Functional properties of whey proteins microparticulated at low pH

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ABSTRACT

The main aim of the study was to assess the effect of microparticulation at low pH on the functionality of heat-denatured whey proteins (WP). Spray-dried, microparticulated WP (MWP) powders were produced from 7% (wt/wt) WP dispersions at pH 3, acidified with citric or lactic acid, and microfluidized with or without heat denaturation. Nonmicroparticulated, spray-dried powders produced at neutral pH or pH 3 served as controls. The powders were examined for their functional and physicochemical properties. Denatured MWP had an approximately 2 orders of magnitude reduction in particle size compared with those produced at neutral pH, with high colloidal stability indicated by substantially improved solubility. The detection of monomeric forms of WP in PAGE also confirmed the particle size reduction. Microparticulated WP exhibited enhanced heat stability, as indicated by thermograms, along with better emulsifying properties compared with those produced at neutral pH. However, MWP powders created weaker heat-induced gels at neutral pH compared with controls. However, they created comparatively strong cold acid-set gels. At low pH, a combination of heat and high hydrodynamic pressure produced WP micro-aggregates with improved colloidal stability that affects other functionalities.

Key words: whey protein, low pH, dynamic high pressure, functional property

INTRODUCTION

Our previous studies showed that microparticulation of whey proteins (WP) using combined heat and high pressure shearing (microfluidization) could successfully modulate and stabilize WP against heat at neutral pH (Dissanayake and Vasiljevic, 2009; Dissanayake et al., 2010). In addition to enhanced heat stability, denatured microparticulated whey proteins (MWP) exhibited

different functionalities from those of native WP, such as improved emulsifying activity and gelling properties (Dissanayake and Vasiljevic, 2009). Although microparticulation produced micro-aggregates by dispersing heat-denatured WP aggregates, the particle size of these aggregates was not small enough to prevent sedimentation. This may have adverse effects on most other WP functionalities, restricting their successful application in various heterogeneous food systems.

Whey proteins at acidic pH are more stable than those at neutral pH against heat-induced aggregation (de la Fuente et al., 2002). Consequently, they form heat-set gels that are relatively brittle and weak. Compared with these gels, those formed at a neutral pH exhibit strong and elastic fracture properties (Havea et al., 2009). The weak and brittle nature of acid gels primarily arises due to the overall positive charge of WP at acidic pH with enhanced intermolecular repulsive electrostatic interactions and inhibition or suppression of thiol group activity (Morr and Ha, 1993; Spiegel and Huss, 2002; Damodaran, 2008). Lactose, a factor that governs the WP gel structure at neutral pH due to its influence on the denaturation rate of β -LG and the particle size of aggregates (Spiegel, 1999), was reported to have little or no effect on gels formed at acidic pH (Spiegel and Huss, 2002). In addition, most WP products contain a considerable amount of calcium, which is associated with the creation of a strong gel network due to additional cross-linking via formation of calcium bridges between protein molecules (Havea and Sing, 2003). Introducing a calcium-chelating agent such as citrate ions may result in relatively weaker WP gels and dispersions with enhanced heat stability (De Rham and Chanton, 1984). Understanding the relative influence of different acidulants on WP gelation would be beneficial for manufacturing of MWP at low pH. The variations in gel quality due to different acidulant effects may be related to specific anion effects, such as size of the ion and the charge density, which may determine their ability to perturb the solvent (water) structure (Resch et al., 2005b). For example, large ions with low surface charge density generally exert the greatest disruption in the hydrogen-bonded structure of water, whereas smaller ions with greater charge density tend to have

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the smallest effect on the disorder of the solvent, which in turn influences the hydrophobic interactions within the protein molecule in solution (Resch et al., 2005b).

The principal aim of the current study was to produce heat-stable MWP powders with improved colloidal stability via reduction of particle size. The study was carried out at low pH to create a WP gel network with relatively weaker molecular linkages that would be easily dispersed by mechanical forces used in high-pressure homogenization or microfluidization. In addition, the influence of change in the ionic environment was assessed by using different acidulants for acidification of samples.

MATERIALS AND METHODS

Materials and Proximate Composition

Whey protein retentate samples used in the study were kindly provided by Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia). They contained approximately 30% total solids and were collected from 2 batches produced on separate days. Initial compositional analysis of these samples was carried out following established AOAC methodology during our earlier studies (Dissanayake and Vasiljevic, 2009). Whey retentates contained an average of 70.10% moisture, 24.53% protein, 1.51% fat, 1.89% lactose, 1.00% ash, and 470.2 mg/L calcium. All chemicals used in the study were of analytical grade.

