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Structure and stability of nanogel particles prepared by internal cross-linking of casein micelles

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Abstract

Cross-linking all caseins within the casein micelles with the enzyme transglutaminase creates nanogel particles consisting of a covalently linked casein network from which micellar calcium phosphate (MCP) can be removed without compromising structural integrity. These casein nanogel particles show similar light, neutron and X-ray scattering behaviour to native casein micelles, indicating similarity in size and substructure. Casein nanogel particles are more stable to heat-induced coagulation, but less stable to acid-induced coagulation than native casein micelles. Changing the MCP content of casein nanogel particles to levels between 0% and 150% of its original concentration strongly affected colloidal stability of the particles. Stability to both heat- and acid-induced coagulation increased with decreasing MCP content. Casein nanogel particles offer applications not only in traditional dairy products, but also in products and applications where the integrity and biocompatibility of the nanogel particle is important.

Keywords: Casein micelle; Nanogel; Transglutaminase; Micellar calcium phosphate

1. Introduction

In bovine milk, caseins constitute the main protein fraction and are present predominantly in the form of hydrated association colloids called casein micelles. Casein micelles contain ~3.4 g H_2O g^{-1} dry matter (Morris, Foster, & Harding, 2000), which consists of ~94% protein and ~6% inorganic materials, referred to as micellar calcium phosphate (MCP; de Kruif & Holt, 2003). Casein micelles have an average radius of ~100 nm (de Kruif & Holt, 2003) and a micelle of this size is thought to contain ~900 structural elements called nanoclusters (Holt, de Kruif, Tuinier, & Timmins, 2003), which contain a core of amorphous calcium phosphate, surrounded by a shell of α_{s1}-, α_{s2}- and β-casein caseins, whose centres of phosphorylation participate in the core via ionic interactions (de Kruif & Holt, 2003; Little & Holt, 2004). The existence of these nanoclusters is supported by data of neutron and X-ray scattering (de Kruif & Holt, 2003; de Kruif et al., in preparation; Holt et al., 2003; Marchin, Putaux, Pignon, & Leonil, 2007; Pignon et al., 2004). The question how nanoclusters associate to form casein micelles is still a matter of debate; cross-linking of nanoclusters by caseins containing multiple centres of phosphorylation and weak protein–protein interactions, including hydrophobic and electrostratic interactions and hydrogen bonding, are likely to be involved (de Kruif & Holt, 2003; Horne, 1998). The micelles are sterically stabilized by what is traditionally termed a 'hairy layer' of κ-casein, and can be considered a polyelectrolyte brush in a medium of high ionic strength (de Kruif & Zhulina, 1996). Colloidal instability of casein micelles can be induced by removal or collapse of the brush and is reviewed by Holt and Horne (1996) and de Kruif (1999).

Due to its abundance and relative ease of isolation, the industrial potential of caseins in food and non-food applications is widespread. In food products, caseins, mostly in the form of caseinates, are used to improve functional properties, such as water binding, viscosity,

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structure, texture, emulsification and foaming (Rollema, 2003). Non-food uses of caseins include casein glue, casein wool, casein bone or ivory and casein photoresist, to produce the so-called shadow masks in TV-tubes (de Kruif, 2003). In this article, we will present a more recent application of caseins, i.e., as biocompatible nanogel particles.

Nanogel (1–1000 nm) and microgel (1–1000 μm) particles are gel particles prepared from synthetic or natural polymers (Hans & Lowman, 2002; Stieger, Pedersen, Linder, & Richtering, 2004a; Stieger & Richtering, 2003; Stieger, Richtering, Pedersen, & Lindner, 2004b). These particles have become an increasingly important area in research in drug delivery, because of their ability to deliver a wide range of drugs to various parts of the body for a sustained period of time (Hans & Lowman, 2002). Other applications of nano- or microgel particles include coatings and adhesives, composite manufacture and low-shrinkage dental polymers. For many of their intended uses, the particles required extremely high stability, which is derived from intra-particle cross-linking of the polymers (Hans & Lowman, 2002).

