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The effect of aggregation into fractals or microgels on the charge density and the isoionic point of globular proteins

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ABSTRACT

Potentiometric titration curves were obtained for aqueous solutions of native whey protein isolate (WPI) and β-lactoglobulin (β-lg) at different NaCl concentrations. The curves crossed at approximately the same pH, which is considered to be the isoionic point (IIP). Titration with NaCl confirmed that the pH was independent of the salt concentration up to 0.1 M at the IIP, which was 4.9 for WPI and 5.0 for β-lg. Fractal aggregates and microgels of different sizes were formed by heating protein solutions at different pH and different concentrations. The titration curves of the aggregates depended on the type of aggregates, but not on their size. The IIP increased by at most 0.2 pH units after aggregation. For a given pH larger than IIP, the charge density of the proteins (α) was reduced after denaturation and aggregation. The reduction was stronger for microgels than for fractal aggregates. Addition of NaCl or increasing the protein concentration mitigated the effect. Comparison between WPI and β-lg showed that the pH dependence of α was almost the same for pH > 5.0 both for native and aggregated proteins.

1. Introduction

Globular proteins have a dense well-defined structure in aqueous solutions. The net charge of proteins (α) is negative at high pH and positive at low pH, but over a wide pH range they contain both negatively and positively charged amino acids. The pH where $\alpha = 0$ in the absence of adsorbed ions is called the isoionic point (IIP) and depends on the type of protein (Bryan, 1978; Cannan, 1942; Longsworth & Jacobsen, 1949; Salis et al., 2011). When globular proteins are heated their relatively rigid structure becomes more mobile, which allows the formation of bonds with other proteins causing aggregation (Amin, Barnett, Pathak, Roberts, & Sarangapani, 2014; Mezzenga & Fischer, 2013). It was found that the morphology of the aggregates depends on the pH (Krebs, Devlin, & Donald, 2007; Langton & Hermannsson, 1992; Nicolai & Durand, 2013). When the net charge of the proteins is high, relatively thin strands are formed, but when the pH is close to the IIP, spherical microgels are formed. At higher protein concentrations, the strands or the microgels will randomly crosslink into self-similar clusters that increase in size when the protein concentration is increased until at a critical concentration a system spanning network is formed (Clark, Kavanagh, & Ross-Murphy, 2001; van der Linden & Foegeding, 2009).

Experimentally, the effect of α on the aggregation process is studied by varying the pH. However, it is long since known that the charge density of proteins at a given pH depends on the ionic strength, the type of ions, and the protein concentration (Bryan, 1978; Cannan, 1942; Cannan, Kibrick, & Palmer, 1941; Longsworth et al., 1949; Nozaki, Bunville, & Tanford, 1959; Sørensen, Linderstrøm-Lang, & Lund, 1927; Tanford & Wagner, 1954). Therefore, when aggregation is compared at different concentrations or ionic strengths at a fixed pH value, α is not kept constant and, consequently, observed effects of the protein or salt concentration on aggregation for a given pH can be caused in part by a change of the charge density. In order to relate the aggregation process of proteins to their net charge density it is important to know how α relates to the pH at the conditions at which the aggregation occurred. In addition, aggregation of proteins may affect their buffer capacity and therefore the relationship between the pH and α. As far as we are aware, no systematic investigation of the effect of heat-induced aggregation on the relationship between the pH and the net charge density of globular proteins has been reported in the literature.

The effect of the pH and the ionic strength on heat-induced aggregation of globular proteins has probably been most intensively investigated for β-lactoglobulin (β-lg) and whey protein

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isolate (WPI) (Guyomarc'h et al., 2014; Kontopidis, Holt, & Sawyer, 2004; Nicolai, Britten, & Schmitt, 2011). The latter contains mainly β-lg, but also other types of whey proteins (De Wit, 1998). Here we present a systematic investigation of the relationship between the pH and the charge density of native and aggregated β-lg and WPI as a function of the NaCl and the protein concentration using potentiometric titration. We will discuss the effect of the pH on aggregation of whey proteins reported in the literature in the light of the results that were obtained in the present study.

2. Materials and methods

The β-lg (Biopure, lot JE 001-8-415) used in this study was purchased from Davisco Foods International, Inc. (Le Sueur, MN, USA). The β-lg powder contained 90.3 wt% protein (measured from Kjeldhal analysis: N 6.38) of which 97% was β-lg with variants A and B in approximately equal quantities. It contained 5 wt% moisture, 0.3 wt% fat, <1 wt% lactose, and 1.8 wt% ash (including 0.02 wt% Ca, 0.09 wt% K, 0.02 wt% Mg, 0.65 wt% Na and 0.51 wt% P, determined by atomic absorption spectroscopy). The WPI powder was purchased from Lactalis (Laval, France). It contained 92% protein of which 70% β-lg and 20% α-lac as determined by size exclusion chromatography. It contained 5 wt% moisture, <0.4 wt% fat, <4 wt% lactose, and 2.0 wt% ash. The powders were dissolved in Milli-Q water while stirring overnight. The protein concentration was determined by UV absorption at 278 nm using extinction coefficients of 0.96 L g^{-1} cm^{-1} and 1.05 L g^{-1} cm^{-1} for β-lg and WPI, respectively.