Production of WP Powders and Sample Preparation

Seven types of MWP powders were prepared with replicates from 7% (wt/wt) WP dispersions. This concentration was the maximum achievable protein concentration that could have been subjected to the described process. The experimental design applied in this study is depicted in Figure 1. Briefly, 4 microparticulated samples were prepared by adjusting the pH of dispersions to 3, using citric or lactic acid, and then spray-drying the dispersions using a pilot-scale spray dryer (SL-10 Mini-Maxi Pilot Spray Dryer, Saurin Enterprises Pty. Ltd., Melbourne, Australia) after 5 passes through a microfluidizer (model 110 Y, Microfluidics, Newton, MA) at 140 MPa, with or without a prior heat treatment of 20 min at 90°C. Three nonmicroparticulated controls were prepared by spray-drying 7% WP dispersions: 2 with the pH adjusted to approximately 3 using citric or lactic acid and the other diluted with Milli-Q water (Millipore, Billerica, MA) without changing the pH (pH ~6). The inlet and the outlet air temperatures of the spray drier were 180 and 80°C, respectively. Subsequently,

hydrated WP dispersions were prepared from spray-dried powders using Milli-Q water with no further pH adjustment at this step. The treatments were designated as follows: untreated control; citric acid-acidified control (**Control-CA**); citric acid-acidified and microfluidized (**M-CA**); citric acid-acidified, heated, and microfluidized (**HTM-CA**); lactic acid-acidified control (**Control-LA**); lactic acid-acidified and microfluidized (**M-LA**); lactic acid-acidified, heated and microfluidized (**HTM-LA**). The analysis of physical, chemical, and thermal properties of the dispersions was conducted as follows.

PAGE

Protein profiling of the samples was carried out by electrophoretic analysis of the powder dispersions according to the method described in Dissanayake and Vasiljevic (2009). Native PAGE was performed to observe the extent of protein denaturation in the samples, whereas reducing and nonreducing SDS-PAGE were performed to observe the types of interactions that prevailed in protein aggregates. β -Mercaptoethanol was used as a reducing agent in reducing SDS-PAGE. The concentration of whey protein samples used in the SDS-PAGE was 1 mg/mL.

Thermal Analysis

Thermal analysis of 12% (wt/wt) WP dispersions was carried out using a differential scanning calorimeter (DSC 7, Perkin Elmer, Norwalk, CT) and the Pyris Manager software (version 5.0002). Samples (approximately 30 μ L) of 12% (wt/wt) WP dispersions were accurately weighed in aluminum pans and hermetically sealed. An empty pan of equal weight was used as a reference. The scanning temperature was increased from 25 to 140°C at a rate of 10°C/min (Dissanayake and Vasiljevic, 2009).

Particle Size Analysis

The particle size distribution pattern of approximately 1% (wt/wt) WP dispersions was obtained using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Approximately 1% (wt/wt) WP dispersions were prepared from the spray-dried powders and hydrated overnight at 4°C. After hydration, dispersions were diluted 1/1,000 with Milli-Q water and vortexed before determination of the particle size. The refractive index of solvent (water) and the dispersed phase (WP) were 1.33 and 1.52, respectively. For each replicate, the average of 2 mean particle diameter readings was obtained.

Figure 1. Experimental design and coding used in the study. WPC = whey protein concentrate; LA = lactic acid; CA = citric acid; M = microfluidized; HTM = heat-treated and microfluidized.

Solubility

Protein solubility was determined by Kjeldahl method using filtered (0.45- μ m filters) supernatant of 5% (wt/wt) protein dispersions after centrifugation (model J2HS, Beckman, Fullerton, CA) for 20 min at $12,000 \times g$ and 20°C (Morr et al., 1985). The applied nitrogen conversion factor was 6.25. Solubility was given by the protein content of the supernatant expressed as a percentage of the total protein content in the initial dispersion. The temperature of 20°C was chosen in assessing solubility of these powders because it approximates the ambient conditions and all characterization experiments of this study were carried out at this temperature.

Heat Stability

Heat Stability via Solubility Method. First, 3-mL aliquots of 5% (wt/wt) protein dispersions in sealed glass vials were exposed to 140°C for 10 s, followed by centrifugation ($12,000 \times g$, 20°C) and determination of protein concentration in the supernatant, which was carried out using the Bradford method. A standard curve ($r^2 = 0.98$) used in the calculations was developed using BSA (Sigma-Aldrich, Chemie GmbH, Steinhelm,

Germany) with Bradford reagent (Sigma-Aldrich, St. Louis, MO). Heat stability was given by the protein content of the supernatant after heating, expressed as a percentage of the protein content of dispersion before heating.

Heat Coagulation Time. Exactly 3-mL aliquots of 5% (wt/wt) protein dispersions were sealed in a glass tube (10 mm diameter and 75 mm length) and heated at 140°C in a shaking oil bath (Ratek, Boronia, Australia). The heat exposure time for the first appearance of visible protein aggregates was considered as the heat coagulation time (**HCT**; Rattray and Jelen, 1996).

