JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Physical Properties, Molecular Structures, and Protein Quality of Texturized Whey Protein Isolate: Effect of Extrusion Temperature

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ABSTRACT: Although extrusion technology has contributed much to increasing the effective utilization of whey, the effect of extrusion conditions on the functional properties of the proteins is not well understood. In this work, the impact of extrusion temperature on the physical and chemical properties, molecular structures, and protein quality of texturized whey protein isolate (WPI) was investigated at a constant moisture content and compared with WPI treated with simple heat only. The Bradford assay methods, sodium dodecyl sulfate−polyacrylamide gel electrophoresis, and reversed-phase high-performance liquid chromatography techniques were used to determine protein solubility and to analyze compositional changes in the two major whey proteins, α-lactalbumin and β-lactoglobulin. Circular dichroism and intrinsic tryptophan fluorescence spectroscopic techniques were applied to study the secondary and tertiary structures of the proteins. This study demonstrated that extrusion temperature is a critical but not the sole determining factor in affecting the functional properties of extruded WPI.

KEYWORDS: extrusion, texturization, whey protein isolate, α-lactalbumin, β-lactoglobulin, molecular structure, temperature, protein quality, CD, fluorescence

INTRODUCTION

Whey, a byproduct of cheesemaking and casein manufacture in the dairy industry, accounts for about 20% (w/w) of the total milk protein. It was estimated[1] that nearly 150 million tons of whey was generated from the production of cheese worldwide in 2005 alone. While the environmental impact of whey is well-known, this dairy stream also represents an excellent source of functional proteins, peptides, lipids, vitamins, minerals, and lactose, which only have been recognized relatively recently.[2−5] Research on whey utilization has largely focused on protein and peptide components of whey and their numerous chemical, physiochemical, and bioactive properties, which has helped greatly transform whey from a waste material that is often undesirable to a valuable dairy commodity containing a multitude of components available for exploitation in the food, biotechnology, medical, and biodegradable material related markets.[3,5−9]

Whey is a complex mixture of globular protein molecules comprised of β-lactoglobulin (β-LG; ∼50 wt %/wt), α-lactalbumin (α-LA; ∼20 wt %/wt), immunoglobulins (IgG; <10 wt %/wt), and bovine serum albumin (BSA; <6 wt %/wt) and other minor protein/peptide components including lactoferrin, lactoperoxidase, lysozyme, and growth factors.[10] Both the three-dimensional molecular structures and the thermal-induced folding/unfolding behaviors of β-LG and α-LA in its pure form have been characterized extensively.[11−13] Whey protein isolate (WPI), one of the most important whey protein products, is a commercial powder with a high protein content (>90 wt %/wt) and is normally manufactured by either ion exchange chromatography or microfiltration, followed by spray drying, a well-established industrial method for converting liquid feed materials into a dry powder form.[14] During the spray-drying process, the liquid feed comes in contact with hot gas, and evaporation takes place to yield dried particles, which are subsequently separated from the gas stream by a variety of methods. Although possible losses in many functional properties of WPI, notably, solubility and gelling, can occur due to high-temperature processing, spray drying is still a preferred method for producing whey protein in powder form.[6,15,16]

Past research[17−22] has yielded a wealth of useful information on heat-induced molecular structural changes in whey proteins in model solutions as well as in whey and milk systems under a wide range of experimental conditions including concentration, pH, and ionic strength. It is generally recognized that in the heated mixture of α-LA and β-LG at neutral pH, both physical changes, for example, ionic, van der Waals, and hydrophobic interactions, and chemical reactions, for example, thiol−disulfide bond scramble and exchange on the intramolecular and intermolecular levels, occur leading to denaturation and aggregation.[15,21,23−26] The sensitive behavior of whey proteins upon heating is of particular interest because when properly controlled, protein denaturation and aggregation often result in novel materials with many potential uses.[27] Micrometer-sized protein aggregates, for example, have been used to improve the creaminess and the texture of dairy-based products and as fat substitutes.[28]

Advances in processing technology have provided options in applying alternative treatments of whey such as extrusion to produce protein-enriched food and nonfood products, which has led to new and economically viable ways of increasing whey utilization. By using a twin-screw extruder, Onwulata et al.[29,30] have successfully incorporated whey protein (in the form of WPI) into snack products to increase their protein content (by ∼20%) and extend their nutritive value. During extrusion, high temperature and high shear force are applied to produce a product with unique physical and chemical characteristics. Product characteristics of extrudates can vary considerably

Received: September 30, 2010
Accepted: March 23, 2011
Published: March 23, 2011

depending on the extrusion processing conditions such as barrel temperature, die geometry, extruder type, feed composition, feed moisture, feed particle size, feed rate, screw configuration, and screw speed.[31] For example,[30] extrusion of WPI at 35 or 50 °C increased their gel strength, which was nearly lost when extruded at 75 or 100 °C. Despite the increased use of extrusion processing on whey proteins to create and to improve the desired functional properties of whey protein dispersions as structuring agents in protein-based foods, it is still difficult to predict structures, textures, and functionality resulting from a extrusion process.[31] Because of the complex and chaotic nature of extrusion processing and the multitude of variables involved, there is little available knowledge on the effect of process parameters on the chemical reactions taking place during extrusion and the structures, textures, and the nutritional implications associated with these changes.[31−33]

The objective of the present work was to investigate the effect of extrusion parameters, particularly temperature (as described in this work) and moisture content of the feed,[34] during extrusion texturization of WPI on the solubility, protein distribution, protein structures on both the secondary and the tertiary levels, and protein quality measured by available sulfhydryl (SH) and primary and secondary amines, as compared with the effect of heating only to gain a better understanding on the relationship between the extrusion conditions and the resulting functionality and protein conformational changes of the WPI extrudates.

