Ethanol-Dependent Heat-Induced Dissociation of Casein Micelles

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The dissociation of casein micelles when heated to ~65 °C in the presence of ethanol [1:1 mixture (v/v) of milk and 65% (w/w) aqueous ethanol] was investigated using L^* values and transmission measurements. Mixtures of milk and ethanol became transparent on heating, which suggests dissociation of casein micelles. Results of experiments using confocal laser scanning microscopy, light scattering (static and dynamic), and dialysis to examine the changes of milk during heating in the presence of ethanol supported the assertion that such treatments result in dissociation of casein micelles, as did studies of model β-casein micellar systems.

Keywords: *Milk; casein micelles; ethanol; dissociation*

INTRODUCTION

The contribution of specific interactive forces to the assembly of casein molecules or submicelles into micelles has been the subject of extensive research for many years. Using specific agents, such as, κ-casein, EDTA, oxalate, urea, citrate, and guanidine, or experimental conditions, such as, dialysis, acidification, enzymology, and high pressure, it has been shown that electrostatic interactions, hydrophobic bonding, and (direct or indirect) calcium phosphate-mediated links contribute to the cohesive interactions responsible for maintaining micellar integrity or, from an other perspective, ensuring protein instability, which in turn is the driving force for the micellarization process (*1*−*7*).

Ethanol is known for its ability to reduce the colloidal stability of casein micelles and coagulate milk. Indeed, the ethanol stability assay was used as an index of the ability of milk to withstand thermal sterilization (*8*). More recently, the ethanol stability assay has been used as a tool with which to investigate micelle morphology, and the stability of micelles in ethanol mixtures is important in determining the shelf life of cream liqueurs (*9*, *10*).

Zadow (*11*) reported that, at elevated temperatures, ethanol solubilized casein micelles. It was proposed that the reversible dissociation of casein micelles, when heated in the presence of alcohol, could be used to incorporate chemicals into the micelles, thereby changing their functional properties (*11*). This paper reports on the stability or existence of casein micelles in ethanol solutions within the temperature range of 5−75 °C.

MATERIALS AND METHODS

Materials. Low-heat skim milk powder (SMP, NILAC) was manufactured at NIZO Food Research, and high-purity β-casein was obtained from Eurial (Eurial, Rennes, France). Dialysis tubing with a 12−14 (T99−T108) and 300 (Spectra/Por CE) kDa nominal molecular weight cutoff was purchased from Medicell International Ltd. (London, U.K.) and Spectrum

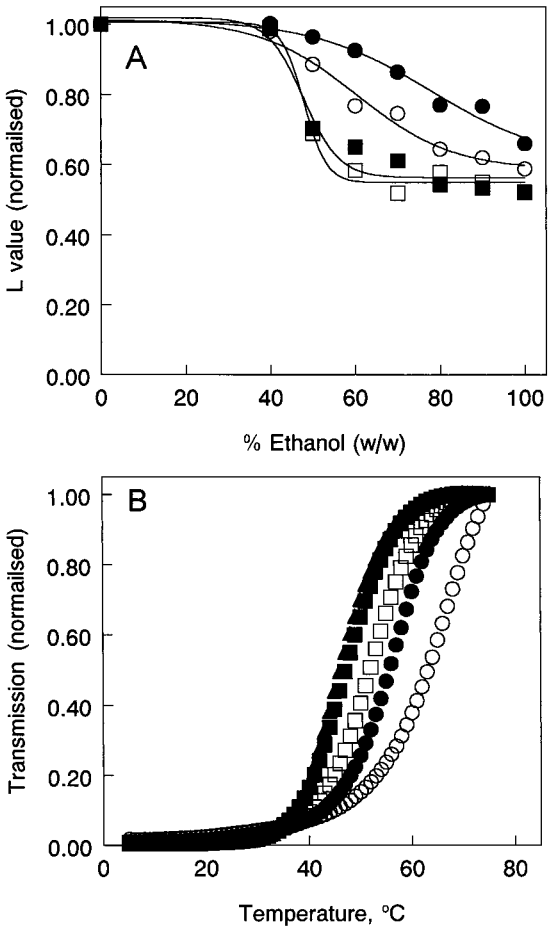

Figure 1. Effect of (A) alcohol on the L value of a 1:1 (v/v) mixture of milk and alcohol at 40 (●), 50 (○), 60 (■), or 70 (□) °C or (B) temperature on the transmission (λ = 600 nm) of a 1:1 (v/v) mixture of milk and 55 (○), 60 (●), 65 (□), 70 (■), or 75% (▲) (w/w) ethanol.