Emulsification

The method described in Dissanayake and Vasiljevic (2009), which was adopted from Pearce and Kinsella (1978), was used to determine emulsifying activity index (**EAI**) and emulsion stability index (**ESI**) to estimate the emulsification capacity of the WP dispersions and the stability of the emulsions formed, respectively. Approximately 80 mL of canola oil and 240 mL of 5% (wt/wt) WP dispersions were mixed and homogenized via microfluidization using 1 pass at 20 MPa to prepare the emulsions. Aliquots for absorbance measurements were obtained from the center of the emulsion samples.

EAI. The EAI (m^2/g) is a function of the turbidity of the emulsions and the oil volume fraction. It is expressed as units of area of interface stabilized per unit weight of protein:

$$EAI = \frac{2 \cdot T}{\phi C},$$

where T is turbidity ($1/\text{m}$), ϕ is the oil volume fraction (dimensionless), and C is the weight of protein per unit volume of aqueous phase in dispersion (g/m^3), before an emulsion is formed.

Turbidity is given by

$$T = \frac{2.303A}{l},$$

where A is the absorbance and l is the path length of the cuvette (m).

The turbidity values were obtained by first diluting the emulsions 1/3,000 with 0.1% SDS and then measuring their absorbance at 500 nm in a cuvette with a 1.5-cm path length. The oil volume fraction (φ) was calculated as

$$\varphi = \frac{C - A - E(B - C)}{C - A + (B - C) \left[\frac{(1 + E)D_o}{(D_s - E)} \right]},$$

where A is the mass of empty pan (g), B is the mass of pan plus emulsion (g), C is the mass of pan plus dry matter of emulsion (g), D_o is the density of oil (g/mL), D_s is the density of protein dispersion (g/mL), and E is the concentration of protein in dispersion (g/mL).

ESI. The ESI was calculated by measuring the turbidity of the emulsions after holding them at 4°C for a defined period (24 h):

$$ESI = \frac{(T \times \Delta t)}{\Delta T},$$

where T is the turbidity value at zero hours, ΔT is change in turbidity, and Δt is the time interval (Pearce and Kinsella, 1978).

Adsorbed Protein

The method described in Dissanayake and Vasiljevic (2009) was used with minor modifications to estimate adsorbed protein. Adsorbed protein content was taken as the weight of protein that would not be separated into the liquid layer after centrifugation of the emul-

sion at 20°C for 30 min at $12,000 \times g$ (model J2HS centrifuge, Beckman). The Kjeldahl method (nitrogen conversion factor = 6.25) was used for all protein estimations. Adsorbed protein was calculated as follows:

$$\begin{aligned} \text{Adsorbed protein (mg/mL)} &= \text{protein in stock} \\ &\text{solution (mg/mL)} - \text{protein in aqueous} \\ &\text{layer of emulsion (mg/mL)}. \end{aligned}$$

Rheology

Viscoelastic properties and flow behavior of 12% (wt/wt) WP dispersions were examined following the methods described previously by Dissanayake et al. (2010) with minor modifications. The pH of the dispersions was first adjusted to 7 using 1 M NaOH before introducing them to the rheometer. The flow behavior of WP dispersions were examined using a CS/CR rheometer (MCR 301, Anton Paar, GmbH, Germany) equipped with proprietary software (Rheoplus/32 v2.81, Anton Paar) and a double-gap-cylinder measuring system (DG26.7-SN7721, Anton Paar). First, the dispersions were pre-sheared for 5 s at a controlled shear rate of 500/s at 20°C and then allowed to equilibrate for 30 s. The viscosity measurements of the protein samples were collected by increasing the shear rate from 0.1 to 100/s within 5 min at the same temperature.

In situ heat gelation was assessed using the same rheological system by applying dynamic small amplitude oscillatory measurements at a constant strain of 1% and frequency of 1 Hz. The rheological measurements were obtained when heating the samples from 20 to 90°C at a rate of $1^\circ\text{C}/\text{min}$ for approximately 70 min and holding them at 90°C for 10 min.

In situ cold-set acid and salt gelation were examined with the same rheometer using either 2% (wt/wt) glucono- δ -lactone powder or 0.1 M NaCl, and a bob-and-cup measuring system (CC27-SN8767, Anton Paar) by applying dynamic small amplitude oscillatory measurements following the procedure described in Dissanayake et al. (2010). Gelation was monitored under a constant strain of 1% and frequency of 1 Hz at 20°C for 150 min. After this period, the frequency sweep from 1 to 10 Hz at 1% shear strain was used to ascertain viscoelastic properties of created gels. The selected shear strain was found to be within the linear viscoelastic region.