MATERIALS AND METHODS

Materials. All reagents including NaCl, 5,5′-dithio-bis(2-nitrobenzioc acid) (DTNB), and ninhydrin reagent used in these studies, unless otherwise noted, were of the analytical grade or ACS certified from Sigma-Aldrich (St. Louis, MO). Tris Ultra Pure was purchased from ICN Biomedicals, Inc. (Cleveland, OH).

The WPI (Provon 190) in both frozen liquid and spray-dried powder forms used in this work were purchased from Glanbia Nutritionals, Inc. (Twin Falls, ID). The components of the WPI, according to the manufacturers' specifications, are as follows: moisture, 2.8%; protein, 89.6%; fat, 2.5; and ash, 3.3%. The frozen liquid form was freeze-dried upon arrival and stored at 4 °C for use as a control.

The total protein content of all of the extruded WPI samples used was analyzed using a standard nitrogen analyzer based on the micro-Kjeldahl method. A nitrogen conversion factor of 6.38 was used for calculation of whey protein contents. The total protein content for all samples was over 89%.

Sample Preparation. The extrusion texturization process for WPI has been described previously by Pordesimo and Onwulata.[35] The spray-dried WPI powder was extruded in a model ZSK30 twin-screw extruder (Krupp, Werner & Pfleiderer Co., Ramsey, NJ) consisting of nine-barrel (900 mm) long zones, each with individual temperature control. The screw elements were selected to provide low shear at 300 rpm; the screw profile was described by Onwulata et al.[36] Feed, the spray-dried WPI powder was conveyed into the extruder with a Series 6300 digital feeder, and water was then subsequently added using type T-35 twin-screw volumetric feeder (K-tron Corp., Pitman, NJ). The feed screw speed was set at 600 rpm, corresponding to a rate of 3.50 kg/h. The texturization temperature was varied at 5, 15, 25, 35, 50, 75, and 100 °C, and the moisture content was controlled at 50% for each extrusion temperature. It should be noted that variability in the moisture level within the extruder is likely present during extrusion. However, our previous work[34] demonstrated a negligible effect of moisture level on the physical properties, molecular structures, and protein quality. An electromagnetic dosing pump (Milton Roy, Acton, MA) was used to add water into the extruder at an input rate of 1.556 L/h (pump set) and pump settings ranging from 20 to 70 (1.3−6 L/h). Samples were collected after 25 min of processing, freeze-dried overnight in a VirTis Freeze Mobile 12XL research-scale freeze dryer (Gardiner, NY), and stored at 4.4 °C until used. All experiments were performed in triplicate.

The control samples were prepared by suspending the nonextruded spray-dried WPI powder (Provon 190) at 20% (w/w) in individual small (10 mL) vials prior to heating at various temperatures, 50, 75, and 100 °C using a quartered RCT magnetic stirring plate (IKA Works Inc., Wilmington, NC) equipped with an Ikatron ETS-D4 fuzzy electronic thermometer (for precision temperature control) for exactly 3 min, which is the same duration time as the extrusion texturization process (as described in this work), followed by lyophilization prior to analyses.

We carefully controlled all variables during each extrusion run and collected samples only after an adequate equilibrium had been established.

Protein Solubility. The protein solubility was determined according to a modified Bradford method.[37] Laboratory-purified β-LG, at least 90% (by SDS analysis), was used as the standard. The extinction coefficient 0.96 L/g cm[38] for β-LG was used to measure accurately the concentrations of the standard solutions. WPI samples were suspended at 3 (for low temperature extrudates, below 50 °C), 6 (75 °C), and 10% (100 °C) (w/w) in deionized Milli-Q water. Suspensions were vortexed for 10 min followed by centrifugation at 10000*g* using a benchtop microcentrifuge for 30 min at room temperature (20 °C). Only the middle portion of the supernatant was removed for analysis. A series of dilutions (1:50, 1:100, 1:200, and 1:250) of the supernatant were made using a solvent containing 50 mM sodium phosphate, 6.0 M urea, 10 mM DTT, and 10 mM sodium citrate, pH 6.75. A standard of 50 μL or diluted sample solutions were incubated in 2.5 mL of dye reagent (Bio-Rad Protein Assay Dye Concentrate, catalog #500-0006, diluted just before use according to instructions) for 30 min, and the absorbance was measured at 595 nm (A_{595}). A standard curve consisting of known β-LG concentrations (mg/mL) was obtained, and only the linear region of the A_{595} vs [β-LG] plot was used to determine the protein concentrations in all WPI samples. The total protein content in WPI samples was determined using the Leco Protein Analyzer model FP-2000 (Leco Corp., St. Joseph, MI), and the nitrogen conversion factor 6.38 was used for whey proteins.[39] The average solubility (%) was obtained over triple repeated sets of assays.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) of the WPI samples was carried out on a Phast System (Pharmacia Corp., Piscataway, NJ) with a phast homogeneous gel containing 20% of acrylamide. Protein samples at appropriate concentrations were solubilized in a solvent system containing 10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, 0.01% bromophenol blue dye, pH 8.0, and 5.0% β-mercaptoethanol when needed for reduction. Gel was stained with coomassie blue dye for 15 min followed by destaining in a solution containing 30% methanol and 10% acetic acid until desired color density level.

