Removal of milk fat globules from whey protein concentrate 34% to prepare clear and heat-stable protein dispersions

Gang Liu and Qixin Zhong¹

Department of Food Science and Technology, The University of Tennessee, Knoxville 37996

ABSTRACT

Whey protein concentrates (WPC) are low-cost protein ingredients, but their application in transparent ready-to-drink beverages is limited due to turbidity caused by fat globules and heat instability. In this work, fat globules were removed from WPC 34% (WPC-34) to prepare heat-stable ingredients via the Maillard reaction. The removal of fat globules by acid precipitation and centrifugation was observed to be the most complete at pH 4.0, and the loss of protein was caused by micrometer-sized fat globules and protein aggregates. Spray-dried powder prepared from the transparent supernatant was glycated at 130°C for 20 and 30 min or 60°C for 24 and 48 h. The 2 groups of samples had comparable heat stability and degree of glycation, evaluated by free amino content and analytical ultracentrifugation, but high-temperature, short-time treatment reduced the color formation during glycation. Therefore, WPC-34 can be processed for application in transparent beverages.

Key words: whey protein concentrate 34%, milk fat globule, acid precipitation, glycation for heat stability

INTRODUCTION

Whey protein ingredients have high contents of EAA and versatile functionalities enabling various applications in the food industry (Vardhanabhuti et al., 2009). Whey protein concentrate 34% (**WPC-34**) is a cost-effective ingredient with about 34% mass being proteins. When compared with WPC-80 and whey protein isolate (**WPI**), fewer purification steps are used in the production of WPC-34 and, therefore, WPC-34 contains big particulates such as milk fat globules (**MFG**) that cause turbidity and precipitation (Hwang and Damodaran, 1995). Conversely, recent studies have found that the MFG membrane (**MFGM**) contains abundant bioactive lipids and proteins that have the potential of inhibiting the growth of cancer cells, lower-

ing cholesterol, inhibiting pathogens, and assisting in the cure of Alzheimer's disease (Kanno, 1990; Spitsberg, 2005). The MFGM has excellent surface activities and can be applied as the carrier material to deliver bioactive ingredients in functional foods (Thompson and Singh, 2006; Thompson et al., 2006). Therefore, processes separating WPC to fractions with and without MFGM may add value to the dairy industry.

Hwang and Damodaran (1995), Damodaran (2010), and Damodaran (2011) selectively precipitated MFGM using various approaches. In an early study (Hwang and Damodaran, 1995), chitosan was used to form complexes with MFGM in Cheddar cheese whey at pH 4.5, and the complexes were removed by centrifugation to form transparent supernatants, resulting in 92% removal of lipids and 6.8% reduction of protein. In a later study, divalent cations at 0 to 50 mM were studied to precipitate MFGM at 30°C and pH 5.2 (Damodaran, 2010). Calcium and magnesium ions were ineffective, whereas complete precipitation was observed for zinc ions above 20 mM. In the latest study, diafiltration of Cheddar cheese whey at pH 4.2 and 35°C for 30 min was also observed to be effective in precipitating MFGM that was speculated to be caused by removal of calcium ions that are critical to the structure of MFGM (Damodaran, 2011). A loss of 14% protein was reported after diafiltration. These studies are important to produce whey protein ingredients in applications requiring visual clarity.

Transparent beverages are possible products containing whey protein ingredients. To produce ready-to-drink beverages, thermal processing is needed to ensure microbiological safety and quality. For acidic beverages with pH <4.6, thermal processing conditions are not regulated, and the beverage industry uses hot-fill processes equivalent to heating at about 88°C for 2 min (Etzel, 2004). For products with pH >4.6, UHT processing can be used (e.g., at 138°C for at least 8 s for dairy products; McGarrah, 1982). Conversely, whey proteins are known to aggregate during heating, especially at acidity near the isoelectric point of whey proteins (~pH 5.0) when the net charge of proteins is low (Bryant and McClements, 1998; Baier and McClements, 2005). Glycation of whey proteins with reducing

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¹Corresponding author: qzhong@utk.edu

saccharides is an effective method to improve the stability of proteins against aggregation during heating, and we recently showed that WPI glycosylated with lactose at 80°C for 2 h enabled transparent dispersions at pH 3.0 to 7.0 with 0 to 150 mM NaCl or CaCl₂ after heating at 88°C for 2 min and 138°C for 1 min (Liu and Zhong, 2013). Because cheese whey and WPC contain lactose, whey protein-lactose conjugates can be produced by integration with the whey protein-manufacturing processes.

