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Pilot-scale formation of whey protein aggregates determine the stability of heat-treated whey protein solutions—Effect of pH and protein concentration

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ABSTRACT

Denaturation and consequent aggregation in whey protein solutions is critical to product functionality during processing. Solutions of whey protein isolate (WPI) prepared at 1, 4, 8, and 12% (wt/wt) and pH 6.2, 6.7, or 7.2 were subjected to heat treatment (85°C × 30 s) using a pilot-scale heat exchanger. The effects of heat treatment on whey protein denaturation and aggregation were determined by chromatography, particle size, turbidity, and rheological analyses. The influence of pH and WPI concentration during heat treatment on the thermal stability of the resulting dispersions was also investigated. Whey protein isolate solutions heated at pH 6.2 were more extensively denatured, had a greater proportion of insoluble aggregates, higher particle size and turbidity, and were significantly less heat-stable than equivalent samples prepared at pH 6.7 and 7.2. The effects of WPI concentration on denaturation/aggregation behavior were more apparent at higher pH where the stabilizing effects of charge repulsion became increasingly influential. Solutions containing 12% (wt/wt) WPI had significantly higher apparent viscosities, at each pH, compared with lower protein concentrations, with solutions prepared at pH 6.2 forming a gel. Smaller average particle size and a higher proportion of soluble aggregates in WPI solutions, pre-heated at pH 6.7 and 7.2, resulted in improved thermal stability on subsequent heating. Higher pH during secondary heating also increased thermal stability. This study offers insight into the interactive effects of pH and whey protein concentration during pilot-scale processing and demonstrates how protein functionality can be controlled through manipulation of these factors.

Key words: whey protein, denaturation, aggregation, concentration

INTRODUCTION

Dairy proteins, and in particular, whey proteins, have been the subject of extensive research due to their excellent nutritional properties, AA profile, and functional properties, and are used in the production and manufacture of nutritional beverages, including infant formula, sports, and lifestyle foods (Playne et al., 2003; Holt et al., 2013). The primary whey proteins in milk are β-LG and α-LA, and their ability to undergo structural changes during heating is of particular importance (i.e., their denaturation/aggregation behavior). Thermal treatment is a critical processing step during the manufacture of most foods and beverages for reduction of microbial load. However, the thermal stability of whey proteins is relatively low, with the formation of whey protein aggregates during heat treatment leading to changes in viscosity, turbidity, particle size, protein precipitation, and gel formation (Ryan et al., 2012).

Both α-LA and β-LG are compact globular proteins, with molecular weights ($\mathbf{M_w}$) of ~14 and ~18 kDa, respectively; α-LA is a monomer at neutral pH whereas β-LG exists as a dimer between pH 5.5 and 7.5 (Mullvihill and Donavan, 1987). The mechanism leading to protein aggregation following thermal denaturation is dependent on several factors, including protein type and concentration, pH, ionic strength, heating temperature, and type of heat treatment (Kehoe and Foegeding, 2011; Dissanayake et al., 2013). A single monomer of β-LG contains 2 disulfide bonds, with one buried free sulfhydryl (**SH**) group (Verheul et al., 1998). This free SH group can become exposed during denaturation/unfolding and aggregate with other β-LG monomers and dimers through disulfide interchange reactions, resulting in the exposure of another free SH group; the reaction is terminated when 2 β-LG monomers or aggregates form a disulfide linkage between their exposed SH groups (Roefs and de Kruif, 1994). However, unlike β-LG, α-LA does not contain any free SH groups, and therefore, in the absence of β-LG, can only aggregate through noncovalent interactions such as electrostatic

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and van der Waals interactions and the hydrophobic effect (Anema, 2009). Previous studies have observed that due to the lack of a free SH group, aggregation of the native α-LA monomer can be reversible if α-LA is heated in isolation, depending on physicochemical and environmental conditions (Boyle et al., 1997). β-Lactoglobulin aggregation, with protein species containing a free SH group or disulfide bridges (or both), is irreversible. Previous research into whey protein denaturation and aggregation has focused on the formation of aggregates through heat treatment of purified whey proteins, often at low concentrations, using laboratory-scale heat treatments, which can be slow to reach final heating temperatures (Verheul et al., 1998; Galani and Apenten, 1999; Croguennec et al., 2004). However, for industrial applications, scientific research using whey protein concentrates and whey protein isolates (**WPI**) at high concentrations are applicable, as these ingredients are similar to the types of ingredients used in the manufacture of infant formula and nutritional beverage products. Previous work has also been carried out, albeit less extensively, using pilot-scale heating equipment, typically in the heat treatment of skim milk (Corredig and Dalgleish, 1996; Oldfield et al., 1998), and to a lesser extent, whey protein concentrates and WPI (Petit et al., 2013; Erabit et al., 2016). Studying the behavior of whey proteins under relevant processing conditions is fundamental to the understanding and optimization of the variables required for the production of stable and viable whey protein products.

The objective of this study was to examine the effects of concentration and pH on heat-induced whey protein denaturation/aggregation, and determine whether soluble aggregate content and size, along with remaining native protein, affect thermal stability of solutions upon subsequent heating. The findings of this study should give further insights into factors affecting the thermal stability of whey proteins during the processing of dairy-based beverages, including infant formula.

MATERIALS AND METHODS

Preparation of Protein Solutions

Whey protein isolate (BiPro), with protein content of 93.3% as determined by Kjeldahl (IDF, 2001), was sourced from Davisco Foods International Inc. (Le Sueur, MN). The mineral content of 0.05% P, 0.04% K, 0.08% Ca, and 0.66% Na was analyzed by inductively coupled plasma mass spectroscopy. Whey protein solutions (2 L) of 1, 4, 8, and 12% (wt/wt; total protein) were prepared by hydration of WPI in distilled water and stirred at room temperature (22°C) for approximately 2 h, before overnight storage at 4°C under gentle agitation, to ensure full rehydration of the powder. Solutions were then re-equilibrated to room temperature and adjusted to the required pH (6.2, 6.7, or 7.2) using 2 *M* NaOH or 4 *M* HCl. Control (unheated) samples were separated from each batch with the remaining solution used for further processing. Heat treatment of solutions was carried out using a MicroThermics laboratory-scale tubular heat exchanger (MicroThermics, Raleigh, NC) with a set flow rate of 1 L/min and holding-tube volume of 0.5 L. Solutions were pre-heated to a temperature of 65°C, with a final heating temperature of 85°C for 30 s, with subsequent cooling to 15°C. Same-day analysis was carried out for measurements of aggregate structure and size to minimize any change to aggregates over time. Each sample batch was replicated 4 times. Throughout this publication, samples will be coded as follows: **H1–6.7** represents a 1% WPI solution heated at pH 6.7.