Densitometry analysis of the resulting gels was accomplished by scanning on a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) followed by processing using the software ImageQuant TL (Ver. 7.0, GE Healthcare Biosciences, Piscataway, NJ). Protein bands were identified using prestained BioRad SDS-PAGE Standards (catalog #161-0304, LMW Markers).

Reversed Phase High-Performance Liquid Chromatography (RP-HPLC). An analytical HPLC, Varian ProStar 230 (Varian, Palo Alto, CA) unit equipped with a Varian ProStar 325 UV−vis Detector, and a binary pump was used for the HPLC analysis. A 20 mL sample was autoinjected into a Vydac 4.6 mm × 250 mm C4 reverse phase column (214TP54, The Separations Group, Hesperia, CA) containing a polystyrene-divinylbenzene copolymer-based packing (particle size 5.0 μm and pore size 300 Å). Gradient elution was carried out with a mixture of two solvents. Eluant A contained 100% acetonitrile and 0.1% trifluoroacetic acid (TFA); eluant B contained 100% water and 0.1% TFA. The elution gradient was set as follows: 0 min 30% A, 0−30 min 30−50% A, 30−40 min 50−90% A, and 5 min for column re-equilibration at 90% A. The flow rate was 0.8 mL/min,

and the column temperature was maintained at 30 °C. All HPLC runs were monitored, and fractions were collected at 214 nm.

Determination of Primary and Secondary Amine Content. The Sigma Aldrich product N 7285 ninhydrin reagent solution (2%)[40] was used to determine the concentration of the primary and secondary amines in the soluble fraction of the texturized WPI samples at concentration ~0.5 mg/mL for all assay reactions. A stock solution of leucine at concentration of 50 μM in 0.05% glacial acetic acid was used to prepare the standard curve according to the reagent instructions, and the absorbance was recorded at 570 nm.

Determination of SH and Disulfide Bond Contents. The assay of total SH groups and disulfide bonds was carried out according to the method of Thannhauser et al.[41] Extruded and heated WPI (Provon 190) samples at varying concentrations of 20−100 mg/mL depending on the solubility were centrifuged at 10000*g* for 30 min at room temperature (20 °C) after a 30 min vigorous vortex in 0.10 M sodium phosphate (pH 7.50) buffer containing 2.0% SDS. A total of 100−200 μL of extract of extrudates (diluted to ~2.0 mg/mL) were pipetted into 3.0 mL of the 1.0 mM DTNB assay solution. The reaction mixture was incubated in the dark for 25 min at room temperature (20 °C), and the absorbance was then recorded at 412 nm against a blank of 3.0 mL of DTNB assay solution and the appropriate amount of solvent. L-Cystine was used as the standard for the determination of disulfide bonds. The content of free SH groups was determined by using Ellman's reagent[42] according to the Pierce method (Immunotechnology Catalog, Pierce, 1993, p E-56), by mixing 100 μL of extract with 100 μL of assay reagent (4 mg/mL Ellman's reagent in 0.1 N sodium phosphate buffer, pH 8.0) and 5 mL of 0.1 N sodium phosphate buffer (pH 8.0). The reaction mixture was allowed to stand for 15 min, and the absorbance was read at 412 nm.

Circular Dichroism (CD) Spectroscopy. Extruded WPI samples (~10.0 mg/mL) were initially suspended in 33 mM sodium phosphate buffer at pH 6.75 (50 mM ionic strength) and room temperature (20 °C) and then centrifuged at 10000*g* for 30 min to remove any sediment precipitate. The soluble portion was filtered through a 0.45 μm pore regenerated cellulose filter and used in the far-UV CD experiments. Appropriate dilutions were made to obtain absorbance values ~1.0 on the UV−vis spectrophotometer. Successive measurements of five repetitive scans, in the far UV region, 190−250 nm, were recorded. All solvents for CD measurements were first filtered through a Millipore 0.22 μm pore filter. CD spectra were recorded on an Aviv model 60DS spectrophotometer (Aviv Associates, Inc., Lakewood, NJ) using 0.5 mm path length cuvettes and a scan time of 4.0 s/nm. The jacketed cells were attached to a circulating constant temperature bath. All spectra are normalized at absorbance unit ~1.0 at 190 nm, corrected for solvent contributions, and expressed in relative ellipticity (far-UV) (mdeg) versus wavelength.

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a SPEX FluoroLog-3 fluorescence spectrometer (JY Horiba, NJ) in a 1 cm path length quartz cell. The temperature was changed and controlled by an F-3004 Peltier controller. All intrinsic tryptophan (Trp) fluorescence experiments were carried out using soluble portion of WPI protein samples in 33 mM sodium phosphate buffer (ionic strength is 50 mM) at pH 6.75 at similar concentrations measured by UV−vis absorption. An excitation wavelength of 295 nm was used to avoid absorption from phenylalanine residues, and fluorescence emission was recorded from 300 to 450 nm.

RESULTS AND DISCUSSION

Effect of Extrusion Temperature on the Protein Solubility of WPI. The Bradford assay[43,44] has long been used as an effective and quantitative method of determining the total amount of soluble protein in dairy samples. In this work, we used laboratory purified β-LG from bovine milk (≥90% by SDS-PAGE) as a standard to determine the protein solubility of texturized WPI samples in water.