The first objective of the present study was to exploit a simple process to prepare transparent samples by acid precipitation of MFG from WPC-34 suspension. The second objective was to evaluate the heat stability and structure of clarified WPC (**cWPC**) and conjugates prepared from cWPC (**gWPC**).

MATERIALS AND METHODS

Materials

The WPC-34 was kindly donated by Grande Cheese Co. (Grande, WI). Precast 15% gradient polyacrylamide gels and protein markers (catalog no. 161-0375) were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Nile Blue A dye was obtained from Sigma-Aldrich Corp. (St. Louis, MO). Bovine serum albumin used in the protein assay was purchased from BioWorld LLC (Atlanta, GA). Other chemicals used in the present study were from either Thermo Fisher Scientific Inc. (Pittsburgh, PA) or Sigma-Aldrich Corp.

Precipitation of MFG from WPC-34 Suspension

The WPC-34 was hydrated at a powder concentration of 2% (wt/vol) in deionized water for about 2 h under mixing on a stir plate until no visible powder was observed. The suspension was adjusted to pH 2.5 to 6.5 with 1.0 N NaOH or 1.0 N HCl. After standing at ambient conditions (21°C) for 4 h, the samples were centrifuged for 15 min at 8,000 × *g* (model Sorvall RC 5B Plus; DuPont, Wilmington, DE). Both the supernatant and precipitates were collected for further analysis. To improve the removal of MFGM, the supernatant was further incubated at ambient conditions for another 24 h and centrifuged again at 4,000 × *g* for 15 min at 10°C. Then, the turbidity of the supernatant was compared for visual appearance by photographing and determining the absorbance at 600 nm (Evolution 201 UV-visible spectrophotometer; Thermo Scientific, Waltham, MA). The protein concentration in the supernatant was measured by the bicinchoninic acid method using an assay kit from Thermo Fisher Scientific Inc. (Rockford, IL), with BSA as a protein standard.

Preparation of cWPC powder

The WPC-34 powder was suspended in deionized water and processed as above for the treatment adjusting to pH 4.0. After centrifugation, the transparent supernatant was collected, adjusted to pH 7.0 with 1.0 N NaOH, and spray-dried (model B-290; Büchi Labortechnik AG, Flawil, Switzerland). The spray dryer was operated at conditions as follows: an inlet air temperature set at 160°C, a compressed air pressure of 600 kPa, an outlet temperature controlled at about 65°C, an air flow rate of 32 m³/h, and a feed flow rate of ca. 4 mL/min. The powder collected from the spray dryer was designated as cWPC and used in further experiments.

SDS-PAGE

The SDS-PAGE was carried out under the reducing conditions with a 15% precast polyacrylamide gel obtained from Bio-Rad Laboratories Inc. The WPC-34 was prepared at 2% (wt/vol) concentration and then subjected to different treatments described in previous sections. Fifty microliters of each collected sample was mixed with 200 µL of the SDS-PAGE loading buffer and then heated at 95°C for 5 min in a water bath. Five microliters of each sample was loaded into each well of gel. Electrophoresis in a Mini Protean Tetra Cell (Bio-Rad Laboratories Inc.) was conducted at a constant voltage of 200 V and a current of 400 mA until the indicator dye reached the gel bottom. After staining with Coomassie Blue for 10 min, the gel was destained until bands became visible.

Characterization of Protein Particles and MFG by Confocal Laser Scanning Microscopy

The microstructural characteristics of MFGM and protein were studied using a Leica SP2 confocal laser scanning microscope (Leica Microsystems GmbH, Mannheim, Germany). The instrument was equipped with an Argon ion laser excited at a wavelength of 633 nm for protein observation and a HeNe laser excited at 543 nm for fat observation. One milliliter of WPC sample [2% (wt/vol) powder suspended in distilled water at pH 7.0] was mixed with 10 µL of a 1.0% (wt/vol) Nile Blue A solution to stain both proteins and lipids before imaging.

Fat, Protein, and Lactose Contents in WPC and cWPC

The total lipid content was determined using the method of AOAC International (2012; method 989.05).