Quantification of Native Protein

Reversed-phase HPLC was used to quantify remaining native protein, in unheated and heat-treated samples, using a Waters (Milford, MA) 2487 dual-wavelength absorbance detector at wavelengths of 214 and 280 nm. A Source 5RPC (150 × 4.6 mm) column (Sigma-Aldrich, Wicklow, Ireland) was used for separation using solvent A (0.1% trifluoroacetic acid in MilliQ water) and solvent B (90% acetonitrile, 0.1% TFA) at a flow rate of 1 mL·min^{-1}. Gradient conditions for elution were described by Kehoe et al. (2011). Samples were diluted to a protein content of 0.25% (wt/wt), in 0.1 *M* sodium acetate buffer at pH 4.6, before centrifugation at 8,000 × *g* (25°C) for 25 min. Supernatants were filtered through a 0.22-μm filter [high-velocity filters, Millipore (UK) Ltd., Durham, UK] before injection. β-Lactoglobulin, α-LA, and BSA standards (Sigma-Aldrich) were used for column calibration. Data were processed using Waters Empower software.

Differential Scanning Calorimetry

Denaturation temperatures of α-LA and β-LG were determined by differential scanning calorimetry (**DSC**). Aliquots of control and heat-treated samples (20 mg) were placed in Tzero Hermetic pans, sealed, and analyzed by a Q2000 DSC (TA Instruments, New Castle, DE). The temperature was gradually increased from 20 to 100°C at a rate of 5°C min^{-1} under nitrogen purged conditions. An empty pan was used as a reference for heat flow to the sample, and denaturation temperatures were expressed as the peak minima of the endotherms

following postlinear integration. Data analysis was carried out using Universal Analysis 2000 software (TA Instruments).

Determination of Molecular Weight of Soluble Aggregates

The formation of soluble aggregates was determined by size exclusion chromatography (**SEC**), using a Waters 2695 separation module HPLC system. Heated and control samples were diluted to 0.25% (wt/wt) protein in 20 m*M* sodium phosphate buffer at pH 7 (mobile phase). Filtration of samples though 0.45 μm low protein binding filters (Sartorius Stedim Biotech GmbH, Germany) ensured removal of larger, non-soluble aggregates. A TSK Gel G2000SW$_{XL}$ run-in series with a G3000SW$_{XL}$, 7.8 × 300 mm column (TosoHaas Bioscience GmbH, Stuttgart, Germany), under isocratic conditions, at a flow rate of 0.5 mL·min^{-1} over 1 h, was used to elute 20 μL of sample to a Waters 2487 dual wavelength absorbance detector at wavelengths of 214 and 280 nm. The following M_w standards were used for column calibration: BSA (~66.5 kDa), α-LA (~14 kDa), β-LG (~18 kDa), aldolase (158 kDa), ferritin (44 kDa), cytochrome c (12 kDa), and carbonic anhydrase (29 kDa). Data analysis and integration were carried out using Waters Empower software. HPLC-grade Milli-Q water was used in the preparation of all buffers and samples. Buffers were vacuum filtered through 0.45-μm high velocity filters [Millipore (UK) Ltd., Durham, UK] before analysis.

Particle Size Analysis

Following heat treatment, the z-average hydrodynamic diameter was determined by dynamic light scattering (**DLS**) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with a HeNe laser with a wavelength of 633 nm and measurement at a scattering angle of 173°. Samples of H4, H8, and H12 were diluted to 1% (wt/wt) using distilled water, before further dilution to 1 in 30 to avoid multiple scattering, and measured in a plastic cuvette at 22°C. The volume distribution was determined using the cumulative method.

Measurement of Turbidity

The absorbance of heated and unheated samples was measured using a HACH 2100N turbidity meter (Hach Company, Loveland, CO) at room temperature (22°C), using a 30-mL sample in a 25-mm glass turbidity tube. The turbidity of samples was expressed in nephelometric turbidity units.

Atomic Force Microscopy

Aggregates formed by heat treatment of 1 and 12% (wt/wt) WPI at pH 6.7 were imaged using atomic force microscopy (**AFM**). Samples were prepared at a concentration of 0.01% protein, deposited onto a surface of freshly cleaved mica, which was fixed to a glass microscopy slide and dried in an incubator at 38°C, before storage in a desiccator (relative humidity ~19%) prior to analysis. Samples were analyzed using an Asylum Research MFP-3DAFM (Asylum Research UK Ltd., Oxford, UK). Images were recorded in AC mode as described previously by Kehoe et al. (2011). All images were processed using AFM imaging software Igor 6.12A with 3-dimensional images compiled using Argyle Light software.

Heat Coagulation Time as a Function of pH

Aliquots of H1–6.7 and H4–6.7 were concentrated to 6% (wt/wt) by dialysis against a polyethylene glycol (**PEG**) solution (8% wt/wt) using dialysis tubing with a 5-kDa M_w cutoff. Polyethylene glycol was used for removal of water from the protein solutions through concentration by osmotic stressing (Bouchoux et al., 2009). The protein content of H8–6.7 and H12–6.7 was reduced to 6% (wt/wt) using Milli Q water.

Aliquots (10 mL) of WPI at 6% (wt/wt) protein were adjusted to create a pH range from 6.4 to 7.2 with 0.1 pH unit increments using 0.1 *M* HCl and 0.1 *M* NaOH. Samples were adjusted initially and stirred for 4 h before final readjustment. Then 3.4 g of each sample was placed into a 4-mL glass tube (120 mm tube length, 10 mm outer radius, 7 mm inner radius; Hettich Benelux BV, Geldermalsen, the Netherlands). Each tube was stoppered using a rubber bung, placed in a steel rack, and submerged in a temperature-controlled silicone oil bath (Hettich ESP oilbaths; Hettich Benelux BV) at 140°C and rocked back and forth at a speed of 7 oscillations per min. Heat stability was determined as the amount of time (min) required for visible flocculation or gelation of proteins to occur. Analysis of heat stability was performed in triplicate.