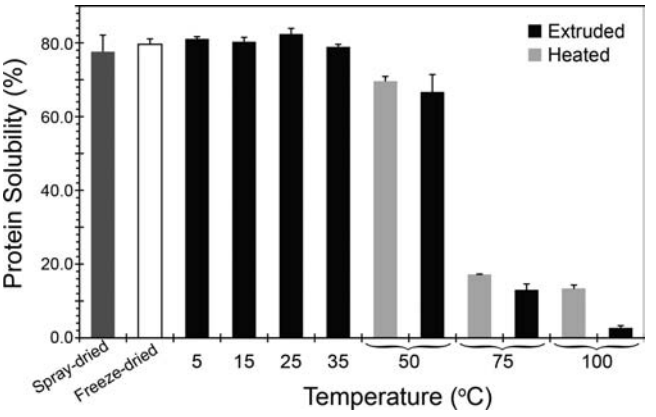

Figure 1. Protein solubility (%) of extruded WPI (at 50% moisture content) in water, determined by the Bradford assay method, shown in black bars. The WPI samples treated by heating only are represented in light gray bars (no frames). Nonextruded spray-dried and freeze-dried WPI samples are compared in dark gray and white-framed bars, respectively. All data were collected in triplicate with standard deviations plotted.

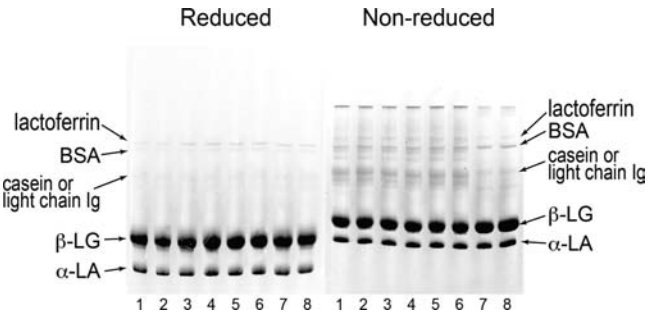

Figure 2. Comparison of SDS-PAGE of texturized WPI samples (at 50% moisture content) under nonreducing and reducing (with β-mercaptoethanol) conditions. Band assignment was made as labeled. Lanes were loaded as the following. Reducing gel (left): 1 and 2, nonextruded spray-dried WPI; 3 and 4, 5 °C; 5 and 6, 15 °C; and 7 and 8, 35 °C. Nonreducing gel (right): 1 and 2, nonextruded spray-dried WPI; 3 and 4, 5 °C; 5 and 6, 15 °C; and 7 and 8, 35 °C.

The results are shown in Figure 1. For extrudates obtained via low temperature texurization (below 35 °C), the protein solubility remained virtually unaffected at ~80%, as compared to that of he nonextruded spray-dried WPI and freeze-dried WPI. Increasing the extrusion temperature decreased the protein solubility, only marginally (~10% reduction) at 50 °C, significantly (by ≥65%) at 75 °C, and nearly completely (to ≤4%) at 100 °C. This reduction in solubility is clearly less pronounced by heating alone, ~70% at 50 °C, ~20% at 75 °C, and 16% at 100 °C, as compared to extrusion treatment at the same temperature. It is evident that extrusion cooking at higher temperature, ≥50 °C, bears far greater impact on reducing protein solubility of WPI than heating alone at the same temperature.

Effect of Extrusion Temperature on the Protein Composition of WPI. SDS-PAGE techniques under both reducing (with β-mecaptoethanol) and nonreducing conditions were applied to study changes in the protein profile and composition as a result of varying extrusion temperature. Figure 2 compares gels of various extruded WPI samples obtained in low temperature range. High molecular weight species were clearly visible in the nonreducing gel (right) as samples migrate into the running gel, indicating the formation of large protein aggregates during extrusion, which can

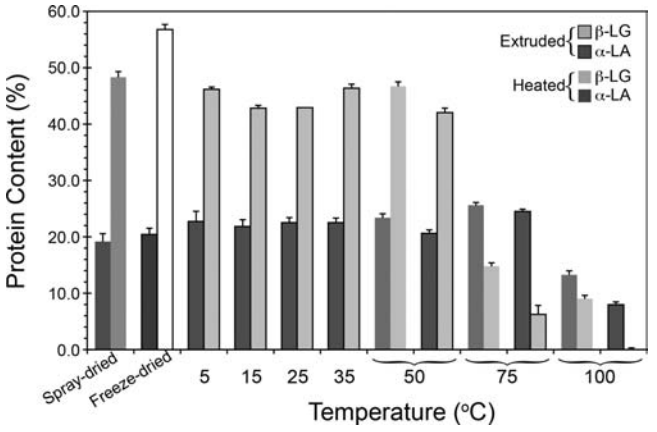

Figure 3. RP-HPLC analysis of α-LA (dark gray-framed bars) and *β*-LG (light gray-framed bars) contents (%) in texturized WPI (at 50% moisture content) as a function of extrusion temperature. The samples treated by heating only were analyzed as a comparison and are represented as nonframed bars. All data were collected in triplicate with standard deviations plotted.

be significantly or completely eliminated through the use of the reducing agent (left gel).

As expected, *β*-LG constitutes the major fraction of the protein in both control samples: nonextruded spray-dried WPI powder and freeze-dried WPI (Provon 190). *β*-LG and α-LA were found to be roughly ∼65 and 35% of the total proteins for the freeze-dried WPI sample in both the nonreducing and the reducing gels by densitometry analysis. These two major whey proteins, on the other hand, were determined to be ∼60 and ∼28% and 70 and 25%, respectively, for the spray-dried sample in the corresponding (nonreducing and reducing) gels. This represents a 10% increase caused by reduction in the amount of *β*-LG caused by spray drying, an indication of heat-induced denaturation and aggregation due to possible formation of the intermolecular S−S disulfide bonds formed through the single free Cys residue in *β*-LG even prior to any further processing treatment,[16,45] including heating and extrusion as reported in this work.