The lactose content was determined using the phenol-sulfuric acid method (Barnett and Tawab, 1957), with pure lactose as a standard. The total protein content was determined using the abovementioned bicinchoninic acid method. Duplicate samples were tested.

Preparation of gWPC

The spray-dried cWPC powder was placed in a container and applied to a layer with ca. 1-mm thickness. The container was then placed on a perforated plate in a desiccator, with a saturated KBr solution below the perforated plate to obtain 79% relative humidity (Akhtar and Dickinson, 2007). The desiccator containing the samples was incubated in an oven pre-equilibrated at 60°C for 24 and 48 h or at 130°C for 20 and 30 min to prepare gWPC.

Free Amino Group Content

The content of available free amino groups was quantified using a previously reported *o*-phthalaldehyde (OPA) method (Nielsen et al., 2001) with slight modifications. The fresh OPA working solution was prepared by diluting the following mixtures to a total volume of 200 mL with deionized water: 2.5 mL of 20% (wt/vol) SDS, 25 mL of 100 mM sodium borate, 400 μ L of β -mercaptoethanol, and 0.16 g of OPA dissolved in 4 mL ethanol. Fifty microliters of protein dispersions [with 1% (wt/vol) powder] was mixed with 3 mL of the freshly prepared OPA working solution. The mixture was vortexed for 10 s and incubated for 2 min in the dark at ambient temperature (21°C) before measuring the absorbance at 340 nm using an Evolution 201 UV-visible spectrophotometer (Thermo Scientific). The OPA working solution was used as a blank. Absorbance data were converted to free amino contents using a calibration curve established with 0.01 to 5.0% (wt/wt) L-lysine standard solutions. Triplicate measurements were conducted for each sample.

Analytical Ultracentrifugation

The molecular weights (MW) of WPC were characterized using a Beckman XL-I analytical ultracentrifuge (Beckman Coulter Inc., Palo Alto, CA). The dispersions were prepared at an overall protein concentration of 1.2 mg/mL and adjusted to pH 3.0. The dispersions were filled into the standard double-sector cell and centrifuged at $182,000 \times g$ for 480 min at 25°C. The UV absorbance data were collected at 280 nm every 4 min at 25°C and analyzed according to the Liu and Zhong (2013). The number of lactose molecules (*n*) glycosylated to 1 whey protein molecule was calculated from the small-

est MW peak of sedimentation profiles (monomeric proteins) using the following equation:

$$n = (M_2 - M_1)/M_3, \quad [1]$$

where M_1 , M_2 , and M_3 stand for the average MW of whey protein before glycation, whey protein conjugate, and lactose (342 Da), respectively.

Heat-Stability Test

Heat stability of WPC dispersions was assessed at 88°C for 2 min in a water bath, simulating the hot-fill process in the beverage industry (Eitzel, 2004), or at 138°C for 1 min in a glycerol bath, simulating UHT processing (McGarrahan, 1982). The dispersions were prepared with powder concentration of 4% (wt/vol) and adjusted to pH 3.0 to 7.0 with 1.0 M NaOH or 1.0 M HCl and 0 to 150 mM NaCl. One or 2 mL of final dispersions was filled in 4-mL glass vials sealed with plastic caps for heat-stability tests.

Color Determination

The color of the protein dispersions was determined on a MiniScan XE Plus Hunter colorimeter (Hunter Associates Laboratory Inc., Reston, VA). Color parameters lightness (L^*), redness (a^*), and yellowness (b^*) were determined for 10-mL samples with 4% (wt/vol) powder. Duplicate analyses of the samples were conducted for each sample.

Statistical Analysis

Data were reported as mean $\pm$ standard deviation from duplicates or triplicates. The statistical analysis was carried out using the SPSS 13.0 statistical analysis system (SPSS Inc., Chicago, IL). One-way ANOVA and a least significant difference test were used to compare statistical differences of the mean at a *P*-value of 0.05.