Aliquots of H4, H8, and H12 WPI at pH 6.2, 6.7, and 7.2 were also analyzed using the same heat coagulation time (**HCT**) method, with each sample standardized to 4% protein, adjusted to create a pH curve ranging from 6.4 to 7.2 with 0.2 pH unit increments.

Rheological Measurements

An AR2000ex controlled-stress Rheometer (TA Instruments, Crawley, UK) fitted with a standard

Deutsches Institut für Normung (DIN) concentric cylinder and cup was used for the analysis of apparent viscosity. The diameters of the bob and cup were 27.5 and 30.0 mm, respectively. Heat-treated (85°C × 30 s) whey protein samples of (1, 4, 8, and 12% (wt/wt)) were measured using a shear rate sweep from 0 to 800 s^{-1} over 4 min, holding at 800 s^{-1} for 1 min, followed by a shear rate decrease from 800 to 0 s^{-1}, over 4 min. Samples were pre-sheared at 200 s^{-1} for 30 s before analysis. Temperature was maintained at 22°C using a Peltier apparatus (±0.1°C). All samples were free of air bubbles and surface foam before analysis, and a tetradecane solvent trap was used to prevent sample evaporation.

Small-amplitude oscillatory shear measurements were carried out using an ARG2 controlled stress rheometer (TA Instruments, Crawley, UK) for the analysis of thermal gelation. A standard DIN concentric cylinder was used as per viscosity measurements. Frequency measurements and strain were kept constant at 0.1 Hz and 0.5%, respectively, with samples pre-heated to 45°C for 5 min. The temperature was then increased from 45°C to 80°C, at a rate of 5°C/min, and held for 90 min at 80°C.

Statistical Analysis

Minitab 17 statistical analysis package (Minitab Ltd., Coventry, UK, 2014) was used for ANOVA (Tukey's honestly significant difference). The level of significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Protein Denaturation

The amount of native protein in heated and unheated solutions, at a protein concentration of 1, 4, 8, and 12%, and pH of 6.2, 6.7, and 7.2, was quantified using reversed-phase (**RP**) HPLC. Samples were diluted in sodium acetate buffer at pH 4.6 and large protein aggregates removed by sedimentation through centrifugation at 12,000 × g for 15 min at 25°C. Samples were subsequently injected onto the HPLC column after filtering. Table 1 shows the amount of native protein present in unheated control samples and samples heat-treated at 85°C for 30 s. A significant ($P < 0.05$) loss in native protein was observed for all samples after heat treatment, compared with the unheated control sample. An increase in protein concentration from H1 to H12 resulted in a decrease in the quantity of native protein. Kehoe et al. (2011) showed previously that an increase in protein concentration resulted in more extensive aggregation in mixed whey protein systems. At higher protein concentration, the probability of collision between protein molecules is increased, resulting in a net increase in the aggregation of unfolded molecules (Kessler, 2002; Wolz and Kulozik, 2015).

Table 1 also shows the amount of native α-LA, β-LG A, and β-LG B protein present in samples as a function of both protein concentration and pH. β-Lactoglobulin A and B are the 2 most common variants of bovine β-LG, although other variants also exist (Sawyer, 2013). Historically, it has been reported that β-LG B is more heat labile compared with β-LG A and undergoes greater levels of denaturation upon heat treatment at pH 6.8 (Gough and Jenness, 1962). However, more recent research has shown that the heat stability of both β-LG variants is dependent on several factors including protein concentration and pH. Nielsen et al. (1996) observed that β-LG samples, heat-treated at concentrations below 5% (wt/wt; pH 7), showed more rapid denaturation of β-LG B, whereas β-LG A was more heat sensitive at concentrations above 5% (wt/wt). O'Kennedy and Mounsey (2006) reported preferential denaturation of β-LG A at acidic pH (pH 5.0–5.5) and β-LG B at more neutral pH (6.5–7.0). In the current study, β-LG A was generally more heat stable, although this effect was less pronounced at pH 6.2. The proportion of native β-LG A remaining was lower at higher protein concentration (H8 and H12), as supported by the findings by Nielsen et al. (1996), although samples still contained a greater proportion of native protein when compared with β-LG B. Qin et al. (1999) studied the structure of β-LG A and B and found that although the 2 variants differ only at 2 AA sites 64 and 118 (variant A has an aspartic acid residue at position 64 and a valine residue at 118, whereas variant B has a glycine and alanine residue at position 64 and 118, respectively), β-LG B is less thermally stable due to differences in structure on the β-strand (V118A), which alters the dynamic properties of the molecule by disrupting the internal hydrophobic packing. In comparison to β-LG and its specific variants, the percentage of native α-LA remaining in heat-treated samples decreased with increasing pH. Law and Leaver (2000) studied the effect of pH on whey protein denaturation in skim milk and found that the rates of denaturation and aggregation of α-LA increase with increasing pH.

The denaturation temperatures of α-LA and β-LG were determined from DSC measurements. Although reversibility of protein denaturation has been previously observed using proteins such as lysozyme (Blumlein and McManus, 2013), no evidence of reversibility for either α-LA or β-LG was observed in DSC thermographs of unheated samples following a second heating cycle (data not shown). The denaturation temperature of α-LA and β-LG corresponded with previous research,

Table 1. Reversed-phase-HPLC results (mean ± SD from triplicate trials) for individual and total native protein in unheated and heat-treated samples prepared at 1, 4, 8, and 12% (wt/wt) at pH (6.2, 6.7, and 7.2)