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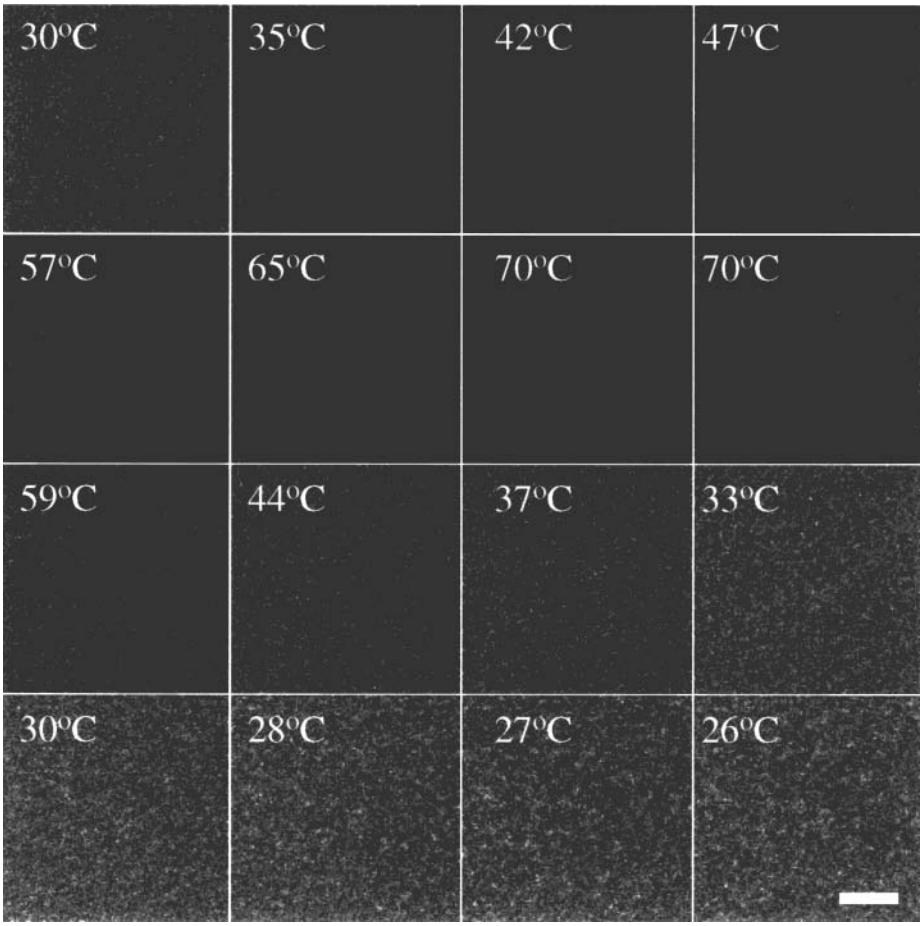

Figure 2. Confocal laser micrographs of a 1:1 (v/v) mixture of milk and 65% (w/w) aqueous ethanol on heating to 70 °C and subsequent cooling.

Medical Industries Inc. (Houston, TX), respectively. Coomassie Plus protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL). All chemicals were of reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO) or Merck (Darmstadt, Germany).

Preparation of Reconstituted Skim Milk and *β*-Casein Solution. SMP was reconstituted by adding 10.45 g of SMP to 100 mL of distilled water. Sodium azide (40 g/L) was added to a final concentration of 200 mg/L. The mixture was stirred at ~45 °C for 1 h, held at ~4 °C for 16 h and then stored at ~4 °C until required (not >4 days).

A stock solution of *β*-casein (50 g/L, in 25 mM sodium phosphate, pH 6.5) was prepared and exhaustively dialyzed (100 mL against 2 × 2 L of 25 mM sodium phosphate, pH 6.5, for 72 h at 4 °C), using dialysis tubing with a nominal molecular weight cutoff of 12−14 kDa. Sodium azide (40 g/L) was added to a final concentration of 200 mg/L. The stock solution was diluted with 25 mM sodium phosphate, pH 6.50, to 5 g/L *β*-casein, as required.

Determination of *L Value.** The *L** value, which is a measure of the lightness of a system, was determined for 1:1 mixtures of milk and ethanol (55−75%, w/w) using a Chromameter (Minolta CR-110, Minolta Corp., Ramsey, NJ). The mixtures were heated in a water bath and rapidly transferred to a Petri dish; the *L** value was then determined.