Quantitative analyses on all protein components of the WPI samples carried out by RP-HPLC, shown in Figure 3, demonstrated a close agreement with the results obtained from the SDS-PAGE experiments for both the freeze-dried and the nonextruded spray-dried WPI samples, with a consistent ∼10% lower *β*-LG content by HPLC. It must be pointed out that the technical difference between the two techniques is attributable to the discrepancy in the exact percentages of each protein. HPLC analysis can only be performed using the HPLC solvent (acetonitrile) soluble portion of the samples for separation and peak identification. Because of the poor solubility of the extruded WPI samples, especially high-temperature extrudates, the HPLC analyses on the relative distribution of each protein component may be underestimated. Nevertheless, Figure 3 clearly showed that the contents of *β*-LG and α-LA remain virtually unchanged at 45 and 22%, respectively, by low-temperature heating and extrusion treatments (below 50 °C). When the temperature was increased to 75 °C, over 50% *β*-LG was lost (as compared to the nonextruded spray-dried WPI) due to heating alone, and a nearly 90% reduction in the amount of *β*-LG due to extrusion was observed, while the α-LA content remained at ∼20% at this temperature. Heating at an even higher temperature, 100 °C only reduced the amount of α-LA marginally but significantly affected *β*-LG, to a lesser extent than extrusion at 75 °C. Extrusion at 100 °C produced nearly an undetectable level of *β*-LG, and only 10% α-LA was measured in the HPLC profile.

Although much remains unknown about the definitive effect of extrusion on the denaturation and aggregation of whey protein, heat-induced changes in the structure and stability of *β*-LG and α-LA both as an individual model system and as a mixture have been well documented.[20,27,46−48] It is often accepted that the characteristics of *β*-LG dominate the behavior of the whey protein aggregates upon heating. However, past studies[25,26] showed that the other whey proteins including both α-LA and BSA might also play an important role in determining the characteristics of the aggregation process largely due to the presence of protein−protein interactions.

β-LG, the most abundant protein in whey, 56−60% total protein, predominantly exists as a dimer at physiological pH and dissociates into native monomers at acidic pH, below 3.5, or basic, above pH 7.5.[49] *β*-LG monomer has two disulfide bridges, Cys66-Cys160 and Cys106-Cys119, a molecular mass of about 18 kDa, and additionally contains a free SH group (SH) Cys121, which is very reactive and often is involved in significant disulfide interchange with the SH group of other whey proteins or casein.[50] α-LA, a Ca^{2+} binding protein with a molecular mass of 14 kDa, contains four intramolecular disulfide bonds, and is far more stable than *β*-LG during heating.[21] It is generally thought that α-LA does not polymerize by itself at neutral pH when heated above 70 °C.[51] *β*-LG, on the other hand, displays a complex thermal-induced denaturation followed by aggregation process involving an initial step of dissociation of native dimers into native/modified monomers at a critical temperature close to 60 °C.[52] The *β*-LG molecule then undergoes conformational changes and partially unfolds, exposing hydrophobic residues and the thiol group that is normally buried at the interface between monomers in the native protein. The thiol group of Cys121 becomes reactive, enabling thiol/disulfide exchange reactions. An irreversible aggregation reaction can be the last step in the heat-induced process as the temperature reaches above 90 °C.[53,54]

Heating the mixture of *β*-LG and α-LA in a model system[25,51,55] can cause additional changes on both the intermolecular and the intramolecular levels through covalent bonding and noncovalent interactions (ionic, van der Waals, and hydrophobic) depending on the temperature, protein concentration, pH, ionic strength, and mineral content. As observed in this work (Figure 3), spray-dried WPI contained ∼10% less in *β*-LG level than freeze-dried, but the amount of α-LA, ∼22%, was about the same in both samples. This loss is presumably caused by the denaturation and aggregation of *β*-LG high-temperature spray-drying process. Although much α-LA remained in the extrudates when heated up to 75 °C, the *β*-LG content showed a nearly 10% decrease. Reductions in both proteins, a noticeable ∼10% in α-LA and a significant ∼40%, were found when extrudates were heated to 100 °C.

Extrusion processing clearly bears a significant impact on both α-LA and *β*-LG contents, especially for the extrudates obtained at high temperatures, ≥75 °C. In addition to heating, extrusion conditions, such as mechanical shear force and the pressure in the die, can cause significant physical and chemical changes in the molecular structures of both proteins by introducing a greater window for possible intramolecular as well as intermolecular interactions and reduce the soluble fractions of both α-LA and *β*-LG drastically, as compared to treatment by heating alone. The *β*-LG level still was

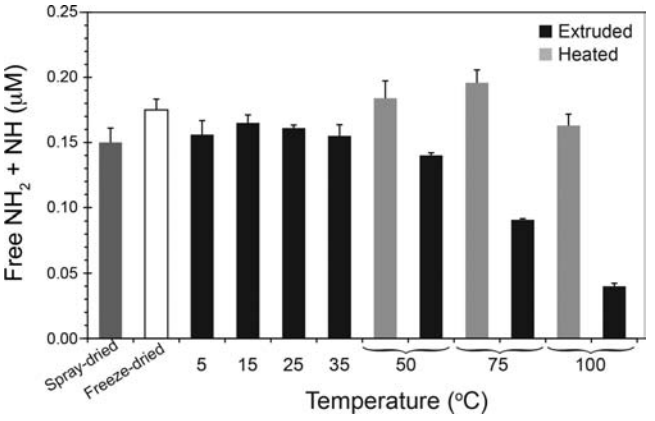

Figure 4. Free primary and secondary amine concentration (mM) of texturized samples (at 50% moisture content), determined by ninhydrin assay method and compared with the control samples by heating only. Nonextruded spray-dried and freeze-dried WPI samples are represented as labeled. All data were collected in triplicate with standard deviations plotted.