Protein content	pH	Native α-LA (%)	Native β-LG A (%)	Native β-LG B (%)	Total native protein (%)
Unheated control		25.83 ± 1.0^{a}	43.71 ± 0.4^{a}	30.46 ± 1.0^{a}	100 ± 1.8^{a}
Heated	6.2				
1		12.52 ± 0.9^{b}	21.58 ± 1.7^{cd}	12.58 ± 4.6^{de}	46.48 ± 2.7^{cd}
4		9.88 ± 1.7^{bc}	23.25 ± 0.1^{c}	13.32 ± 3.6^{de}	46.45 ± 1.9^{cd}
8		11.01 ± 3.3^{b}	13.40 ± 2.9^{fgh}	7.25 ± 2.6^{fg}	31.66 ± 4.9^{ef}
12		10.77 ± 1.9^{bc}	8.78 ± 4.0^{h}	8.77 ± 2.6^{fg}	28.03 ± 6.1^{fg}
	6.7				
1		7.81 ± 1.8^{bc}	28.77 ± 3.6^{b}	14.85 ± 0.9^{d}	51.44 ± 3.9^{bc}
4		1.44 ± 0.8^{cd}	12.24 ± 0.5^{fgh}	26.33 ± 2.5^{b}	39.01 ± 3.4^{de}
8		11.24 ± 2.5^{b}	15.13 ± 3.7^{ef}	6.95 ± 4.0^{fg}	33.32 ± 3.1^{ef}
12		5.62 ± 1.0^{cd}	9.60 ± 2.3^{gh}	4.25 ± 2.4^{g}	19.48 ± 1.8^{g}
	7.2				
1		10.23 ± 2.3^{bc}	30.67 ± 0.5^{b}	19.47 ± 0.9^{c}	60.37 ± 6.2^{b}
4		7.88 ± 1.5^{bc}	17.02 ± 1.0^{def}	20.23 ± 1.4^{c}	45.14 ± 2.4^{cd}
8		9.54 ± 3.3^{bc}	14.17 ± 3.3^{fg}	4.95 ± 2.6^{fg}	28.65 ± 3.4^{fg}
12		8.37 ± 2.8^{bc}	19.84 ± 2.3^{cde}	9.02 ± 2.6^{ef}	37.23 ± 4.3^{def}

$^{a–h}$Values within a column with different superscripts are statistically different at $P < 0.05$.

with temperatures ranging from 64.5 to 66.5°C and 75.5 to 78.5°C, respectively. At pH 6.2 and 6.7, a significant ($P < 0.05$) decrease occurred in the denaturation temperature of β-LG at higher whey protein concentrations (8 to 12%). For pre-heated solutions, a decrease in denaturation temperature occurred, although the decrease occurred at a lower denaturation temperature and was not statistically significant. It has previously been observed that increasing the protein concentration of β-LG results in a decrease in its denaturation temperature (Qi et al., 1995), with comparable results also observed in mixed whey protein systems (Boyle and Alli, 2000; Murphy et al., 2014).

Characterization of Aggregates Formed

Size Exclusion Chromatography. Size exclusion chromatography HPLC was used to quantify the M_w distribution of soluble aggregates produced during heating. Aggregates too large to pass though the SEC columns (i.e., insoluble denatured/aggregated material) were excluded by prior filtration through 0.45-μm filters. This resulted in a maximum M_w range cutoff between ~1 and ~600 kDa. Figure 1A depicts a typical chromatogram of unheated and heated (85°C × 30 s) WPI. Figure 1B shows both the percentage area of insoluble protein and distribution of soluble aggregates detected by SEC-HPLC. Although an effect of concentration was evident for samples heat-treated at pH 6.7 and 7.2, this effect was not observed for samples heat-treated at pH 6.2. No significant difference was observed in the percentage area of insoluble material or soluble aggregates formed at this pH. It appears that the interactive effects of pH and concentration are dominated by diminution of charge repulsion effects at the lower pH examined. In contrast, samples heat-treated at pH 6.7 and 7.2 showed a more pronounced concentration effect, with an increase in the percentage area of soluble aggregates formed in the M_w range of 280 to 600 kDa with increasing protein concentration, and a concomitant decrease in lower M_w species (<24 kDa). These findings are in agreement with previous measurements of soluble aggregate formation in β-LG solutions. Mehalebi et al. (2008) also observed an increase in the formation of large soluble aggregates with increasing β-LG concentration (pH 8.0), and a decrease at pH 6.0. In contrast, Schmitt et al., (2007) reported that 1% whey protein, heat-treated at 85°C for 15 min, resulted in fewer soluble aggregates with increasing pH. This was attributed to reduced protein attractive interactions as a result of increased overall negative charge. Ryan et al. (2013) reported that small, compact, and highly charged soluble aggregates are resistant to changes in ionic strength and can improve the thermal stability of whey protein-containing beverages during heat treatment.

Dynamic Light Scattering. The particle size of aggregates in unfiltered solutions, as determined by dynamic light scattering (**DLS**), was significantly ($P < 0.05$) different at each WPI concentration (H1, H4, H8, and H12) heat-treated at pH 6.7 and 7.2. Increasing protein concentration (from H1 to H8) resulted in a significant decrease ($P < 0.05$) in z-average diameter (Figure 2A). The polydispersity index (**PdI**) is used as a measure of the breadth of the M_w distribution of polymer samples (Rane and Choi, 2005). All particle size distributions measured in this experimental work presented were monomodal distributions. It is noted

that the PdI for samples of H1–6.2 and H4–6.2 are lower than the equivalent samples heat-treated at pH 6.7 or 7.2 (Table 2). This result suggests that although these samples are still polydisperse in their distribution, they are less polydisperse than the equivalent samples heat-treated at pH 6.7 or 7.2.

Samples heat-treated at pH 6.2, overall, had a greater z-average particle diameter than samples heat-treated at higher pH. The isoelectric point of α-LA and β-LG is in the range of pH 4.6 to 5.2 depending on the conformation of the individual protein (native/non-native; El-Salam et al., 2009); in comparison, the pH of rehydrated WPI solutions (dependent on protein concentration) is typically in the range of pH 6.6 to 7 with fresh raw milk having a pH of 6.8 (Mullvihill and Donavan, 1987). A decrease in pH toward the isoelectric point causes a reduction in overall net negative charge (at a pH above the pI) and increases the probability