Statistical Analysis

The study of functionalities of MWP powders was organized as a randomized full factorial design with

acidulant, microfluidization, and heat treatment as the major factors and replicates as blocks. Replication of all experiments was carried out twice with subsequent subsampling ($n = 4$). Results were analyzed using a general linear model of SAS statistical program (SAS Institute, 1996) and the level of significance was pre-set at $P = 0.05$. Tukey's Studentized range (Honestly Significant Difference) test was used for multiple comparisons of means of functional and colloidal properties.

RESULTS AND DISCUSSION

In this study, different types of MWP powders were produced at low pH using 2 acidulants, citric acid and lactic acid, followed by microfluidization and spray drying of these (7% wt/wt) protein dispersions (Figure 1). All spray-drying parameters were constant for all the samples.

Electrophoretic Analysis of MWP at Low pH

As depicted in Figure 2A, in the native PAGE gel, the protein bands corresponding to native states of the major WP, β -LG and α -LA, were clearly visible (lanes 1, 2, 3, 5, 6, and 7), whereas a very small proportion of aggregated proteins was also present on top of the stacking gel. The 2 protein standards used in this study were commercial β -LG and α -LA products (Sigma-Aldrich, Chemie GmbH) with monomeric molecular weights of approximately 18 and 14 kDa, respectively (Fox, 2003). Importantly, those protein bands were absent in heat-treated WP (lanes 4 and 8) regardless of acidulant type, confirming the complete denaturation of WP upon heating.

Furthermore, the presence of monomeric protein bands in nonreducing SDS-PAGE gel of the HTM-CA sample (Figure 2B, lane 4) indicates that noncovalent interactions were responsible for the aggregation. According to de la Fuente et al. (2002), at very acidic pH values, such as pH 2 to 3, the principal whey protein β -LG possesses only a positive charge and as a result the covalent disulfide bond interchange is very unlikely due to the high stability of the thiol group at low pH. However, in both reducing and nonreducing SDS-PAGE gels with lactic acid-acidified samples, such bands (related to HTM-LA; Figure 2B and 2C, lane 8 in both) were relatively obscure. This could be due to a possible ion-specific aggregation of lactate ion with reducing agents, although we expected to observe more resolved bands under reducing conditions. Formation of β -LG and surfactant (SDS) complexes under acidic pH conditions, and the perturbation of actual molecular characteristics due to these interactions, have been identified and are attributed to the surfactant type and

concentration and the environmental conditions (Jung et al., 2008). Furthermore, the monomeric β -LG band of the M-LA sample under SDS-PAGE nonreducing conditions (Figure 2B, lane 7) showed greater intensity than that of other samples. However, the interpretation of these observations is rather difficult because the influence of SDS on cleaving WP aggregates appears to be different at low pH. Another interesting observation was that the reducing SDS-PAGE gels, particularly with citric acid, clearly displayed 2 protein bands, possibly corresponding to the 2 genetic variants (A and B) of α -LA (Fox, 2003).

Thermal Analysis of MWP Obtained at Low pH

Table 1 and the thermograms shown in Figure 3 describe the thermal behavior of different WP samples prepared from MWP at low pH and corresponding controls. Analysis by differential scanning calorimetry (DSC) primarily assists in understanding the extent of prior WP denaturation as well as the heat stability of proteins, based on the shifts of main features of denaturation profiles. The extent of protein denaturation is related to the enthalpy change associated with protein unfolding. In addition, the unfolding of globular WP is an endothermic heat process (De Wit, 1990; Paulsson and Dejmek, 1990). The DSC thermograms of WP samples obtained in the current study clearly indicate that the denatured MWP (HTM-CA and HTM-LA) samples did not contain endothermic peaks in the region of WP denaturation. Similar results were obtained for the denatured MWP used in our previous study conducted at neutral pH (Dissanayake and Vasiljevic, 2009). This could be due to enhanced heat stability of the WP resulted from irreversible denaturation during the heating process. In contrast, the undenatured WP samples created broad endothermic peaks. We observed a broad endothermic peak with peak height located around 77°C related to the denaturation temperature of β -LG at native conditions, which is consistent with other reports (De Wit, 1990; Patel and Kilara, 1990; Fitzsimons et al., 2007). Similarly, 2 broad peaks in Figure 3A, with peak temperature around 82°C, are from undenatured samples acidified with citric acid to pH 3 (control-CA and M-CA). In addition, the broad peaks in Figure 3B from the samples acidified with lactic acid (control-LA and M-LA) were visible, with their peak heights corresponding to denaturation of globular protein around 88°C. The higher denaturation temperatures of WP at low pH confirmed their enhanced heat stability. This occurrence may be a consequence of the prevalence of stronger hydrogen bond assisted network at acidic pH (Resch et al., 2005a), inhibition of thiol group activation, and purely repulsive electrostatic

Figure 2. (A) Native, (B) nonreducing SDS-PAGE, and (C) reducing SDS-PAGE patterns of whey protein (WP) powders produced by spray drying of 7% (wt/wt) WP dispersions in citric or lactic acid at pH 3 after different treatments as follows. Lanes (from left to right): MWM = molecular weight marker; α -LA = α -LA standard; β -LG = β -LG standard; 1 = untreated control; 2 = citric acid-acidified control; 3 = citric acid-acidified and microfluidized; 4 = citric acid-acidified, heated, and microfluidized; 5 = untreated control; 6 = lactic acid-acidified control; 7 = lactic acid-acidified and microfluidized; 8 = lactic acid-acidified, heated, and microfluidized.

