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Acid-responsive properties of fibrils from heat-induced whey protein concentrate

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ABSTRACT

The heat-induced fibrils of whey protein concentrate (WPC) have demonstrated an acid-responsive property; that is, the fibrils went through formation-depolymerization-reformation as pH was adjusted to 1.8, 6.5, and back to 1.8. We investigated the microstructure, driving force, and thermal stability of 3.0% (wt) WPC nanofibrils adjusted between pH 6.5 and 1.8 twice. The results showed that the nanofibrils had acid-responsive properties and good thermal stability after reheating for 10 h at 90°C and adjusting pH from 1.8 to 6.5 to 1.8. The content of WPC fibril aggregates was not much different with the prolongation of heating times during pH variation. Although the nanofibrils' structure could be destroyed only by changing the pH, the essence of this destruction might only form fiber fragments, polymers that would restore a fibrous structure upon returning to pH 1.8. A described model for the acid-responsive assembly of fibrils of WPC was proposed. The fibrils went through formation-depolymerization-reformation by weaker noncovalent interactions (surface hydrophobicity) as pH changed from 1.8 to 6.5 back to 1.8. However, the fibrils lost the acid-responsive properties because much more S-S (disulfide) formation occurred when the solution was adjusted to pH 6.5 and reheated. Meanwhile, fibrils still possessed acid-responsive properties when reheated at pH 1.8, and the content of fibrils slightly increased with a further reduction of α-helix structure.

Key words: whey protein concentrate, fibrils, microstructure, acid responsive

INTRODUCTION

The study of protein aggregation has been important in relation to food production and biotechnology (Bauer et al., 2000). Many globular proteins have shown the ability to form long, thin fibrillar aggregates at pH 2.0 and low ionic strength, such as β-LG (Kavanagh et al., 2000; Ikeda and Morris, 2002; Veerman et al., 2002, 2003), whey protein concentrates (**WPC**; Wang et al., 2013), and whey protein isolates (**WPI**; Durand et al., 2002; Gosal et al., 2002; Arnaudov et al., 2003), after heating at high temperature for several hours. Arnaudov et al. (2003) described fibrils of β-LG at acidic pH in 3 main stages: an initial unfolding step, a step of linear fibrillar aggregation via nucleation and growth, and finally a step of random association of the fibrils. Ionic strength and pH also affect both the kinetics of fibril formation and the morphology of fibrils (Aymard et al., 1999; Bolder et al., 2006; Arnaudov and de Vries, 2007). With heat treatment at 80°C, added NaCl substantially accelerated β-LG denaturation at pH 2.5 (Schokker et al., 2000). Flexible fibrillar networks are formed at pH 3.35, and more branching is observed with an increase in $CaCl_2$, equating to an increase of ionic strength of 60 mM (Mudgal et al., 2009). Fibrils become shorter and more flexible and have a lower critical percolation concentration of β-LG fibril gel with increasing ionic strength (Mudgal et al., 2011). Surface hydrophobicity plays a dominant role in the formation of fibrils aggregates. Except the hydrophobic interactions, other noncovalent interactions such as ionic bonds, van der Waal's force, and hydrogen bonds also stabilize the formation of fibrils, whereas disulfide interchange reactions have been shown to be inhibited at low pH (McKenzie et al., 1972). Disulfide bonding between β-LG molecules does not occur to any significant extent because cysteine residues are predominantly protonated (Otte et al., 2000; Alting et al., 2002). The formation of heat-induced nanofibrils is due to noncovalent interactions; therefore, its aggregation properties might be different from normal protein aggregates. The objective of the present study was to investigate the variation of properties between nanofibrils and normal protein aggregates of WPC through adjusting pH (pH 1.8, 6.5) and reheating (90°C, 10 h). Finally, a described model for the assembly and depolymerization of WPC fibrils was proposed at pH between 1.8 and 6.5.

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MATERIALS AND METHODS

Materials

The WPC-80 (76.93% protein, 1.4% fat, 5.6% lactose, 4.62% ash) was purchased from Hilmar Cheese Co. (Hilmar, CA). Thioflavin T and 1-anilino-8 naphthalene sulfonate were purchased from Sigma-Aldrich (St. Louis, MO). 2,2'-Dinitro-5,5′-dithiodibenzoate (**DTNB**) was purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were of analytical grade. All the reported results were averages of 3 separate experiments.