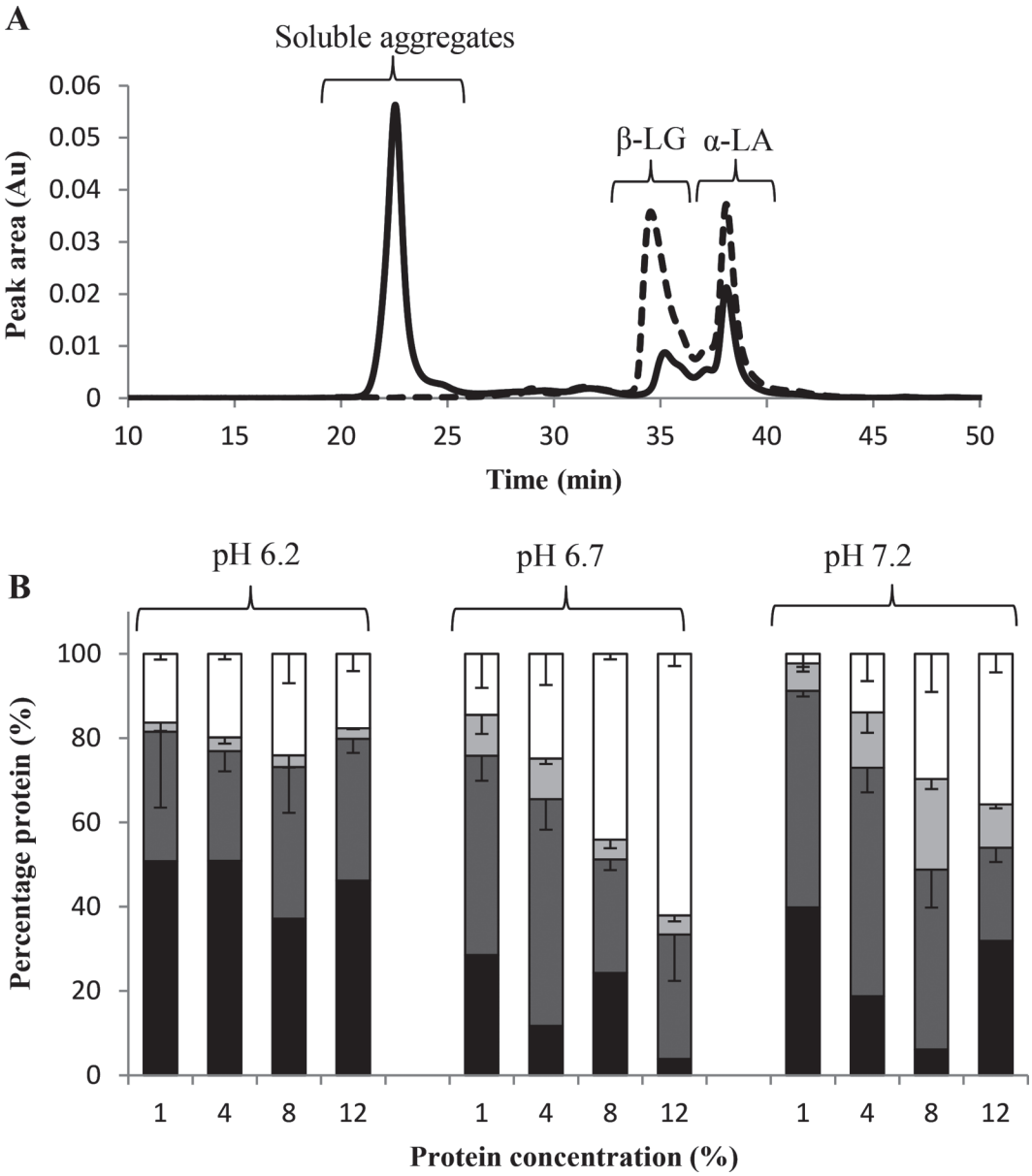

Figure 1. (A) A representative size exclusion chromatography (SEC)-HPLC chromatogram of an unheated (control = broken line) and heat-treated (85°C × 30 s = unbroken line) whey protein sample at 8% (wt/wt) at pH 6.7. (B) Molecular weight distribution of soluble protein aggregates and insoluble material as determined by SEC after samples (1, 4, 8, and 12% wt/wt) underwent heat treatment of 85°C × 30 s at pH 6.2, 6.7, and 7.2. Distribution represented as insoluble material (black), ≤24 (dark gray), 24 to 280 (light gray), and 280 to 600 kDa (white). Error bars represent SD.

Table 2. Polydispersity index (mean ± SD from triplicate trials) of each heat-treated (85°C × 30 s) whey protein solution prepared at 1, 4, 8, and 12% (wt/wt) at pH (6.2, 6.7, and 7.2) as determined by dynamic light scattering

Protein content (%)	pH	Polydispersity index
	6.2	
1		0.06 ± 0.01
4		0.06 ± 0.01
8		0.17 ± 0.03
12		0.34 ± 0.05
	6.7	
1		0.10 ± 0.03
4		0.08 ± 0.01
8		0.25 ± 0.02
12		0.25 ± 0.01
	7.2	
1		0.23 ± 0.02
4		0.29 ± 0.06
8		0.27 ± 0.01
12		0.26 ± 0.01

of protein aggregates forming. Increased association of proteins can ultimately lead to the formation of covalently bonded aggregates (Brodkorb et al., 2016), and an increase in the average particle size of aggregates, compared with whey proteins heat-treated at a pH further away from the isoelectric point (Cornacchia et al., 2014). Consequently, samples heat-treated at pH 6.2 do not demonstrate a concentration effect, as pH dominates with SEC-HPLC results supporting this observation.

However, samples heat-treated at higher pH (6.7 and 7.2) showed a decrease in z-average particle diameter with increasing protein concentration. In protein systems, as the protein concentration is increased, the number of primary protein particles available for denaturation and aggregation increases (Kessler, 2002).

Turbidity. The turbidity of heat-treated whey protein solutions varied as a function of both pH and protein concentration (Figure 2B); a positive relationship was evident between the z-average particle size distribution, and turbidity, for samples heat-treated at pH 6.7. A significant decrease ($P < 0.05$) in turbidity at pH 6.7 was observed with an increase in protein concentration from H1 to H8. A significant increase ($P < 0.05$) in turbidity was also observed at each protein concentration, when samples heat-treated at pH 6.7 and 7.2 are compared with samples heat-treated at pH 6.2 (insert of Figure 2B). Again, when compared against results for DLS, samples heat-treated at 1% had significantly higher ($P < 0.05$) turbidity, with a greater effect of pH at this protein concentration due to the electrostatic repulsions previously mentioned. It is not clear whether increased turbidity at this protein concentration is due to larger aggregates or a greater proportion of aggregates. However, when turbidity and DLS data are observed together, it is possible to categorize the samples into 2 groups, the first of which contains more turbid samples (samples heat-treated at pH 6.2), with larger average particle sizes and the second of which contains less turbid samples (samples heat-treated at higher concentration at pH 6.7 and 7.2) containing aggregates with smaller overall particle sizes.