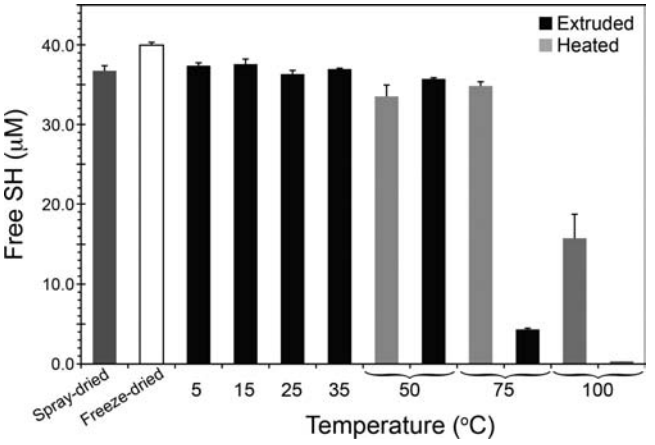

Figure 5. Free SH contents (μM) of texturized WPI samples (at 50% moisture content), determined by DTNB method, and compared with samples treated by heating only. Nonextruded spray-dried and freeze-dried WPI samples are also represented as labeled. The level of free SH in pure β-LG was determined to be 67.4 $\pm$ 4.5 μM (2.0 wt %/wt) and used as an independent control of the assay. All data were collected in triplicate with standard deviations plotted.

affected the most, to a negligible level at 100 °C extrusion texturization, and only ~10% α-LA remained.

Protein Quality of the WPI Extrudates. Chemical methods including DTNB and ninhydrin assays were used to measure available free SH and primary and secondary amine concentrations, respectively, and to assess the effect of temperature (by heating and extrusion) on protein quality of the WPI extrudates. The results are shown in Figures 4 and 5. Spray drying clearly caused losses in the levels of available primary and secondary amines as well as free SH as compared to freeze-dried WPI. Heating alone did not appear to affect the available primary and secondary amine level even at 100 °C but reduced the SH level by half, demonstrating that temperature plays an important role in S−S bond formation. The primary and secondary amines are mostly located on the side chains of the proteins and, thus, may not cross-link upon heating only. Extrudates obtained at low to medium temperature (≤50 °C) maintained the protein quality at

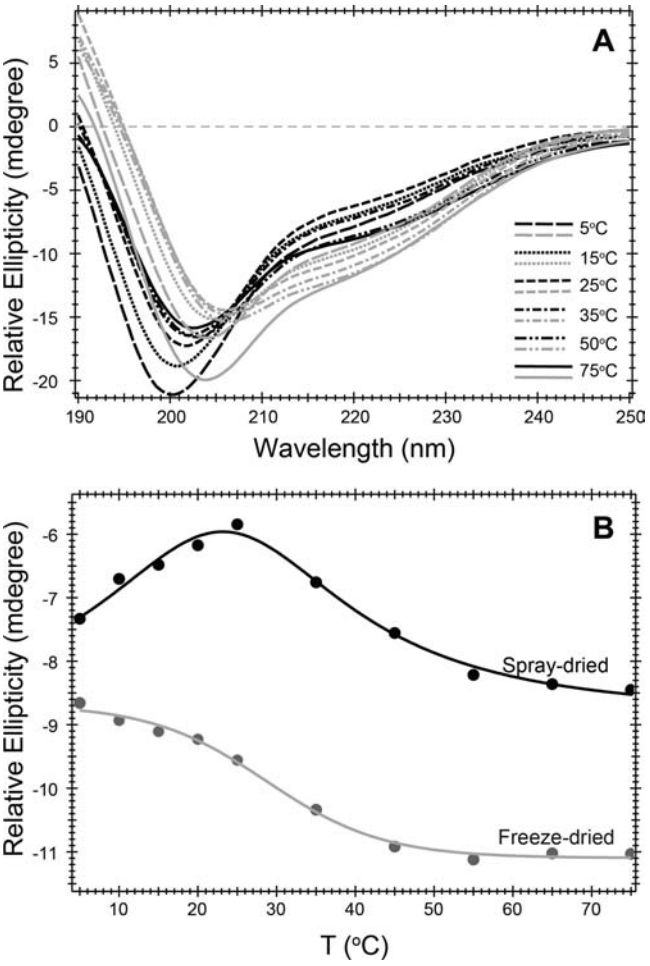

Figure 6. (A) Far-UV CD spectra of spray-dried (black lines) and freeze-dried WPI (gray lines) in 33 mM phosphate buffer (I = 50 mM), pH 6.75, at temperatures as indicated. (B) Relative ellipticity at 222 nm as a function of temperature (°C) for spray-dried (black) and freeze-dried WPI (gray). Both sets of data were fitted with a sigmoid function.

the nonextruded level. As the extrusion temperature reaches above 75 °C, the combined effect of all extrusion variables resulted in a significant level of reduction in the available free amine content (Figure 4) and a near complete elimination of free SH in the extrudates (Figure 5).