RESULTS AND DISCUSSION

Acid Precipitation of WPC-34 Suspensions

Effects of pH (2.5–6.5) on precipitation properties of WPC-34 were first studied. After adjusting pH and incubation for 24 h, precipitation was observed at all pH conditions, most significantly at pH 4.0, followed by pH 4.1 (Figure 1A). After centrifugation, the supernatants of these 2 samples had lower absorbance than other samples (Figure 1B) and were transparent (not shown). The protein content in the supernatants ranged from 0.425 to 0.561% (wt/vol), corresponding

to 17.5 to 37.5% precipitation of total protein (Figure 1C). The lowest protein content was at pH 4.0, agreeing with the most precipitated MFG (Figure 1A). When analyzed using SDS-PAGE for selected samples (Figure 2), the precipitated mass had bands corresponding to major whey proteins such as β -LG, α -LA, and BSA and differed slightly from the supernatant after centrifugation. Most notably, the band at around 100 kDa, corresponding to the heavy chain of IgG (Damodaran, 2010), was more intense in the precipitate (Figure 2) than in the supernatant (Figure 3). The supernatants obtained from the samples adjusted to different pH conditions showed similar SDS-PAGE profiles (Figure 3), indicating that pH did not have significant effects on the precipitation of major whey proteins.

The results suggest that transparent WPC dispersions can be obtained by precipitating MFG at appropriate acidity. It is likely that MFG in WPC-34 have a net charge close to 0 at pH 4.0 and precipitate due to hydrophobic attraction (Damodaran, 2010). The observations agree with the fact that phosphate has a pK_1 of pH 4.0 and is the only ionizable group of MFGM phospholipids (Moncelli and Becucci, 1995; Damodaran, 2010). The optimum acidity of precipitating MFG (pH 4.0) in the present study is slightly different from an earlier study at pH 4.2 (Damodaran, 2011), possibly because of different starting materials and different precipitation temperatures (21 vs. 35°C). The loss of proteins during acidic precipitation was also higher than 6.8 to 14% observed by Hwang and Damodaran (1995), Damodaran (2010), and Damodaran (2011) who used liquid whey as the starting material. In our case, using commercial WPC-34, it is possible that some proteins were denatured during production and some were not hydrated sufficiently, which caused significant losses after precipitation and centrifugation, as evidenced by major whey proteins all present in the precipitate. The rest of this paper is based on samples precipitated and centrifuged at pH 4.0.

Microstructures of WPC-34 Suspensions

To further understand precipitation properties of WPC-34 suspensions, confocal laser scanning microscopy was used to characterize microstructures of particulates. As displayed in Figure 4, lipid particles contained a significant quantity of proteins, and some micrometer-sized protein particles were also present. The average diameter of the particles observed in microscopy was about 2.1 μm , with the biggest particle being ca. 3.6 μm . These particles are much bigger than the <10-nm size of individual whey protein molecules (Wang et al., 2013; Zhong et al., 2013), which are too small to be imaged by confocal laser scanning micros-

Figure 1. Appearance of suspensions with 2% (wt/vol) whey protein concentrate 34% (WPC-34) after adjusting to pH as shown on vial caps and incubation for 24 h at room temperature (A). Panels B and C show the absorbance and percentage of precipitated protein after centrifuging the samples at $4,000 \times g$ for 15 min at 10°C. Au = absorbance units. Color version available in the online PDF.

copy. The protein microparticles likely are created due to denaturation and aggregation of proteins during cheese manufacturing and WPC production. Further-

Figure 2. Sodium dodecyl sulfate PAGE analysis of the precipitated mass after 24 h incubation at pH 4.0 (lane 1) and 3.6 (lane 2), with comparison with the precipitated mass after centrifuging the pH 3.5 sample (lane 3). Color version available in the online PDF.

more, the mean diameter of fat globules in raw milk is about 3.4 μm , which is reduced to about 0.6 μm after homogenization (Walstra et al., 2005). Based on the dimension of particulates on microscopy images, the milk likely was homogenized. The fat globules likely had aggregated with some whey proteins, as observed in the SDS-PAGE, which showed significant amounts of whey proteins in the sediments (Figure 2).

