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Heat induced formation of beta-lactoglobulin microgels driven by addition of calcium ions

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ABSTRACT

Stable suspensions of spherical protein particles (microgels) can be formed by heating beta-lactoglobulin solutions in the presence of calcium ions. The conditions for the calcium induced microgel formation were studied at different pH between 5.8 and 7.5 and different protein concentrations between 5 and 100 g/L after heating at 85 °C for 15 h. The results showed that a critical molar ratio of calcium to proteins (R) is needed to form microgels independent of the protein concentration. R decreases with decreasing pH. The microgels have a hydrodynamic radius ranging from 100 to 300 nm and their internal protein concentration ranges from 0.2 to 0.45 g/mL. The amount of calcium bound to the proteins was determined and the results suggest that the crucial parameter for microgel formation is the net charge density of the native proteins. The microgel suspensions are stable in a narrow range of R but the microgels aggregate at higher calcium concentrations.

1. Introduction

Beta-lactoglobulin (β-lg), which is the major whey protein in cow's milk, is a globular protein with a molar mass of 18.2 kg/mol, a radius of about 2 nm and an iso-electric point pI = 5.2 (Hambling, Mc Alpine, & Sawyer, 1992). In aqueous solution, β-lg denatures when heated and aggregates or even forms a gel if the concentration is sufficiently high. Heat-induced aggregation of β-lg has been studied extensively in past, see for a recent detailed review (Nicolai, Britten, & Schmitt, 2011). The morphology of the aggregates depends strongly on the pH. In salt free solutions large rigid rod-like aggregates are formed at pH 2–3, whereas closer to the iso-electric point the aggregates are spherical. At pH > 6.3 small strands are formed at low concentrations that associate at higher protein concentrations into larger randomly branched aggregates with a self-similar structure (Aymard, Gimel, Nicolai, & Durand, 1996; Pouzot, Nicolai, Visschers, & Weijers, 2005).

At and very close to pI, β-lg aggregates do not form stable suspensions, but associate and eventually precipitate. However, it was found that in a narrow range of the pH just above pI (around pH 5.9) and just below pI (around pH 4.6) stable suspensions of rather monodisperse spherical particles were formed with a radius of about a hundred nanometres (Schmitt et al., 2009; Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008; Donato, Schmitt, Bovetto, & Rouvet, 2009; Moitzi et al., 2011). These spherical particles were called microgels and can also be formed by whey protein isolate (WPI) that contains a majority of β-lg together with other globular proteins such as α-lactalbumin (Schmitt et al., 2010). In a recent study (Phan-Xuan et al., 2011), we have shown that the pH is a crucial parameter for the formation of stable microgel suspensions in pure water. Stable suspensions are only obtained if the pH is set between 5.75 and 6.2 before heating and involves a process of self-stabilization.

The presence of calcium ions enhances heat induced aggregation of β-lg (Sherwin & Foegeding, 1997; Xiong, Dawson, & Wan, 1993), but the specific interaction between Ca^{2+} and β-lg is still poorly understood at the molecular level (Simons, Kosters, Visschers, & de Jongh, 2002). It has been suggested that three effects or a combination of them might be responsible for calcium-induced protein aggregation:

- Intermolecular cross linking of adjacent negatively charged or carboxylic groups by the formation of protein-Ca^{2+}-protein complexes (Bryant & Mcclements, 1998; Hongsprabhas, Barbut, & Marangoni, 1999; Xiong et al., 1993)
- Reduction of the net negative charge of the proteins by binding of calcium ions (Hongsprabhas & Barbut, 1997)

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- Ion induced conformation changes, which lead to altered hydrophobic interactions and aggregation at elevated temperatures (Kinsella, Whitehead, Brady, & Bringe, 1989; Wang & Damodaran, 1991).

However, (Xiong et al., 1993) demonstrated that the role of Ca^{2+} in the formation of intermolecular bridges was unlikely and that the main effect of Ca^{2+} is to reduce the net protein charge. Excess Ca^{2+} may even have an inhibitory effect on the protein aggregation rate (Roefs & Peppelman, 2001).

Sherwin & Foegeding (1997) demonstrated that aggregation rates were affected by $CaCl_2$/protein stoichiometry rather than the Ca^{2+} and protein concentrations separately. Simons et al. (2002) suggested that calcium was bound to carboxylates with a threshold affinity. Subsequent site specific screening of surface charges resulted in protein aggregation, driven by partial unfolding of β-lg at elevated temperatures, which was facilitated by the absence of electrostatic repulsion.

The aim of the study presented here was to investigate the effect of adding calcium ions on the formation of stable β-lg microgel suspensions. We will first discuss in detail the effect of adding $CaCl_2$ on heat induced aggregation at pH 6.9 and then show the influence of the pH between 6.0 and 7.5. The size and the density of the microgels formed at different conditions were determined using light scattering techniques. Finally, we will discuss the mechanism of the formation and stable β-lg microgel suspensions in the presence of calcium ions and propose a process of self stabilization analogous to that in pure water at lower pH.