Transmission Measurements. Transmission-temperature profiles for 1:1 mixtures of milk and water or ethanol (55−75%, w/w) were carried out using a Cary 1E spectrophotometer (Varian Australia Pty Ltd., Mulgrave, VIC, Australia). The sample path length was 2 mm, and the transmission wavelength was 600 nm. The samples were heated from 5 to 75 or 80 °C at 0.4 °C min^{-1}. The apparent dissociation temperature was assigned as the temperature at which the inflection point in the temperature−transmission profile existed.

Confocal Laser Scattering Microscopy (CLSM). A 1:1 (v/v) mixture of milk and 65% (w/w) ethanol at temperatures in the range 20−70 °C was examined by CLSM using a Zeiss LSM310 confocal scanning laser microscope (Carl Zeiss Ltd., Hertfordshire, U.K.) fitted with a ×63 oil immersion objective lens. Helium−neon laser excitation at 633 nm in combination with a 760−810 band-pass filter and a FT580 dichroic mirror was used. A general protein dye (Fast Green FCF, 100 *μ*L of a 1% w/v aqueous solution) was added to 50 mL of the 1:1 (v/v) mixture of milk and 65% (w/w) ethanol and mixed. Approximately 200 *μ*L were then transferred to a silicone spacer gasket (*12*) on a Linkam C102 warm stage (Linkam Scientific Instruments Ltd., Surrey, U.K.) attached to the microscope. The temperature was increased to 70 °C at a rate of 1 °C min^{-1}, held at 70 °C for 5 min, and cooled to 26 °C. Gray level images (256 × 256 pixels) were digitally acquired at selected temperatures during the heating−cooling cycle.

Dialysis Experiment. A 1:1 (v/v) mixture of milk and 65% (w/w) aqueous ethanol (1.5 mL) at 20 or 65 °C was transferred rapidly to dialysis tubing with a nominal molecular weight cutoff of 300 kDa and dialyzed against 2 L of 35% (v/v), at 20 or 65 °C, for 7 h. After dialysis, the protein content of the solution within the dialysis sac was determined using a standard protein determination kit.

Light Scattering. Static light scattering (SLS) measurements of a 1:40 milk and 65% ethanol mixture were made in the temperature range 20−70 °C, and the scattered light was measured at angles in the range of 50−100°. The radius of gyration (R_G) was determined by a Guinier plot of ln I_θ versus Q^2, where Q is $(4\pi/\lambda)\sin(\theta/2)$ and the slope of the line is $R_G^2/3$.

Dynamic light scattering (DLS) measurements on 1:1 (v/v) mixtures of 0.5% *β*-casein in 25 mM sodium phosphate, pH 6.5, or milk and alcohol solutions (0−60%, w/w) at 0 to 60 °C,

were performed using a Spectra Physics 275 mW Ar laser (λ = 514.5 nm). Scattered light was detected, at 90°, by a photomultiplier, which was interfaced to a digital ALV 5000 correlator. Measuring times varied with the intensity of scattered light but were carried out for a sufficiently long period so as to collect 10^7 photons.

The data were analyzed using a second-order cumulant on a double-exponential fit as outlined by O'Connell and de Kruif (*13*).

RESULTS AND DISCUSSION

The data in Figure 1A support the report of Zadow (*11*) that, on heating a 1:1 (v/v) mixture of milk and ethanol, the mixture became less turbid, as determined by L^* values, and the casein micelles appear to dissociate. The system was also studied by measuring the transmission of the milk−ethanol mixture as a function of temperature (Figure 1B). When the mixture was cooled, the casein particles appeared to reassociate into micelles (results not shown). As shown, increasing the ethanol content (from 55 to 75%, w/w) of the alcohol solution caused a commensurate decrease in the apparent dissociation temperature (inflection point in the temperature−transmission profile; Figure 1B). When a 1:1 mixture of milk and 100% ethanol was prepared, a precipitate formed instantly but, surprisingly, when this mixture was heated to 70 °C, with gentle stirring, the precipitated protein redissolved, giving a transparent solution, which, when subsequently cooled on ice, formed an opaque gel. The authors opine that this phenomenon is similar, or related, to the effect of 2,2,2-trifluoroethanol (TFE) on casein micelles reported by Horne and Davidson (*14*). Addition of TFE at low levels precipitates casein, but at higher levels the protein is redispersed and the mixture appears to be "much less turbid" than the mixtures at lower TFE concentrations (*14*).

The alcohol-dependent temperature-induced dissociation of the micelles was further investigated by three approaches: CLSM, dialysis experiments, and light scattering techniques.