Compositional Changes of WPC-34 After Removing Fat Globules

After removing MFG, the transparent supernatant was spray-dried to produce cWPC powder. The lipid content decreased from $3.30 \pm 0.71\%$ in WPC-34 to $0.94 \pm 0.07\%$ in cWPC. The protein content also decreased from $37.8 \pm 0.7\%$ in WPC-34 to $35.2 \pm 0.9\%$ in cWPC-34. This agreed with the presence of proteins in the precipitates (Figure 2). As lactose remained in the

Figure 3. Sodium dodecyl sulfate PAGE analysis of the supernatants obtained after centrifuging 2% (wt/vol) whey protein concentrate 34% (WPC-34) suspensions adjusted to pH 3.0, 3.9, 4.0, 4.1, 4.2, 5.0, and 6.0 and incubated for 24 h at 21°C. Color version available in the online PDF.

supernatant after precipitating fat globules, the content of lactose increased from $55.1 \pm 0.0\%$ in WPC-34 to $63.8 \pm 1.5\%$ in cWPC.

Heat Stability of Glycated cWPC

After redispersing spray-dried cWPC at 4% (wt/vol) powder [1.4% (wt/vol) protein], the dispersions were transparent at pH 3.0 to 7.0 and 0 to 150 mM NaCl (Figure 5A). After heating at 88°C for 2 min (Figure 5B) or 138°C for 1 min (similar appearance as Figure 5B; not shown), only the dispersion at pH 3.0 with ≤ 50 mM NaCl remained transparent, whereas the other samples became turbid. For the gWPC prepared by heating the spray-dried cWPC powder at 60°C for 24 h, the dispersions were clear at pH 3.0 to 4.0 and 0 to 150 mM NaCl, and pH 5.0 and 50 to 150 mM NaCl after heating at 88°C for 2 min (Figure 5C). When the glycation at 60°C was extended to 48 h, transparent dispersions were observed at pH 3.0 to 7.0 and 0 to 150 mM NaCl but had dark brown color (Figure 5D).

To decrease the color formation during glycation, 2 other gWPC samples were prepared at the same humidity but at a much higher temperature (130°C) and a shorter duration (20 or 30 min). Sample appearance after heating is shown in Figure 5E to H, with the quantified color parameters presented in the next sec-

Figure 4. Confocal laser scanning microscopy images of whey protein concentrate 34% (WPC-34) suspension stained using Nile Blue A dye and excited at 633 and 543 nm to show proteins in green (A) and lipids in red (B), respectively. Scale bar = 35 μ m. In the overlaid picture (C), the analysis is shown for an example of a fat globule with both proteins and lipids. Color version available in the online PDF.

tion. The gWPC prepared at 130°C for 20 min remained transparent after heating at 88°C for 2 min when dispersions were prepared with 4% (wt/vol) powder, pH 3.0 to 6.0, and 0 to 150 mM NaCl or pH 7.0 and 0 mM NaCl (Figure 5E); after heating at 138°C for 1 min (Figure 5F), dispersions at pH 5.0 and 0 mM NaCl, pH 6.0 and <100 mM NaCl, and pH 7.0 and 0 to 150 mM NaCl became turbid. In contrast, the gWPC prepared at 130°C for 30 min became turbid only after heating the pH 7 and 50 to 150 mM NaCl samples at 138°C for 1 min (Figure 5G and H).

It is well known that the Maillard reaction involves multiple stages of reactions. The initial stage begins with a condensation reaction between the amino group of protein and the carbonyl group of reducing saccharides (van Boekel, 2006). The covalent attachment of

reducing saccharides onto the protein could improve some functional properties of proteins (e.g., solubility, emulsification, foaming, and heat stability; Chevalier et al., 2001). The later stages of the Maillard reaction include the polymerization and degradation reactions originating from the Amadori rearrangement, and some advanced reaction products are responsible for the color formation (Ajandouz et al., 2001; Martins and Van Boekel, 2005; Liu and Zhong, 2012). The similar heat stability suggests similar degrees of conjugation at the initial stage of Maillard reaction for the gWPC produced at 2 temperatures (130 vs. 60°C) but different durations (20 or 30 min vs. 24 or 48 h). The lighter color of gWPC produced at 130°C indicates the reduced later stages of reactions at a shorter time. This trend agrees with our earlier study where WPI was glycated with

Figure 5. Appearance of aqueous dispersions of clarified whey protein concentrate (cWPC; A and B) and the conjugate (C–H) before (A) and after heating at 88°C for 2 min (B, C, D, E, and G) or 138°C for 1 min (F and H). The cWPC was prepared by precipitation at pH 4.0, and the obtained supernatant was neutralized and spray-dried to produce powdered cWPC. The cWPC powder was glycated at 60°C for 24 (C) and 48 h (D), or at 130°C for 20 (E and F) and 30 min (G and H). All vials contained 4% (wt/vol) powder and were adjusted to pH 3.0 to 7.0 with 0, 50, 100, and 150 mM NaCl (vials from left to right in each image) before the heat-stability test. Color version available in the online PDF.