Effect of Extrusion Temperature on the Secondary Structures of the WPI Extrudates. In this work, we used CD spectroscopy to study changes in the secondary structural elements of WPI as a function of temperature and extrusion processing. Figure 6A shows the far-UV CD spectra of both freeze-dried and nonextruded spray-dried WPI at neutral pH as a function of temperature, and changes in ellipticity at 222 nm as a function of temperature are plotted in Figure 6B. By the CD method, a double negative dip (ellipticity maxima) at 205 and 222 nm signifies the existence of typical α-helical structures, such as in native α-LA (not shown for brevity), while a broad dip encompassing the 210−220 nm region usually represents largely β-sheet structure motif, as in native β-LG (not shown for brevity). The CD spectrum of WPI should resemble a mixture of both β-LG and α-LA, and its change upon heating would reflect changes in the secondary structure contents, that is, denaturation process. As discussed above, spray drying causes protein denaturation, in a fashion as shown in Figure 6A. All CD spectra of

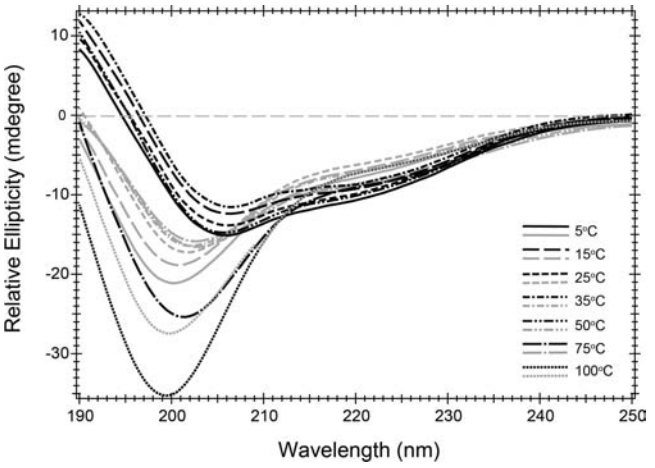

Figure 7. Far-UV CD spectra of WPI extrudates obtained at the temperatures (and 50% moisture content) as indicated (black lines) in 33 mM phosphate buffer (I = 50 mM), pH 6.75, as compared with that of nonextruded spray-dried WPI samples (Provon 190) cooled or heated at the corresponding temperatures (gray lines).

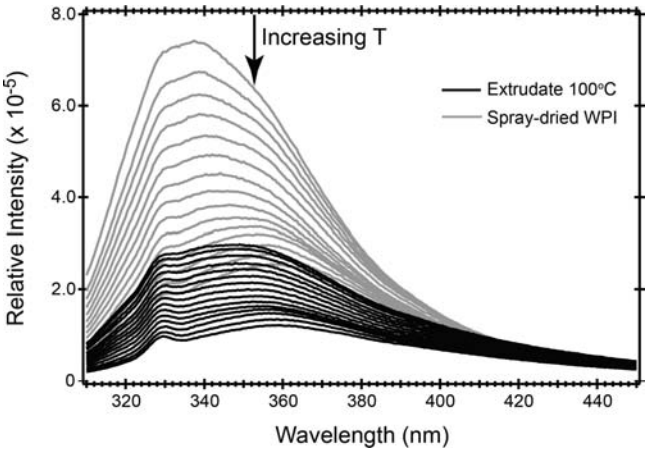

Figure 8. Trp intrinsic fluorescence spectra of nonextruded spray-dried WPI (gray lines) as compared to the WPI extrudates obtained at 100 °C and 50% moisture content (black lines) as a function of increasing temperature in the direction of the arrow.

spray-dried WPI samples (black lines) exhibited not only less pronounced CD peak at 222 nm but also blue-shifted, from 205 to 200 nm, relative to those of freeze-dried samples (light gray lines), indicative of a loss in the α-helical structural content.

The ellipticity at 222 nm for both nonextruded spray-dried and freeze-dried WPI as a function of temperature (Figure 6B) demonstrated difference and similarity in their secondary structural elements at each temperature. In the temperature range 5−25 °C, nonextruded spray-dried WPI appeared to undergo a loss in ellipticity, which is normal for an unfolding process. Freeze-dried WPI, on the other hand, behaved in an opposite fashion with an increasing trend in ellipticity at 222 nm, which is attributable to the structural transition, from predominately β-sheet to α-helical structure, as β-LG is known to be a β-sheet protein but has high α-helical propensity.[56] At a temperature above 25 °C, both proteins reacted similarly and became rather insensitive to a temperature change at ∼45 °C. It should be noted that the difference in magnitude mainly is due to the difference in protein concentration in solution as the ellipticity cannot be normalized as per residue in this work; all concentrations were made to be as close as possible by weight and by absorbance at 280 nm.

The CD spectra of the WPI extrudates studied in this work (black lines) were shown in Figure 7 and compared with that of the spray-dried WPI heated at respective temperatures (gray lines). Interestingly, the CD spectra of the extrudates obtained by extrusion at low and extreme low temperature (5−50 °C) bear a strong resemblance to that of freeze-dried WPI, that is, native proteins, deviating from those of the spray-dried samples by heating treatment in comparison. This suggested a likely reversal of the denaturaion in WPI (caused by spray-drying) brought about by the extrusion texturization treatment, which has not been reported previously to our best knowledge. The changing course of the CD spectra of spray-dried WPI as a function of temperature approximates to that of a typical globular protein denaturation process.