Solution Preparation

The WPC powder was dissolved in double-distilled water, and, after dissolving, the solution was adjusted to pH 2.0 by adding 6 *M* HCl. To remove any undissolved protein, the solution was centrifuged at 19,000 × *g* (GL-21M centrifuge, Shanghai Precision Instruments Co. Ltd., Shanghai, China) for 30 min at 4°C. The nitrogen content of supernatants were determined by Kjeldahl analysis (N × 6.38; KDN-102C, Shanghai Qian Jian Instruments Co. Ltd.) and the supernatant was diluted into 3.0% (wt) solution; to adjust pH to 1.8 (6 *M* HCl, 0.1 *M* HCl), solutions were heated at 90°C for different periods of time (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h) in a water bath. After heat treatment, the samples were immediately cooled to room temperature by immersing in ice bath to form WPC nanofibrils (Ko and Gunasekaran, 2006; Bolder et al., 2007; Akkermans et al., 2008). Normal protein aggregates (pH 6.5) were formed by the same methods, except for adjusting pH (before centrifuging and heating treatment).

Transmission Electron Microscopy

The microstructure of heated protein dispersions was investigated by transmission electron microscopy (H-7650, Hitachi High-Technologies Corporation, Krefeld, Germany) according to the procedure of Krebs et al. (2009), with some modifications. Protein dispersion was diluted to 0.3% (wt) in Millipore water (Simplicity Purification System, Millipore, France), and a droplet of the diluted sample was loaded onto an amorphous carbon film supported by a copper grid. After 15 s, the droplet was removed with a filter paper. The samples were studied by transmission electron microscopy (**TEM**), operating at 80 kV.

Turbidity

The turbidity of dispersions was measured using a UV spectrophotometer (TU-1800, Beijing Precision Instruments Co. Ltd., Beijing, China) according to the procedure of Kurganov (2002), with some modifications; the path length of sample cell used was 10 mm. The protein samples were diluted to the concentration of 1.5% (wt) in double-distilled water, vortexed, and had absorbance measured at 400 nm and room temperature; the double-distilled water was used as the blank and turbidity values were represented through absorbance values. All measurements were performed in triplicate.

Protein Surface Hydrophobicity

The surface hydrophobicity of the protein samples was determined by the method of Tubio et al. (2004). Protein dispersions were diluted with 0.01 *M* phosphate buffer (pH 6.7), containing samples of varying total WPC (0.02, 0.01, 0.005, and 0.0025%, wt/vol) added to aliquots (20 μL) of 1-anilino-8 naphthalene sulfonate (8.0 mmol/L in the same buffer), vortexed, and kept in the dark for 20 min. The fluorescence emission intensities at 470 nm (whereas excitement was 390 nm) for each protein concentration were measured with fluorescence spectrophotometer (F-4500, Hitachi High-Technologies Corporation). The initial slope of the plot of fluorescence intensity versus protein concentration, which was calculated by linear regression (in all cases, R^2 >0.95), was used as an index of the surface hydrophobicity of the protein sample evaluated. All measurements were performed in triplicate.

Determination of Free Sulfhydryl Group

Free sulfhydryl group (SH) contents of protein samples were determined by the method of Shimada and Cheftel (1989), with some modifications. For free sulfhydryl group content determination, 5 mL of the Tris-Gly buffer (0.086 *M* Tris, 0.09 *M* glycine, 0.004 *M* EDTA, pH 8.0) containing 8 *M* urea were added to 0.3-mL protein samples (30 mg/mL). Then, 20 μL of DTNB (Merck) was added and absorbance was measured at 412 nm with UV spectrophotometer (UV-2401 PC, Shimadzu Corporation, Kyoto, Japan) after 15 min. The supernatants in buffer without DTNB were used as blanks. The calculation was as follows:

$$\mathrm{SH}(\mu\mathrm{mol/g}) = \frac{(73.53 \times A_{412} \times D)}{C}, \quad [1]$$