Atomic Force Microscopy. The size and shape of aggregates formed following heating of H1–6.7 and H12–6.7 solutions were visualized using AFM (Figure 3). A positive correlation was observed between the size and height (y-axis) of large aggregates in AFM images, and the z-average diameters determined by DLS (Figure 3A, image 4). Although the drying of particles in air during the AFM preparative process can affect both particle size and shape, analysis of the images for 1 and 12% protein demonstrated differences in aggregate formation and distribution as a function of concentration. The 3-dimensional images (Figure 3, image 3) show

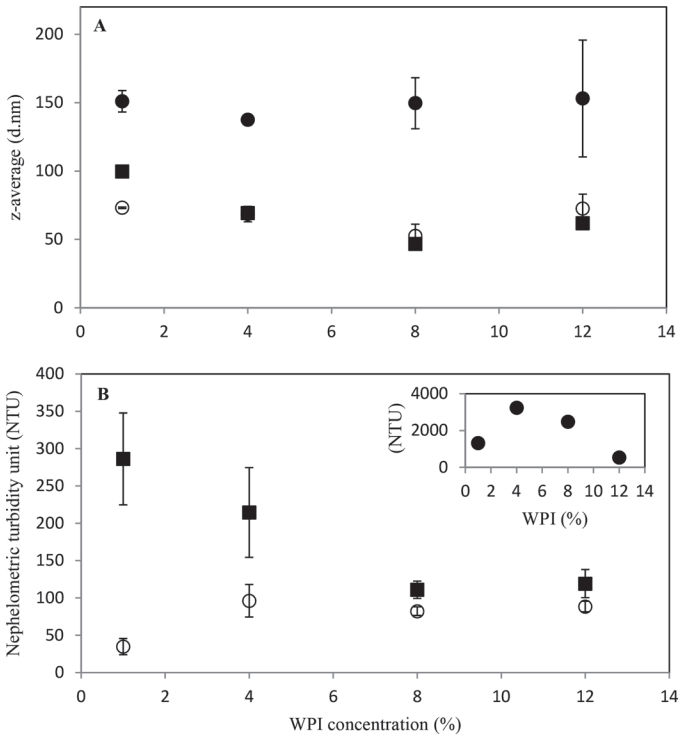

Figure 2. Particle size and turbidity measurements of heated whey protein samples (1, 4, 8, and 12% wt/wt protein heat treated at 85°C × 30 s). Graph A represents the particle size diameter (d.mm) of the whey protein samples at different concentrations: pH 6.2 (●), pH 6.7 (■), and pH 7.2 (○). Graph B represents the turbidity of whey protein samples at different concentrations: pH 6.2 (●), pH 6.7 (■), and pH 7.2 (○). The insert in graph B represents the turbidity of samples heat treated at pH 6.2. WPI = whey protein isolate. Error bars represent SD.

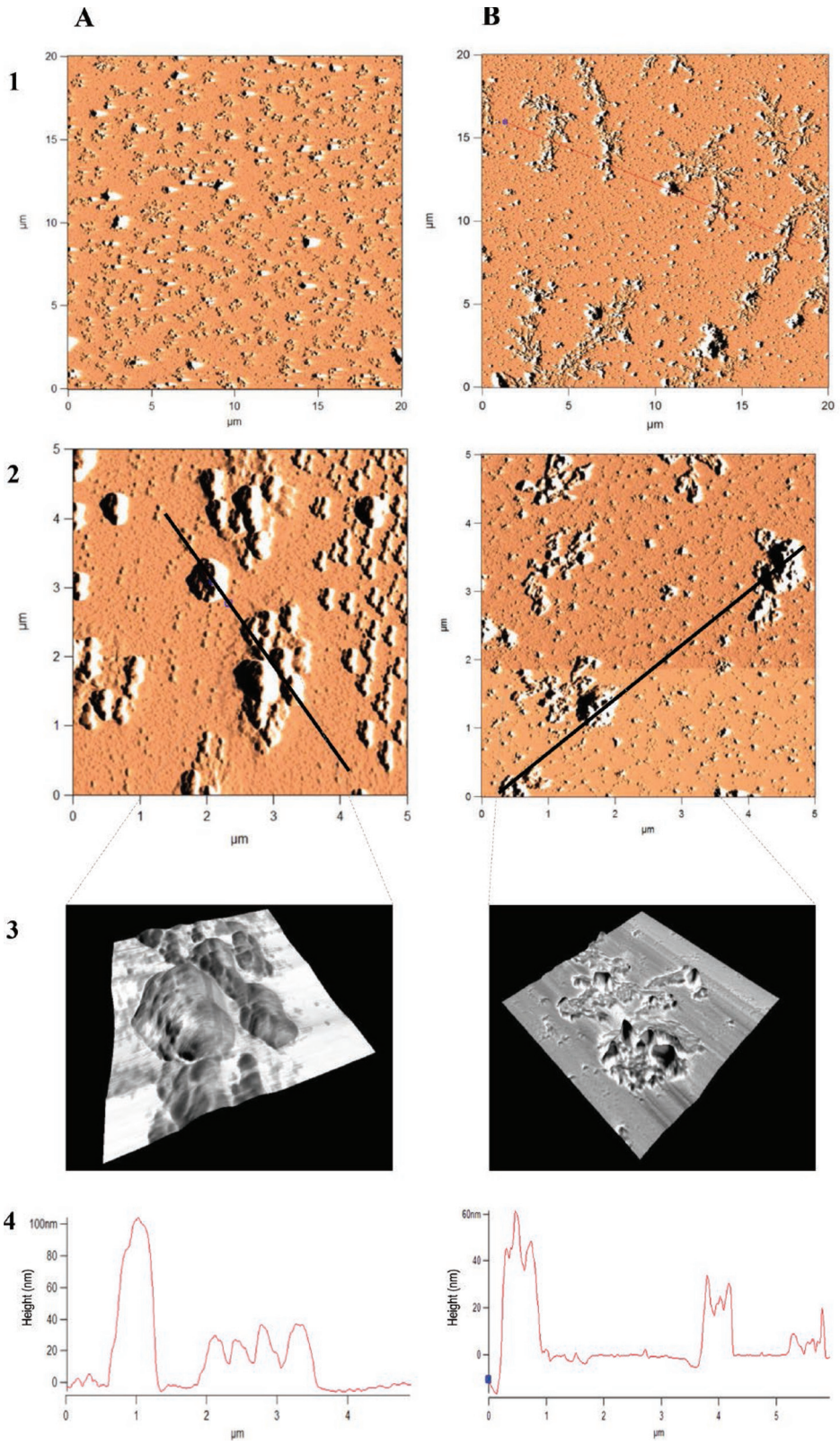

Figure 3. Atomic force microscopy images (recorded in air) of aggregates formed after heat treatment at pH 6.7 (85°C × 30 s) of (A) 1% whey protein and (B) 12% whey protein. Amplitude images of areas of 20 µm × 20 µm and 5 µm × 5 µm are shown in image 1 and 2, respectively, with 3-dimensional images of individual aggregates given in image 3. The bottom graph (4) shows the height profile of the cross-section displayed in image 2.