In this article, we studied nanogel particles prepared by enzymatic cross-linking of micellar caseins. Due to their low level of secondary and tertiary structure, caseins form excellent substrates for cross-linking by transglutaminase (TGase), which can covalently link protein-bound glutamine and lysine residues. The rate of cross-linking of caseins is higher in sodium caseinate than in skim milk (Lorenzen, 2000). In sodium caseinate, where the caseins exist in small solvent-mediated aggregates, the susceptibility of caseins to cross-linking decreases in the order κ-CN > α_s-CN > β-CN (Tang, Yang, Chen, Wu, & Peng, 2005), whereas in milk, where casein micelles predominate, the rate of cross-linking decreases in the order κ-CN > β-CN > α_s-CN (Hinz, Huppertz, Kulozik, & Kelly, 2007; Sharma, Lorenzen, & Qvist, 2001; Smiddy, Martin, Kelly, de Kruif, & Huppertz, 2006). Enzymatic cross-linking of milk proteins is reviewed in detail by Jaros, Parschefeld, Henle, and Rohm (2006a). Cross-linking progressively increases the intra-micellar stability (O'Sullivan, Kelly, & Fox, 2002b; Smiddy et al., 2006) and cross-linking of all caseins converts casein micelles from association colloids to nanogel particles (Huppertz, Smiddy, & de Kruif, 2007). The studies presented in this article provide further information on the structure and colloidal stability of these casein nanogel particles.

2. Materials and methods

2.1. Sample preparation

Skim bovine milk was prepared by reconstituting Nilac low-heat skim milk powder (NIZO Food Research, Ede, The Netherlands) in demineralised water at a level of 9 g 100 g^{-1}. Serum protein-free milk was prepared by reconstituting serum-protein free skim milk powder, prepared by diafiltration (see Huppertz et al., 2007), in demineralised water at a level of 8.4 g 100 g^{-1}. Milk dialysate was prepared by exhaustively dialysing a 50 g L^{-1} lactose solution against 2 × 20 volumes of reconstituted skim milk for 48 h at 20 °C. Sodium azide (0.5 g L^{-1}) was added to all samples to prevent microbial activity.

For dynamic and static light scattering and small-angle X-ray scattering measurements, residual lipid globules were removed from the serum protein-free micelle suspensions. For this purpose, casein micelles were pelleted by ultracentrifugation, resuspended in milk dialysate, followed by adjustment of the casein content to 2.5 g 100 g^{-1}. This procedure is outlined in detail by Huppertz et al. (2007). Samples prepared as such will be referred to as casein micelle suspensions.

2.2. Enzymatic cross-linking

Samples of milk, serum protein-free milk or casein micelle suspensions were pre-warmed to 30 °C and Activa TG TGase (declared activity 1000 unit g^{-1}; Ajinomoto, Hamburg, Germany) was added at a level of 0.5 g L^{-1}. Samples were incubated at 30 °C for 24 h prior to inactivation of the TGase by heating at 70 °C for 10 min. Samples were subsequently cooled to 20 °C in ice-water. A control sample, without added TGase, was treated in the same manner in all cases. SDS–PAGE under reducing conditions was used to determine the level of residual monomeric casein in the sample.

2.3. MCP-adjustment

For adjustment of the MCP content, TGase-treated samples were cooled to 5 °C, followed by adjustment of their pH to ~4.5 or 5.5, 6.0 with 1 M HCl or to 8.0 or 9.0 with 1 M NaOH; a control sample was kept at its original pH of ~6.6. Samples were subsequently subjected to dialysis against 2 × 20 volumes of skim milk for 48 h at 5 °C. The concentration of total, micellar and non-micellar calcium was determined by atomic absorption spectroscopy, as described by Huppertz et al. (2005).

2.4. Dynamic and static light scattering

Following 100-fold dilution in milk dialysate, filtration (5 μm pore size) and centrifugation (1000 × g for 5 min), suspensions of native and cross-linked casein micelles were analysed simultaneously by dynamic light scattering (DLS) and static light scattering (SLS) using an ALV Compact Goniometer System (ALV-Laser; Vertriebsgesellschaft Gm-bH, Langen, Germany) equipped with four detector units (ALV/GCS-4) and two ALV-5000/E multiple tau digital correlators. A Coherent Verdi V2 diode-pumped laser (Coherent, Inc., Santa Clara, CA, USA) was used, operating with vertically polarised light with a wavelength of $\lambda = 532.0$ nm. Measurements were performed at 16 or 32 scattering wave vectors in the range