known that the presence of alcohols in aqueous phase significantly modifies molecular structure and aggregation of globular proteins (Cinelli et al., 1997; Clark & Smith, 1989; Elysee-Collen & Lencki, b, 1996a). One might expect that the inherent disorder resulted in high solvent accessibility of b-CN structure making it even more susceptible to the environmental effects. Until now many attempts have been made to understand the interconnection between the secondary structure, micellization and solvent properties of b-CN (Faizullin et al., 2013; Horne & Davidson, 1987; Horne, 2002; Mikheeva et al., 2003; O'Connell, Kelly, Auty, Fox, & de Kruif, 2001a; O'Connell, Kelly, Fox, & de Kruif, 2001b; O'Connell et al., 2003; Portnaya et al., 2006, 2008; Stroylova et al., 2013; Trejo & Harte, 2010; Ye & Harte, 2013; Zadow, 1993). There is a strong opinion (Corsaro, Maisano, Mallamace, & Dugo, 2013; D'Angelo, Onori, & Santucci, 1994; Dolenko et al., 2015; Onori, 1988; Sarkar et al., 2015; Sato, Chiba, & Nozaki, 1999; Wakisaka & Ohki, 2005; Wakisaka & Matsuura, 2006) that the peculiarities of various physicochemical properties of water-alcohol mixtures are governed by their microphase separation. It means that after dissolution of small amphiphilic protic molecules such as alcohols in water the microdomains enriched either by water or by alcohol are formed. The resulting solvent microheterogeneity is determined by subtle interplay between hydrophobic and hydrophilic interactions and can modulate in turn the solution properties of the solute, e.g. protein (Konnova, Faizullin, Haertlé, & Zuev, 2013; Takamuku, Hatomoto, Tonegawa, Tsutsumi, & Umecky, 2015). However, the possible impact of microheterogeneity in water-ethanol solutions on both the secondary structure and micellization properties of b-CN remains to be taken into consideration.

In the present work we applied dynamic light-scattering (DLS), fluorescence spectroscopy (FS) and circular dichroism (CD) techniques to study molecular structure and colloid properties of b-CN in a wide range of temperatures and alcohol concentrations.

2. Material and methods

2.1. Sample preparation

Native b-CN extracted from bovine milk by rennet coagulation was purchased from Lactalis (Laval, France). Measurements were performed with 0.5 mg/ml b-CN water/ethanol solutions containing 0–50% (v/v) of ethanol. b-CN aqueous solution without ethanol was used as a control. For preparation of samples the 5 mg/ml protein stock solution was made dissolving b-CN in Na-phosphate buffer (50 mM, pH 7.4) overnight at 4 °C. Then after filtration through 0.22 μm Millipore membrane the protein concentration was measured spectrophotometrically at 280 nm using absorbance coefficient 0.486 (1 g/L) for b-CN (http://web.expasy.org/protparam/). Samples were prepared on the day of measurement and used after 1 h incubation at room temperature.

2.2. Particle size measurements

The particle size analysis was conducted by DLS using a Zetasizer Nano-S ZEN1600 instrument (Malvern Instruments, Malvern, UK) equipped with He-Ne laser operating at $\lambda = 633$ nm. Scattered light was detected in the backscattering regime at the scattering angle 173° due to its less susceptibility to the presence of large particles comparing with scattering angle 90° (Achieving high sensitivity at different scattering angles with different optical configurations, Version 4 Technical Note, Malvern Instruments Limited, 2014). Solutions of b-CN were transferred to the low-volume spectroscopic plastic cells and placed in the temperature controlled chamber regulated from 5 to 60 °C (some measurements were made up to 80 °C) by steps of 5 °C. The duration of temperature stabilization was 15 min. The hydrodynamic diameter of b-CN was measured five times at each temperature. Every measurement corresponds to auto-correlation functions recorded during 40 s. For water-ethanol solutions the refractive indexes and viscosities at different temperatures were taken from tabulated data (Herráez & Belda, 2006; Pires, Costa, Ferreira, & Fonseca, 2007). The refractive index of protein was accepted to be 1.45 independent of the temperature. The CONTIN method (Malvern DTS software) was used to calculate the diameter from the autocorrelation data. Size distribution by the number was used for the analysis of results.

2.3. Fluorescence spectroscopy

Fluorescence spectra were obtained with a Hitachi F-4500 spectrofluorimeter equipped with a right angle holder and a Peltier thermostat. Both emission and excitation slit widths were set to 5.0 nm. The emission spectra of a single b-CN tryptophan (Trp) residue were recorded between 300 and 400 nm every 0.5 nm with excitation at 295 nm. The scan speed was set at 60 nm/min. Protein solutions were placed into the quartz cuvette with 1 cm optical pathway. The spectra were collected in triplicate at temperatures varying from 10 to 50 °C. Before every measurement the temperature was stabilized for 15 min. The spectra were corrected for Raman scattering of buffer. The wavelength position of fluorescence emission maxima were determined using Peak Fit software (Systat Software, UK).

2.4. Circular dichroism measurements

Far-UV CD spectra were recorded by Jasco J-810 Spectropolarimeter (Jasco Incorporated) in quartz cuvette Hellma 106-QS with the pathways 0.02 cm for b-CN concentration 0.5 mg/ml and 1 cm for 0.01 mg/ml one. CD spectra were obtained in temperature range 10–50 °C every 10 °C. Each spectrum represents the average of 3 successive scans, acquired with a scan rate 8 s/nm in the spectral range 190–250 nm with a step of 1 nm. Protein spectra were corrected by subtracting solvent ellipticity and expressed as mean residue ellipticity (degrees cm^2/dmol). The protein secondary structure content was calculated using the program CONTIN (http://dichroweb.cryst.bbk.ac.uk) and the basic set of proteins №7 (Whitmore & Wallace, 2004, 2008). One should note that in the accessible spectral range it is doubtable to obtain viable results in case of intrinsically unstructured b-CN. Thus, the composition of the secondary structure obtained through deconvolution of CD spectra should be considered as semi-quantitative estimation of the protein structural changes.

3. Results

The dynamic light scattering technique has been employed to study the temperature dependent self-association of b-CN in mixed solvents. Fig. 1 depicts changes in the hydrodynamic diameter of b-CN particles in water and water-ethanol solutions upon heating. In water up to 20 °C b-CN exists in monomeric form with a narrow size distribution around 8 nm. The heating above 40 °C provokes the formation of b-CN micelles with an average size 17–18 nm. In