the structure and shape of individual aggregates, with H1–6.7 having a smoother appearance than aggregates formed at H12–6.7. However, this result must be taken with caution as the individual aggregates observed by AFM may not be representative of all aggregates formed during the heating process.

Samples heated at H1–6.7 appeared to be more uniform (monodisperse) in appearance, which corresponds to its lower PdI value (Table 2). When compared with samples heat-treated at H12–6.7, a more distinctive network structure can be observed, with aggregates clustered in often linear fragments indicating a greater extent of particulate interaction.

Heat Stability as a Function of Aggregate Size and pH

Correlation between aggregates formed from heat treatment of H4, H8, and H12, at pH 6.2, 6.7 and 7.2 (standardized to 4% (wt/wt) protein) and the thermal stability of heat-treated solutions, was carried out using the HCT method over a pH range from pH 6.4 to 7.2 (Table 3). The HCT method analyzes the heat stability of liquid samples by placing glass tubes (rotated) in a silicone oil bath at a set temperature (140°C) until visual aggregation is observed. Solutions heat-treated at 1% (wt/wt) protein were highly heat stable. No aggregation was apparent after 40 min, which was chosen as an arbitrary cutoff point for HCT in this experimental work.

It is apparent from Table 3 that an increase in pH resulted in an increase in HCT, when samples are compared at the pH at which they were pre-heat-treated at (85°C × 30 s). This effect is more evident as the pH is increased from 6.4 to 7.2 during the HCT experiment. For samples previously heat-treated at pH 6.2, higher protein concentration resulted in an increase in HCT. In contrast, samples previously heat-treated at pH 6.7 and 7.2 generally tended to show an opposite trend. Samples previously heat-treated at pH 6.7 and 7.2 were deemed thermally stable on subsequent heating at pH 7 and 6.8, respectively. When correlated with DLS results, solutions H8 and H12 at pH 6.7 and 7.2 also had statistically ($P < 0.05$) lower particle size and a higher ($P < 0.05$) proportion of large soluble aggregates (280 to 600 kDa), compared with respective samples with prior heat treatment at pH 6.2. Typically, the pH range between 6.8 and 7.2 is a target pH for the manufacture of nutritional beverages containing whey ingredients, including infant formula. The maximum HCT observed in this study also lay within this pH range.

To further understand the influence of whey protein denaturation/aggregation on the heat stability of a protein solution, HCT analysis was also performed on samples heat-treated at pH 6.7 and protein concentrations of 1, 4, 8, and 12% (wt/wt). Samples were then

Table 3. Heat coagulation time (HCT) data (mean ± SD from triplicate trials) of pre-heated (85°C × 30 s) whey protein solutions prepared at 4, 8, and 12% (wt/wt) at pH (6.2, 6.7, and 7.2) after being placed in a silicone oil bath at 140°C

		HCT (min)		
Protein content (%)	pH[1]	pH 6.2	pH 6.7	pH 7.2
	6.4			
4		13.50 ± 3.40^{b}	18.24 ± 1.33^{a}	21.74 ± 2.57^{ab}
8		20.44 ± 1.16^{a}	16.73 ± 0.35^{a}	23.42 ± 1.12^{a}
12		17.18 ± 2.29^{a}	13.22 ± 1.68^{b}	18.66 ± 2.80^{b}
	6.6			
4		15.05 ± 1.46^{b}	15.77 ± 1.33^{a}	21.36 ± 7.04^{a}
8		22.86 ± 2.02^{a}	22.70 ± 1.45^{b}	15.70 ± 1.12^{b}
12		24.34 ± 0.88^{a}	23.03 ± 1.35^{c}	23.13 ± 1.54^{a}
	6.8			
4		18.67 ± 0.70^{c}	31.13 ± 6.15^{a}	32.9 ± 6.17^{a}
8		35.74 ± 1.20^{a}	25.11 ± 1.79^{b}	>40[2]
12		20.58 ± 1.83^{b}	27.25 ± 1.16^{ab}	31.17 ± 1.17^{a}
	7.0			
4		22.34 ± 1.00^{c}	>40[2]	>40[2]
8		29.52 ± 1.26^{a}	35.08 ± 1.74^{a}	>40[2]
12		28.27 ± 2.38^{a}	30.67 ± 0.70^{b}	>40[2]
	7.2			
4		22.93 ± 2.29^{c}	>40[2]	>40[2]
8		38.34 ± 0.70^{a}	>40[2]	>40[2]
12		31.17 ± 1.02^{b}	14.45 ± 1.36	>40[2]

$^{a–c}$Values within a column with different superscripts are statistically different at $P < 0.05$.

[1]pH at which previously heated samples were buffered before HCT analysis.

[2]Samples were stable for up to 40 min after which the HCT method was interrupted.