Although CLSM is not the method of choice with which to examine particles of the size of casein micelles, it is possible to study alcohol-dependent temperature-induced dissociation in vitro. The micrographs show the progressive disappearance of the protein aggregates when the 1:1 (v/v) mixture of milk and 65% (w/w) aqueous ethanol was heated (Figure 2). At 70 °C few, if any, protein aggregates were evident, which indicates that the casein micelles had dissociated at elevated temperatures. The aggregates present at elevated temperatures were possibly residual milk fat globules. As shown, the protein particles appear to reassociate on cooling into colloidal aggregates of larger apparent size than those in the initial (unheated) system.

The second approach to studying the dissociation of casein micelles when heated in the presence of ethanol was by dialysis. A 1:1 mixture of milk and 65% ethanol was dialyzed against a 35% aqueous ethanol solution at either 20 or 65 °C using dialysis tubing with a nominal molecular weight cutoff of 300 kDa. When dialyzed at 20 °C, there was little, if any, loss of protein from the dialysis sac, whereas when dialysis was carried out at 70 °C, all of the protein had diffused out into the bulk phase within 7 h of dialysis. From these data it can be reasonably adduced that the majority, if not all, of the casein micelles had dissociated.

The dissociation of micelles when heated in the presence of alcohol was also studied by light scattering.

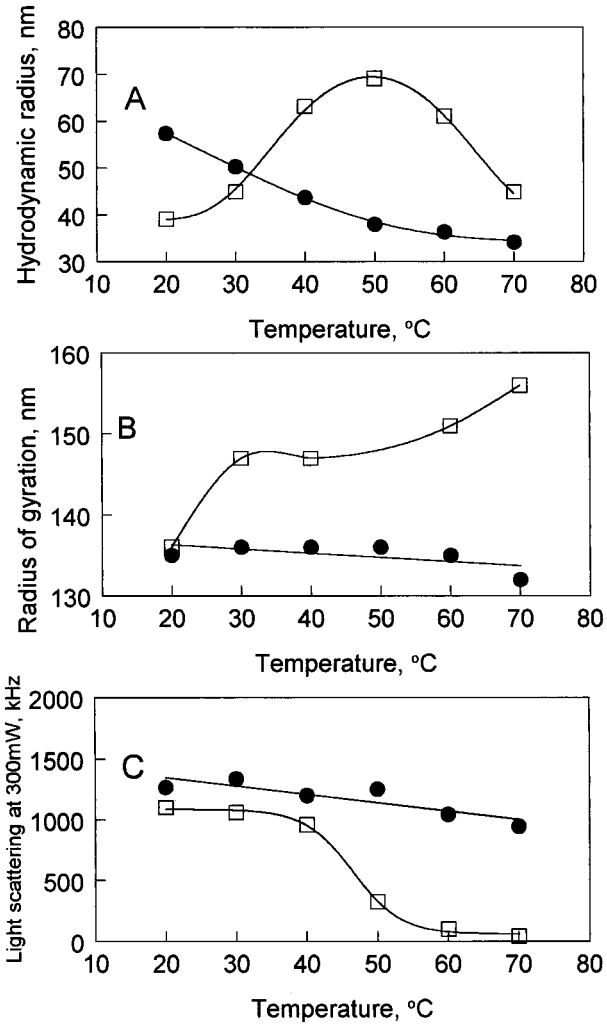

Figure 3. Effect of temperature on the (A) hydrodynamic radius, (B) radius of gyration, or (C) intensity of light scattering of casein micelles in a 1:1 (v/v) mixture of milk and water (●) or 65% (w/w) ethanol (□).

The effect of alcohol at different temperatures on the hydrodynamic radius of casein micelles was determined by DLS. As shown in Figure 3A, there was a progressive increase in the apparent hydrodynamic size of the micelle with temperature up to ~50 °C, above which the size decreased to a size marginally larger than native casein micelles at 20 °C. These results are similar to those of Horne and Davidson (*14*), insofar as they can be compared; that is, the apparent micelle size in "dissociated" alcohol mixtures was slightly larger than native micelles. In an attempt to elucidate the state of casein particles in the milk−ethanol systems at elevated temperatures, additional light scattering techniques were used to determine the radius of gyration of the casein micelles. In agreement with the DLS results, the radius of gyration increased in the "dissociated system" (Figure 3B). It is noteworthy that the intensity of scattered light decreased markedly at temperatures >40 °C; it is proposed that this is due to dissociation of the principal scattering species in milk, that is, the casein micelles. Considering the results for the hydrodynamic radii, in conjunction with the intensity of scattered light as affected by temperature (Figure 3C) and the results of the dialysis experiments, it is proposed that casein micelle dissociation occurs between 40 and 50 °C. The hydrodynamic radii at temperatures >40 °C represent