Table 1. Thermal properties of microparticulated whey protein powders produced at pH 3 using citric and lactic acids detected by differential scanning calorimetry

Treatment ¹	Onset temperature (°C)	Endset temperature (°C)	Peak temperature (°C)	ΔH^2 (J/g)
Control	66.0	84.5	78.1	2.7
Control-CA	67.2	92.0	81.5	5.3
M-CA	65.7	91.7	81.2	3.1
HTM-CA		Significant peaks not detected		
Control-LA	78.3	97.7	88.5	2.6
M-LA	75.6	97.1	88.5	3.0
HTM-LA		Significant peaks not detected		

¹Treatments: CA = citric acid-acidified; M-CA = microparticulated, citric acid-acidified; HTM-CA = heat-treated, microparticulated, citric acid-acidified; LA = lactic acid-acidified; M-LA = microparticulated, lactic acid-acidified; HTM-LA = heat-treated, microparticulated, lactic acid-acidified.

² ΔH = enthalpy of denaturation (J/g).

interactions between monomers upon protein unfolding (de la Fuente et al., 2002), all of which could have created unfavorable conditions for subsequent protein aggregation.

The endothermic total enthalpy of protein denaturation is primarily linked to the disruption of different endothermic and exothermic molecular associations (Damodaran, 2008). Therefore, as indicated by difference in the area of each peak, decrease in denaturation enthalpy of M-CA sample compared with the control-CA sample as well as increase in denaturation enthalpy of M-LA samples compared with the control-LA sample may be a consequence of applied high-pressure shearing on these WP (Table 1). Furthermore, the different peak temperatures and enthalpy values observed for thermograms of WP in citric and lactic acids showed the variable effects of acidulant (Table 1). This may indicate that the specific influence of lactate ions on heat stability of WP to be more prominent compared with the effect of calcium chelation (imparted by citrates) on the heat stability of WP.

Particle Size Distribution Pattern of MWP at Low pH

Figure 4 and Table 2 present the patterns of particle size distribution of MWP powders produced at low pH. As Figure 4 indicates, the most significant observation was the confinement of average particle size of all WP samples to a submicron level. In addition, the average particle size of denatured MWP was significantly ($P < 0.05$) smaller than that of the control at neutral pH (Table 2). This is a greater reduction of particle size of denatured WP compared with that of MWP obtained at neutral pH in our previous study (Dissanayake and Vasiljevic, 2009), which reported an average particle size of around 10 μm . In addition, both types of MWP derived from lactic acid samples (M-LA and HTM-LA) had significantly ($P < 0.05$) reduced particle size compared with that of control-LA. Similarly, micropar-

tication further significantly ($P < 0.05$) reduced the particle size of M-CA. Moreover, the relative particle size of heat-treated MWP (HTM-CA) was not significantly ($P < 0.05$) different from that of the control-CA sample, although the former sample had undergone aggregation during heat denaturation. In general, these findings indicate that microparticulation has a direct effect on particle size reduction of WP powder dispersions, particularly those created under low pH conditions.

The application of high-pressure shearing during microfluidization may have affected WP in different ways. The process would result in simultaneous collision, compression, shearing, and flowing with the contraction and, more importantly, stretching and elongation of protein molecules (Altmann et al., 2004; Bouaouina et al., 2006; Akkermans et al., 2008). In addition, the WP gels prepared under acidic conditions possess weak brittle characteristics mainly acquired by relatively weaker linkages, which may be confined only to non-covalent interactions under highly repulsive conditions. High-pressure shearing, on the other hand, may have easily disrupted these weak linkages and caused possible conformational rearrangements, finally generating smaller WP particles. In addition, the results indicate no significant ($P < 0.05$) effect via the calcium-chelating ability of citrate ions on gel formation. Although we expected that masking of calcium ions might lead to further reduction of particle size of WP by creating a weaker gel network, the instability of citrate ions at extremely low pH may have prevented chelation of calcium.

Solubility, Heat Stability, and Emulsifying Capacity of MWP Powders

Table 2 summarizes the major findings with regard to changes in selected physical functionalities as affected by microparticulation of WP at low pH adjusted with

Figure 3. Differential scanning calorimetry thermograms of 12% (wt/wt) whey protein dispersions prepared from microparticulated whey proteins and controls (A = citric acid-acidified samples, B = lactic acid-acidified samples). Control = untreated control; Control-CA = citric acid-acidified control; M-CA = citric acid-acidified and microfluidized; HTM-CA = citric acid-acidified, heated, and microfluidized; Control-LA = lactic acid-acidified control; M-LA = lactic acid-acidified and microfluidized; HTM-LA = lactic acid-acidified, heated, and microfluidized.