Effect of Extrusion Temperature on the Tertiary Structures of the WPI Extrudates. Intrinsic Trp fluorescence was used to assess tertiary structural changes in the WPI extrudates as a function of temperature. Both α-LA and β-LG contain Trp residues, four and two, respectively, and their fluorescence can be used as a probe to study environmental changes. It has been shown[57] that thermal denaturation of α-LA results in a shift of the fluorescence spectrum maximum from 327−331 to 344−346 nm. β-LG native protein at neutral pH gave an emission with a λ_{max} of about 332 nm,[58] which is expected for Trp residues in an apolar environment.[59] After it was heat treated,[60] λ_{max} shifted to a longer wavelength but about 10 nm less than that of a fully exposed Trp residue.

In this work, we used temperature as a perturbation tool to assess any residual tertiary structural contacts in the WPI extrudates and to investigate the effect of extrusion temperature on these contacts. Figure 8 showed that temperature imposed a significant impact on the Trp fluorescence of extrudate obtained at the highest temperature used in this work, 100 °C, and the nonextruded spray-dried WPI was also examined as a comparison. Clearly, there is a great loss in the quantum yield of Trp fluorescence in the extrudate due to extrusion treatment. In addition, a gradual reduction in intensity accompanied by a red shift in fluorescence maxima in both cases, from ∼340 to ∼360 nm in the nonextruded spray-dried WPI and from ∼350 to ∼360 nm in the extrudate, were observed as the temperature was increased. To quantitatively compare the effects of extrusion processing and heating treatment on the environment of Trp residues, we plotted the relative fluorescence intensity at its maximum and the wavelength where the maximum occurred as a function of temperature in Figure 9. If the freeze-dried WPI was used as the reference for having near native tertiary structural contacts, then all extruded WPI obtained by low temperature extrusion (5−50 °C) were positively identified to possess similar structural contacts with corresponding response to temperature perturbation. The elevated extrusion temperature undoubtedly diminished or completely destroyed these structural contacts.

In conclusion, spray drying, much like heating, can reduce some functional properties of WPI such as protein solubility, protein compositions, and protein quality, and denature the globular structures of whey proteins. Extremely low to low temperature extrusions, 5−50 °C, and high moisture content (50%) not only can maintain these functional properties of the extrudates at the nonextruded spray-dried levels but also are capable of reversing the denaturation in the molecular structures caused by spray drying and refolding the proteins to near their

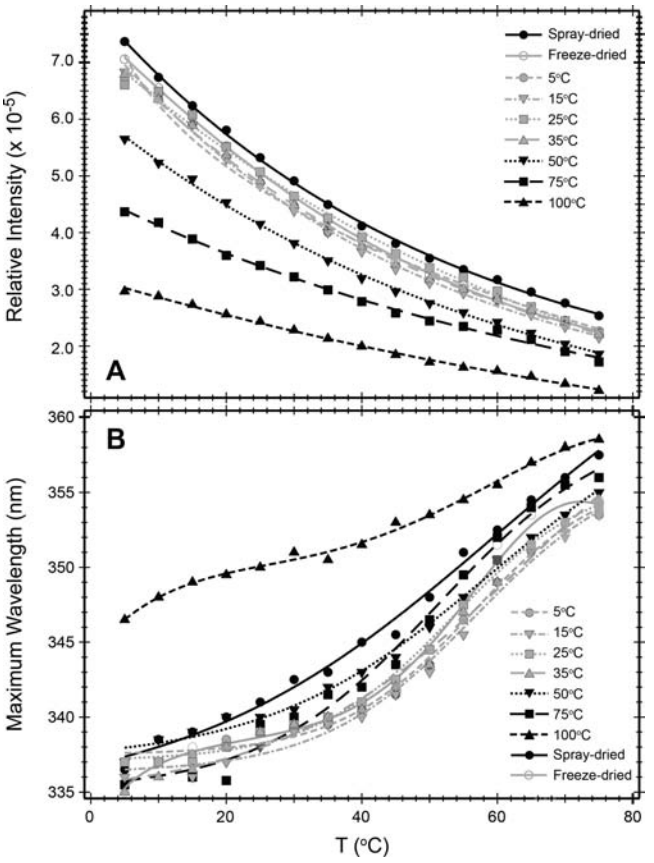

Figure 9. Maximum Trp intrinsic fluorescence of WPI samples as a function of temperature. (A) Relative fluorescence intensity at its maximum vs temperature (T, °C) and (B) wavelength (nm) of relative fluorescence at its maximum vs temperature (T, °C). The samples are shown as labeled.

native states. Extrusion processing at medium to high temperature, 75 °C, produced extrudates with reduced functional properties as well as significant denaturation of the molecular structures as compared to treatment by heating only. Moreover, the WPI extrudate particles obtained under these conditions showed an even and compact network arrangement as visualized by AFM, suggesting a possible application as a biomaterial. WPI extrudates obtained at high-temperature extrusion, 100 °C, displayed poor solubility, undetectable levels of free SH and amines, a minimal amount of secondary structural elements, and nearly nonexistent tertiary contacts in the molecules.

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DISCLOSURE

Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

ACKNOWLEDGMENT

We acknowledge the technical contributions by Edward D. Wickham (Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture) in the protein solubility, SDS-PAGE electrophoresis, RP-HPLC, CD, fluorescence spectroscopy, and all chemical assay experiments and the technical assistance by Audrey Thomas-Gehring (Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture) in the extrusion texturization of WPI samples used in this study.

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