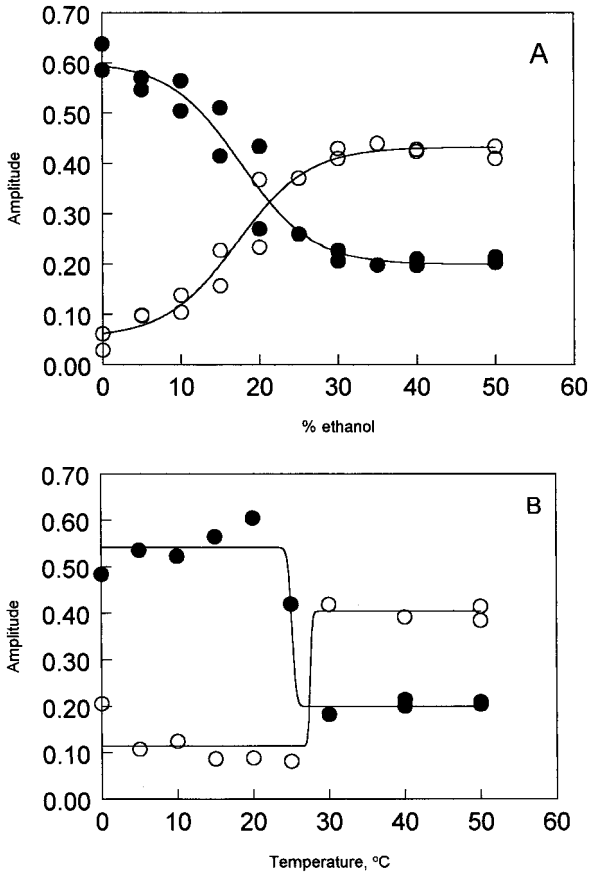

Figure 4. Effect of (A) ethanol concentration on the micelle (●) or monomer (○) amplitude in a 1:1 mixture of 0.5% β-casein in 25 mM sodium phosphate, pH 6.5, and ethanol (0−50%, w/w) at 60 °C or (B) temperature on the micelle (●) or monomer (○) amplitude of a 1:1 mixture of 0.5% β-casein and 50% ethanol. Amplitude is proportional relative concentration of each species (monomer or micelles).

a small fraction of micelles that are in a transient state between original micelles and dissociated casein particles. As has been proposed for the behavior of casein micelles under other dissociating conditions, it is likely that these ghost or skeleton micelles exist in equilibrium with monomeric casein molecules (*1*, *3*). This concept is consistent with the results of Horne and Davidson (*14*), who, using sequential/differential centrifugation, showed that extensive dissociation of the micelles occurred in the milk−TFE mixtures at high concentrations of the latter in the mixture.

Alcohol-dependent dissociation of micelles at elevated temperatures was also studied using a model β-casein micellar system. β-Casein has a unique amphiphatic primary structure, with a hydrophobic C-terminal and a polar, highly charged N-terminal, and undergoes an endothermic temperature-dependent self-assembly process into soaplike micelles (*5*, *6*). In the current study the amplitude (which is proportional to concentration) of β-casein monomers and micelles at 60 °C, as affected by ethanol, was measured. As shown in Figure 4A, the addition of ethanol promoted monomerization of β-casein at 60 °C, but it appears that this effect was markedly temperature dependent (Figure 4B). Ethanol caused polymerization of β-casein at temperatures at which the monomers predominate in an aqueous milieu (i.e., <20 °C). These results indicate that there is a critical temperature above or below which ethanol has marked stabilizing or destabilizing effects, respectively.

CONCLUSION

From the data presented, it is concluded that, on heating of a 1:1 (v/v) mixture of milk and 65% (w/w) ethanol, the casein micelles dissociate. The aforementioned effect appears to be similar, or related, to the dissociating effect of TFE as reported by Horne and Davidson (*14*). From the results of the effect of ethanol on a model micellar system (β-casein) it appears that this phenomenon is due to protein−solvent interactions and an increase in solvent quality on the addition of ethanol at elevated temperatures. In the accompanying paper the molecular state (mobility/solubility) of the caseins in the presence of alcohol is examined in further detail and a mechanism for the effect of alcohol is presented.

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