2 acidulants. Solubility is usually one of the key indicators of protein functionality. In addition, the solution conditions such as pH, ionic environment, and temperature are essentially important in controlling solubility, because it is affected by protein–water and protein–protein interactions (Alquicira, 2006). The results in Table 2 indicate that applied treatment (acidification, heating, microparticulation) significantly ($P < 0.05$) reduced the solubility of resulting MWP compared with controls. On the other hand, solubility among treated samples was similar ($P > 0.05$). Whey proteins are

Figure 4. Particle size distribution pattern of microparticulated whey proteins obtained by different treatment at low pH (A = citric acid-acidified samples, B = lactic acid-acidified samples). Control = untreated control; Control-CA = citric acid-acidified control; M-CA = citric acid-acidified and microfluidized; HTM-CA = citric acid-acidified, heated, and microfluidized; Control-LA = lactic acid-acidified control; M-LA = lactic acid-acidified and microfluidized; HTM-LA = lactic acid-acidified, heated, and microfluidized.

highly soluble over a wide range of pH (Damodaran, 2008). However, at very low pH (2 to 3), WP should possess a net positive charge because their isoelectric point (pI) is around pH 5. Therefore, some of the WP molecules may partially unfold or deviate from their existing conformation due to high intramolecular repulsions that may lead to aggregation and subsequent partial loss of solubility (de la Fuente et al., 2002). This loss of solubility may also be a consequence of modulated hydration properties resulting from the combined effect of treatments on whey proteins. Heat-induced protein aggregation generally reduces the aqueous solubility of proteins (de la Fuente et al., 2002; Considine et al.,

Table 2. Colloidal and interfacial properties of microparticulated whey protein powders produced at pH 3 using citric and lactic acids

Treatment ¹	Solubility (%)	Heat stability (%)	Emulsion activity index (m ² /g)	Emulsion stability index (h)	Adsorbed protein (mg/mL)	Average particle size (nm)
Control	86.4 ± 2.9 ^a	26.0 ± 1.5 ^c	6,521 ± 651 ^c	25.8 ± 0.2 ^a	7.5 ± 0.9 ^c	131 ± 6.1 ^a
Control-CA	67.4 ± 2.7 ^b	34.2 ± 0.5 ^b	9,062 ± 2,011 ^b	25.2 ± 0.1 ^b	14.9 ± 1.3 ^a	112 ± 11.5 ^{abc}
M-CA	70.4 ± 0.4 ^b	35.2 ± 2.2 ^a	5,625 ± 628 ^c	25.3 ± 0.1 ^b	12.8 ± 0.4 ^b	84 ± 4 ^d
HTM-CA	67.3 ± 0.0 ^b	19.7 ± 1.6 ^d	6,566 ± 806 ^c	26.0 ± 0.1 ^a	15.0 ± 0.4 ^a	101 ± 19.3 ^{bcd}
Control-LA	70.0 ± 1.2 ^b	17.6 ± 1.1 ^f	9,366 ± 1,530 ^a	25.2 ± 0.1 ^b	14.1 ± 1.0 ^{ab}	123 ± 5.7 ^{ab}
M-LA	68.8 ± 3.5 ^b	19.0 ± 0.6 ^e	8,183 ± 1,378 ^{abc}	24.4 ± 0.3 ^c	12.2 ± 0.6 ^b	91 ± 1.8 ^{cd}
HTM-LA	68.9 ± 1.0 ^b	10.9 ± 1.0 ^g	7,546 ± 191 ^{abc}	25.9 ± 0.3 ^a	15.0 ± 1.0 ^a	100 ± 6.3 ^{cd}

^{a-g}Means within a column with different superscripts differ significantly ($P < 0.05$).

¹Treatments: CA = citric acid-acidified; M-CA = microparticulated, citric acid-acidified; HTM-CA = heat-treated, microparticulated, citric acid-acidified; LA = lactic acid-acidified; M-LA = microparticulated, lactic acid-acidified; HTM-LA = heat-treated, microparticulated, lactic acid-acidified.

2007). Although low compared with the control (Table 2), solubility of denatured MWP powders obtained at low pH was considerably improved compared with the denatured MWP produced at neutral pH. As shown in our previous study (Dissanayake and Vasiljevic, 2009), the solubility of MWP produced at neutral pH was only 31.2%. This effect stems directly from the combination of high-pressure shearing with the changes in processing conditions.