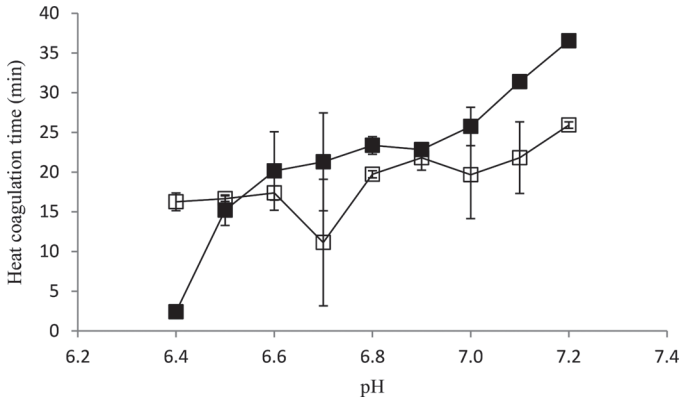

Figure 4. Heat coagulation time (HCT) curves for samples of 1% (□) and 8% (■) whey protein solutions, heat treated at pH 6.7 at 85°C (×30 s). Samples used for HCT analysis were standardized to 6% wt/wt protein before HCT measurements. Heat coagulation time was carried out in a paraffin oil bath at 140°C. Error bars represent SD.

standardized to 6% (wt/wt) protein, which allowed for the inclusion of pre-heat-treated 1% (wt/wt) samples, the protein concentration of which was too dilute for visual detection in previous HCT analysis. At pH 6.4, solutions H1–6.7 were significantly more stable than H8–6.7 samples (Figure 4). However, when the pH of both H1–6.7 and H8–6.7 solutions was increased to 7.0 or above, solutions H8–6.7 had significantly greater ($P < 0.05$) heat stability, with an overall maximum stability of 36.5 min. From DLS and HPLC measurements it is clear that increasing the protein concentration results in an overall decrease in particle size in combination with an increase in large (280 to 600 kDa) soluble aggregates resulting in increased thermal stability. Recent research has focused on the use of soluble whey protein aggregates to stabilize whey protein systems, in particular model systems, for the production of high concentration whey protein beverages (Ryan and Foegeding, 2015). From SEC-HPLC results it was observed that the presence of significant ($P < 0.05$) quantities of large-sized soluble aggregates (280 to 600 kDa), combined with the stabilizing effect of pH, helped produce more heat-stable whey protein systems at pH values greater than 7.0.

Rheological Measurements

The apparent viscosity of WPI dispersions was analyzed immediately postprocessing. Measurements demonstrated that an increase in protein concentration to 12% (wt/wt) resulted in an increase in viscosity, with pH having no significant effect (Figure 5). Results obtained were similar to those of previous research carried out on non-Newtonian WPI solutions (Bazinet et al., 2004). Samples at H12 had significantly higher ($P < 0.05$) apparent viscosity than dispersions prepared at lower concentrations [1, 4, and 8% (wt/wt) protein]. By increasing protein concentration, a greater quantity of protein aggregates are formed upon heating, leading to

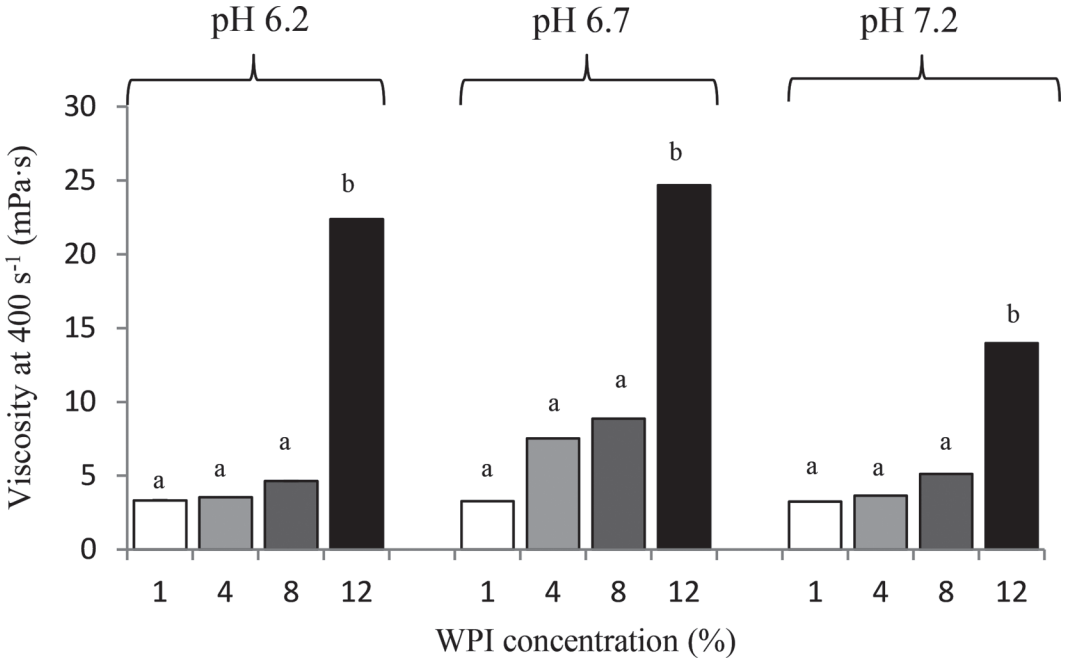

Figure 5. Apparent viscosity of heat-treated whey protein samples (85°C × 30 s) at corresponding pH (6.2, 6.7, and 7.2). Sample points displayed were taken at 400 s^{-1} at 25°C. Letters differing within each pH differ significantly ($P < 0.05$). WPI = whey protein isolate.

greater particle interactions resultant in higher viscosities (Verheul et al., 1998; Kessler, 2002).

Oscillatory rheology (data not shown), used to measure gel formation and strength (after heat treatment at 80°C for 90 min), showed that only H12–6.2 WPI dispersions underwent gelation on prolonged heating. Whey protein gels formed between pH 4 and 6 are sometimes referred to as aggregate or particulate gels, as opposed to fine stranded gels, which are formed at higher pH (Stading and Hermansson, 1990). Gelation of this specific sample was most likely due to the quantity and type of aggregates produced following heat treatment. In order for gel formation to occur, certain parameters must be met in relation to the pH and ionic environment. A critical protein concentration and heating temperature are normally needed depending on environmental conditions, with both of these parameters tending to increase on moving away from the isoelectric point (Lazidis et al., 2016).

CONCLUSIONS

This study demonstrated the effect that whey protein concentration and pH have on protein aggregate formation and subsequent thermal stability. The WPI solutions heat-treated at pH 6.7 and 7.2 had a greater proportion of soluble protein material in the range 280 to 600 kDa, lower overall protein aggregate size, and increased thermal stability when compared with solutions heat-treated at pH 6.2. Increasing the protein concentration from 1 to 12% (wt/wt) in solutions heat-treated at pH 6.7 and 7.2 resulted in a concomitant reduction in turbidity and particle size, and an increase in thermal stability. The findings of this study demonstrate the importance of protein concentration in controlling protein aggregation and the subsequent functionality of whey ingredients and nutritional formulations in which they are used.

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