lactose, maltodextrin, or glucose at 60°C (for 24–72 h) and 80°C (for 2 h; Liu and Zhong, 2013). It was also noted that heat stability of gWPC at pH 7.0 was not as good as lower pH when heated at 138°C, which is different from WPI-lactose conjugates (Liu and Zhong, 2013) and opposite to the expectation that whey proteins should have more negative charges at pH 7.0 and are expected to be more stable. We are not sure what

caused this exception, as WPC and WPI production conditions are proprietary information. The structures of gWPC are presented below.

Color Development in WPC Glycated at 60 or 130°C

The evolution of reddish-brown color of the samples is commonly used as an indicator for the progress of the

Table 1. Color parameters of 4% (wt/vol) powder dispersions (pH 7.0) constituted with clarified whey protein concentrate (cWPC) and its conjugates prepared at various conditions¹

Glycation conditions	Redness (a*) ²	Yellowness (b*) ³	Lightness (L*) ⁴
None (cWPC)	-1.16 ± 0.01 ^e	3.48 ± 0.01 ^e	69.48 ± 0.02 ^a
60°C for 24 h	-1.76 ± 0.03 ^d	23.84 ± 0.22 ^b	66.26 ± 0.03 ^d
60°C for 48 h	-4.11 ± 0.04 ^a	50.645 ± 0.12 ^a	57.97 ± 0.05 ^e
130°C for 20 min	-2.09 ± 0.03 ^c	8.705 ± 0.02 ^d	68.71 ± 0.02 ^b
130°C for 30 min	-2.51 ± 0.02 ^b	15.53 ± 0.35 ^c	67.82 ± 0.04 ^c

^{a-e}Different superscript letters within a column indicate significant differences in the mean ($P < 0.05$).

¹Numbers are mean ± SD from 2 replicates.

²Negative values indicate green and positive values indicate red.

³Negative values indicate blue and positive values indicate yellow.

⁴L* = 0 yields black and L* = 100 indicates diffuse white.

Maillard reaction (Bekedam et al., 2006). Table 1 lists the L*, a*, and b* values of dispersions with 4% (wt/vol) powder at pH 7.0, constituted with cWPC and its conjugates pretreated at various conditions. The most drastic increase for cWPC before and after glycation was the b* value, which represents the yellowness of the conjugate samples and the major color change (browning) during the Maillard reaction (Jaeger et al., 2010; Purlis, 2010). The browning was more significant at longer glycation duration and was less for samples glycated at 130°C than those at 60°C. This observation, in agreement with the visual appearance (Figure 5), supports the discussion above that later stages of the Maillard reaction were not extensive during glycation at 130°C for a short time. The decrease in the a* and increase in b* values, as observed for all gWPC, indicate that the glycation process yielded the samples with less redness and darker appearance (Figure 5). The changes in a* and b* values were also found to correlate with glycation conditions, showing that HTST glycation resulted in smaller changes of both a* and b*. As for the L* value, although the improved solubility after glycation was observed to increase the L* value (Akhtar and Dickinson, 2007), all conjugate treatments showed a reduced L* value. This is likely because the cWPC is already transparent after removing MFGM. The advantage of short-time glycation at a high temperature, therefore, is significant in terms of reducing pigment formation (Table 1) while achieving heat stability (Figure 5).

Structure of Glycated cWPC

The free amino groups are reacted in the early stage of the Maillard reaction (Laroque et al., 2008; Gu et al., 2009). Thus, the loss of available amino groups can be used as an indicator to compare the degree of glycation. As shown in Table 2, the content of free amino

groups decreased in all gWPC samples. No significant differences ($P > 0.05$) were observed for the glycation conditions of 60°C for 24 h and 130°C for 20 min, as well as 60°C for 48 h and 130°C for 30 min. The results in Table 2 verify the higher glycation rate at 130°C than at 60°C. The data also generally agree with the heat stability of gWPC in Figure 5. The data further confirm that differences in color developments at the 2 different glycation temperatures are due to later stages of the Maillard reaction (Brands and van Boekel, 2002; Oliver et al., 2006).