Both the denatured MWP samples acidified with citric and lactic acids exhibited high heat stability, with HCT greater than 3 min, compared with the non-denatured MWP at acidic pH, which showed visible aggregates after approximately 1.5 min. In addition, the HCT of native control and denatured MWP at neutral pH were 9 s and 1.7 min, respectively, as reported by Dissanayake and Vasiljevic (2009). These high HCT values are consistent with the outcomes of DSC analysis for acidified samples, which also showed absence of endothermic peaks or higher denaturation temperatures compared with native controls. However, the reduced heat stability of all samples is contradictory and may have resulted from specific acidulant effects.

Emulsifying properties of MWP powders were assessed via 2 well-established parameters: EAI and ESI. Important factors that control EAI are the molecular flexibility, which determines the degree of unfolding of proteins, the extent of interactions of proteins with other molecules, and surface hydrophobicity, which influences the affinity of proteins to the oil-water interface (Monahan et al., 1993). According to Pearce and Kinsella (1978), EAI also depends on the entire experimental setup. Thus, the EAI values obtained in the current study were highly specific to the parameters, equipment, and materials used. As shown in Table 2, acidified controls (control-CA and control-LA) showed significantly ($P < 0.05$) higher EAI values compared with the untreated control. However, microfluidization substantially ($P < 0.05$) reduced these values for

citric acid-acidified samples. On the other hand, microparticulation had only a minor effect on lactic acid-acidified samples (M-LA and HTM-LA). Therefore, the variability in EAI of MWP prepared with different acidulants may have resulted from specific anion effects. These effects would mainly involve perturbation of the solvent structure, which may consequently cause conformational changes in the protein structure, leading to alterations of their functional attributes (Resch et al., 2005b).

Initial heat treatment should reduce the emulsifying characteristics of proteins via protein aggregation (de la Fuente et al., 2002). However, heat-treated samples did not show significant ($P > 0.05$) differences in EAI compared with the native control (Table 2). This could be due, in part, to particle size reduction of denatured protein aggregates during microfluidization (Figure 4) that resulted in enhanced emulsification properties of proteins via fragmentation of aggregates. Similarly, it could have resulted from exposure of reactive sites during denaturation or due to a modification of protein secondary conformation via high-pressure shearing. The same factors would have resulted in elevated concentration of adsorbed protein on oil droplet surfaces, as shown in Table 1. Change of protein structure during microfluidization may result in exposure of previously buried reactive sites, such as hydrophobic groups that are favorable to the interface. The exposure of such groups may cause proper alignment of hydrophilic and hydrophobic residues, consequently resulting in improvements of interfacial properties. The EAI depends on the interfacial area that can be coated by proteins (Pearce and Kinsella, 1978).

Emulsion stability depends on the steadiness of the interface, which is governed mostly by different molecular properties. One such property is the net charge of the protein molecule, which presents a barrier to the close approach of oil droplets and thereby prevents coalescence (Klemaszewski and Kinsella, 1991). In ad-

dition, proteins form coherent monolayers via involvement of intermolecular disulfide bonds (Damodaran, 2008). Coalescence and creaming appear to be the main factors responsible for ESI (Pearce and Kinsella, 1978). However, the turbidity measurements were obtained from the center of the emulsion sample to maximize the uniformity of the results. As shown in Table 2, denatured, microparticulated samples from both acidulants created significantly ($P < 0.05$) more stable emulsions. The same samples also exhibited significantly ($P < 0.05$) greater ability to adsorb to the surfaces of oil droplets compared with other acidified samples. The emulsion stability of native controls was comparable to that of denatured samples, although the former had significantly ($P < 0.05$) lower adsorbing properties (Table 2).

Flow Behavior of WP Dispersions

Figure 5 presents the apparent viscosity of 12% (wt/wt) WP preparations derived from MWP powder samples at adjusted pH 7. All dispersions exhibited non-Newtonian and shear thinning behavior. This may arise due to favorable orientation of protein molecules to the major axes of the direction of flow with the possible dissociation of weakly held oligomers into their smaller species (Damodaran, 2008). More importantly, the denatured MWP samples had comparatively higher yield point and maintained higher viscosity levels throughout the shear rate range. The bulk rheology of a colloidal dispersion is determined by a combination of various forces, which mainly depend on the particle size of dispersion. The average particle size of these WP varied around 100 nm, and hence, the flow behavior depends on hydrodynamic forces, which arise from the relative motion of particles to the surrounding fluid, Brownian motions, which are ever-present thermal randomizing forces, and on the elastic interparticle forces (Genovese et al., 2007). In addition, particle shape, size and size distribution, particle deformability, and liquid polarity could affect the flow behavior; for example, nonspherical particles cause increase in viscosity due to extra energy dissipation during flow. Furthermore, for a given particle concentration, viscosity decreases with increasing polydispersity, as indicated by particle size distribution width (Genovese et al., 2007). The increase in apparent viscosity of denatured MWP may have resulted from much higher water-holding capacity, especially under a net positive charge where hydrogen bond formation is most likely, or via a possible partial protein unfolding due to elevated intramolecular repulsion and consequent formation of physical cross-links (Resch, 2004). In addition, high-pressure shearing may have caused further conformational rearrangements,