2. Materials and methods

2.1. Materials

The β-lactoglobulin (Biopure, lot JE 001-8-415) used in this study was purchased from Davisco Foods International, Inc. (Le Sueur, MN, USA). The powder contained about 89.6 wt% protein (Kjeldahl, N x6.38) and the protein composition was 55.4% and 41.6% of the variants A and B, respectively, and less than 2% of other whey proteins (based on HIC-HPLC analysis) (Donato et al., 2009). The powder was dissolved in salt free Milli-Q water with 200 ppm NaN_3 added to avoid bacterial growth. The solutions were dialysed against the solvent for a period of 8 h with 4 exchanges of the solvent. The pH was set to the desired value by drop wise addition of 0.1 M HCl or NaOH under vigorous stirring and aliquots of 0.1 M or 0.5 M $CaCl_2$ were added to reach the desired concentration. Because addition of $CaCl_2$ reduces the pH of the solution, we used small amounts of 0.1 M NaOH to bring the pH back to the initial value. The amount of NaOH used to readjust the pH was noted for the calculation of the net charge of protein. The solutions were filtered through 0.2 μm pore size Anotop filters before heating.

The native protein concentration was measured after filtration by UV absorption at 278 nm using an extinction coefficient of 0.96 $Lg^{-1}cm^{-1}$(Townend, Winterbottom, & Timasheff, 1960). Solutions were heated at 85 °C for a period between 5 and 15 h in air tight cylindrical glass vials (7 mL) with a diameter of 10 mm using a thermostat water bath. The heating rate was fast as the set temperature was reached within 4 min. The samples were cooled rapidly to 20 °C by holding the vials under running tap water. The fraction of residual native protein after heating was determined by precipitation at pH 4.6 and measuring the UV absorption of the supernatant. We checked by light scattering that no aggregates were present in the supernatant.

Heating led to a small decrease of the pH when it was set above 6.9 (from 7.5 to 7.1–7.3 and from 7.2 to 7–7.15) and a small increase of the pH when it was set below 6.9 (from 6 to 6.4–6.6, from 6.2 to 6.6–6.7 and from 6.4 to 6.7–6.9) as was reported for salt free solutions by (Donato et al., 2009)

2.2. Dynamic and static light scattering

Dynamic and static light scattering measurements were done using a commercial apparatus (ALV-Langen, Germany). The light source was a He–Ne laser with wavelength $\lambda = 632$ nm. The temperature was controlled by a thermostat bath to within ±0.2 °C. Measurements were made at angles of observation (θ) between 12 and 150°. The relative scattering intensity (I_r) was calculated as the intensity minus the solvent scattering divided by the scattering intensity of toluene at 20 °C.

In dilute solutions I_r is related to the weight average molar mass (M_w) and the scattering wave vector (q) dependent structure factor ($S(q)$) of the solute (Nicolai, 2007) (Brown, 1996):

$$I_r/KC = M_w S(q) \tag{1}$$

with K an optical constant depending on the refractive index increment. We have used for the refractive index increment 0.189 mL/g. The structure factor describes the dependence of the intensity on the scattering wave vector (q) and depends on the structure and the size of the solute. The z-average radius of gyration (R_g) can be determined from the initial q-dependence of $S(q)$:

$$S(q) = \left[1 + \frac{q^2 R_g^2}{3}\right]^{-1} \quad qR_g \leq 1 \tag{2}$$

The intensity autocorrelation function was measured with dynamic light scattering (DLS) (Berne & Pecora, 1993). In all cases the correlation functions could be described in terms of a narrow relaxation time distribution. The average relaxation rate (Γ) was found to be proportional to q^2. In dilute solutions the relaxation is caused by self diffusion of the particles and Γ is related to the diffusion coefficient (D): $\Gamma = (q^2 \cdot D)$. The average hydrodynamic radius (R_h) may be calculated using the Stokes–Einstein equation:

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot R_h} \tag{3}$$

with η the viscosity, k Boltzman's constant and T the absolute temperature. The density of the particles was calculated from M_w and R_h by assuming that they were spherical:

$$\rho = \frac{3M_w}{4 \cdot \pi \cdot R_h^3 N_{av}} \tag{4}$$

with N_{av} Avogadro's number.

2.3. Confocal Laser Scanning Microscopy (CLSM)

CLSM was used in the fluorescence mode. Observations were made with a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany). A water immersion objective lens was used HCxPL APO 63x NA = 1.2 with theoretical resolution of 0.3 μm in the x–y plane. A small fraction of β-lg was labelled with the fluorochrome rhodamine B isothiocyanate, by adding a small amount of a concentrated rhodamine solution (5 ppm) to the β-lg solutions before heat treatment. No effect of labelling on the aggregation process was observed.