Analytical ultracentrifugation was used to study molecular structures of cWPC and gWPC prepared at various conditions, with the sedimentation profiles shown in Figure 6 and the analyses listed in Table 3. After glycation at 60 or 130°C, the smaller MW peak showed an increase in MW, which indicates the conjugation between lactose and protein. The portion of the higher MW peak area increased but the MW decreased after glycation (Figure 6; Table 3). The MW of the larger mass peak was more than twice of that of the smaller mass peak, which suggests the cross-linking of proteins during glycation, as previously reported (Jiménez-Castaño et al., 2005; Lillard et al., 2009; ter Haar et al., 2011). The reduced MW of the higher mass peak af-

Table 2. Contents of free amino group in clarified whey protein concentrate (cWPC) and its conjugates prepared at various conditions¹

Glycation conditions	Free amino content (%)
None (cWPC)	10.0 ± 0.8 ^a
60°C for 24 h	7.8 ± 0.3 ^b
60°C for 48 h	5.9 ± 0.2 ^c
130°C for 20 min	7.6 ± 0.4 ^b
130°C for 30 min	5.5 ± 0.3 ^c

^{a-c}Different superscript letters within a column indicate significant differences in the mean ($P < 0.05$).

¹Numbers are mean ± SD from 2 replicates.

Table 3. Molecular weight (MW) characteristics of clarified whey protein concentrate (cWPC) and its conjugates prepared at different conditions determined by analytical ultracentrifugation

Glycation conditions	Peak 1		Peak 2		No. of lactose glycated to protein ¹
	Area (%)	Average MW (kDa)	Area (%)	Average MW (kDa)	
None (cWPC)	96.1	17.0	3.9	82.2	—
60°C for 24 h	90.6	18.2	9.4	81.7	3.5
60°C for 48 h	87.8	19.7	12.2	45.5	7.9
130°C for 20 min	90.4	19.0	9.6	77.9	5.8
130°C for 30 min	85.9	20.2	14.1	50.3	9.3

¹Calculated from peak 1, based on the following equation: $n = (M2 - M1)/M3$, where n = the number of lactose molecules glycated to 1 whey protein molecule and $M1$, $M2$, and $M3$ stand for the average MW of whey protein before glycation, whey protein conjugate, and lactose (342 Da), respectively.

ter glycation may be caused by the improved solubility of initially aggregated proteins in cWPC. Based on the smaller mass peak (monomeric proteins; Equation [1]), the number of lactose molecules glycated to each whey protein molecule was estimated to be 3.5 and 7.9 for glycation duration of 24 and 48 h at 60°C, respectively, and that of gWPC prepared at 130°C for 20 and 30 min was 5.8 and 9.3, respectively. The results are consistent with a previous study demonstrating multiple reducing saccharides bound to 1 whey protein molecule based on mass spectroscopy (Hiller and Lorenzen, 2010). Therefore, glycation of a sufficient number of lactose molecules on whey proteins is needed to obtain heat stability (Figure 5).

Figure 6. The best-fit sedimentation coefficient [s , in Svedberg unit (S)] distributions [$c(s)$] from analytical ultracentrifugation of clarified whey protein concentrate (cWPC; black solid line) and its conjugate dispersions constituted to 0.12% (wt/vol) protein and pH 3.0. The zoom-in view of curves at s -values of 3.0 to 6.5 S is shown in the inset. OD = optical density. Color version available in the online PDF.

CONCLUSIONS

A simple approach was developed to prepare transparent whey protein samples from turbid WPC-34 by acid precipitation of MFG at ambient conditions followed by centrifugation. The optimal pH to produce the most transparent cWPC sample was observed at pH 4.0. The gWPC prepared from spray-dried cWPC powder showed the dramatically improved heat stability from cWPC at various acidic and ionic conditions. The conjugates prepared at 130°C for 20 and 30 min showed a lighter color but had similar heat stability enhancement compared with those prepared at 60°C for 24 and 48 h, which agreed with comparable degrees of glycation. Findings from this study may be used to directly prepare protein ingredients from WPC-34 for applications such as transparent ready-to-drink protein beverages.

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