Fractal whey protein aggregates were formed by heating salt-free WPI solutions at 80 °C and neutral pH until the reaction was finished. Stable suspensions of fractal aggregates of two different sizes were obtained by heating protein solutions at C = 62 g/L and C = 90 g/L. WPI microgels of two different sizes were obtained by heating WPI solutions at C = 40 g/L at pH 5.8 and at pH 6.1. In all cases the amount of HCl or NaOH needed to set the pH was carefully recorded and used to calculate the effect on the charge density. The average molar mass (M_w) and hydrodynamic radius (R_h) were determined with static and dynamic light scattering using the same methodology as described by Phan-Xuan et al. (2011).

Titration experiments were conducted at room temperature using an automatic titrator (TIM 856, Radiometer Analytical) equipped with a combined pH electrode and a temperature probe. The pH electrode was calibrated by a three-point calibration using standard buffers. When the pH of protein solutions was checked with a different pH-meter and a different probe it was found to be the same within 0.05 units. The pH of all samples was first set to 8.0 by adding a standard solution of NaOH (0.1 M or 1 M, depending on the protein concentration) and then titrated to pH 4 with a standard solution of HCl (0.1 M or 1 M). A number of protein solutions at set pH values close to the IIP were titrated with a 2 M NaCl solution. Titrations were done at a rate of 0.1 mL per min, but no significant difference was observed when a lower rate was used.

The net charge density of the proteins as a function of the pH was deduced as follows. The number of H^+ bound or released per protein was calculated from the volume of the added NaOH and HCl solutions (V):

$$\Delta\alpha = \frac{V(HCl) \times [HCl] - V(NaOH) \times [NaOH]}{m/M_w}, \quad (1)$$

where m is the mass of titrated whey proteins, [HCl] and [NaOH] are the concentrations of the HCl and NaOH solutions and M_w is the average molar mass of the whey proteins ($M_w = 1.75 \times 10^4$ g/mol for WPI and 1.86×10^4 g/mol for β-lg). Notice that the amount of H^+ in the water is negligible in the pH range covered in the experiment. The dependence of the pH on α was subsequently calculated by taking α = 0 at the IIP:

$$\alpha = \Delta\alpha - \Delta\alpha(\mathrm{IIP}). \quad (2)$$

3. Results

3.1. Native proteins

β-lg solutions at C = 10 g/L with different NaCl concentrations ([NaCl]) were titrated as described in the materials and methods and were found to cross at approximately pH 5, see Fig. 1a. Crossing of the titration curves at a single value of the pH has earlier been reported for pure β-lg (Cannan, Palmer, & Kibrick, 1942), but also

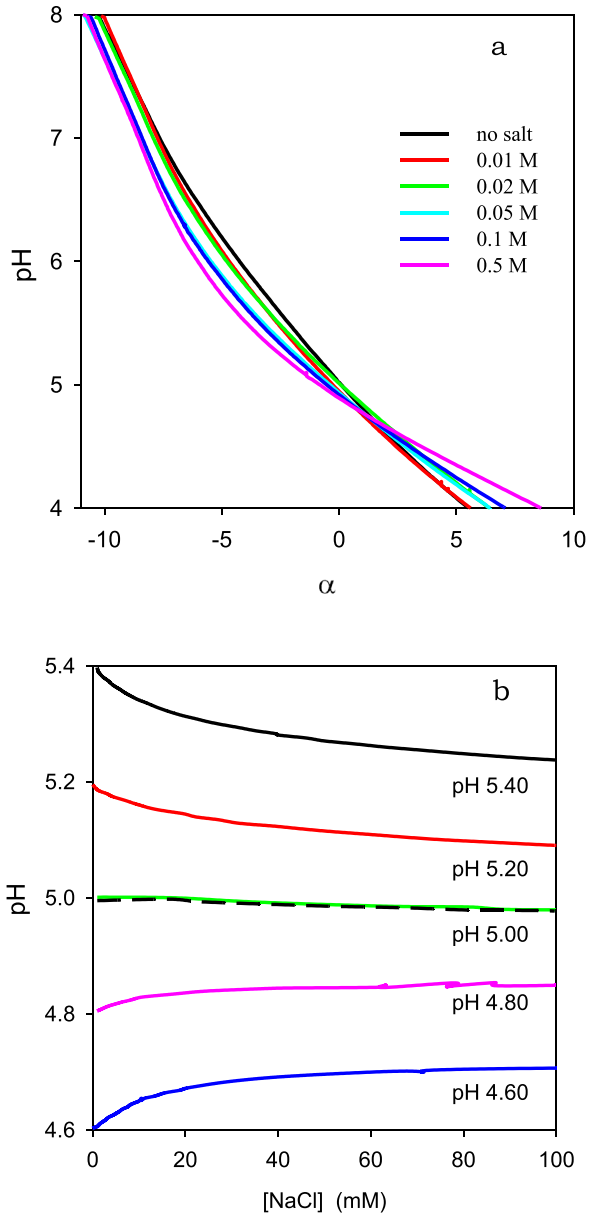

Fig. 1. a. pH as a function of the net charge density for β-lg solutions at C = 10 g/L at different NaCl concentrations indicated in the figure. Fig. 1b. pH as a function of the NaCl concentration for β-lg solutions at C = 10 g/L set at different pH before titration. The dashed line represents result for C = 20 g/L.