causing alterations in surface charges of denatured MWP, which in turn could affect protein–protein and protein–solvent interactions, leading to viscosity increments. The particle size distribution pattern of these WP preparations revealed that the average particle size of MWP was significantly ($P < 0.05$) smaller than that of the native control (Table 2). Therefore, the greater secondary electroviscous effects, which can arise due to increased overlapping of the electrical double layer and raised interparticle potential of smaller particles compared with larger colloids, may have imparted greater viscosity (Resch, 2004; Daubert et al., 2006). Additionally, high-pressure shearing did not impose a noticeable effect on the apparent viscosity of undenatured WP samples except for the dispersions prepared from control-LA. That sample exhibited higher viscosity than the other samples during the shear rate ramp, which may be a consequence of an anion-specific effect. Resch et al. (2005a) reported that the β -LG dispersions derived from powders prepared with lactic acid showed greater viscosity with higher water-holding capacity compared with those prepared with citric acid. In addition, powders prepared with citric acid have been shown to exhibit extreme difficulty in redispersing in water, which is consistent with the findings of the current work.

Gelling Properties of MWP

Heat-induced gelation of MWP produced at low pH was studied with small amplitude oscillatory rheology. Figure 6 presents the changes in storage modulus (G') during heating, holding, cooling, and aging of 12% (wt/wt) WP powder dispersions at constant strain and frequency. Results suggest that all previously unheated WP samples gelled upon heating, with the native control recording the highest G' , which indicates the creation of relatively more cross-linked viscoelastic gel networks compared with acidic samples. During heat-induced gelation, initial unfolding and subsequent aggregation of proteins take place, and the mechanical nature of the resulting gel network is governed by the extent of attractive and repulsive forces between protein–protein and protein–water molecules (Hudson et al., 2000). In general, at pH $>$ pI, fine-stranded, strong elastic gels are formed, as opposed to pH $<$ pI, where fine-stranded, weak brittle gels are created (Foegeding, 2005). Compared with the firmer network structure of untreated control with higher G' value, the acidic controls possessed relatively weak gel networks.

Although the pH of the whey protein systems was adjusted to 7 before measurements, these findings indicate a possible influence of citrate and lactate ions, together with other ions present in the medium, in structure

Figure 5. Apparent viscosity of 12% (wt/wt) whey protein dispersions with pH adjusted to 7 and a shear rate control sweep (0.1 to 100/s) conducted at 20°C. Control = untreated control; Control-CA = citric acid-acidified control; M-CA = citric acid-acidified and microfluidized; HTM-CA = citric acid-acidified, heated, and microfluidized; Control-LA = lactic acid-acidified control; M-LA = lactic acid-acidified and microfluidized; HTM-LA = lactic acid-acidified, heated, and microfluidized.

formation during heating. Ionic strength, after reaching a certain level, can create a repulsive molecular environment, leading to a weak viscoelastic network. In addition, among acidic samples, the control-LA sample had the highest G' value (Figure 6B), but high-pressure shearing conveyed a negative effect on the gel strength, as shown by evolution of G' of the M-LA sample during heating. These results are consistent with the flow behavior of lactic acid-acidified samples. Meanwhile, microfluidization imparted greater gelling ability to citric acid-acidified samples (Figure 6A), with higher G' values reported for M-CA compared with control-CA.

Figures 6A and 6B indicate that citric acid samples started gelling before lactic acid samples during heat gelation. The onset of denaturation and subsequent aggregation of citric acid samples occurred approximately 10 min before those of lactic acid. The elevated denaturation temperatures of WP with lactic acid (Figure 3B and Table 1) present an additional explanation for the weak gelling behavior and limited instant thickening ability. These results are in agreement with previously reported data for gelling behavior of derivatized β -LG powders produced with citric acid and lactic acid (Resch et al., 2005a). Apparently, the citric acid-acidified sample rapidly underwent gelation at 80°C and formed large disordered aggregates, in contrast to slower network formation of lactic acid gels with an ordered filament aggregation mechanism.

Conversely, the heat-induced gels formed by pre-denatured samples (Figure 6) were weaker compared with

Figure 6. Changes in storage modulus (G') of 12% (wt/wt) whey protein dispersions (A = citric acid-acidified samples, B = lactic acid-acidified samples) during heating (20 to 90°C) and holding (90°C/10 min) at constant strain (1%) and frequency (1 Hz). Control = untreated control; Control-CA = citric acid-acidified control; M-CA = citric acid-acidified and microfluidized; HTM-CA = citric acid-acidified, heated, and microfluidized; Control-LA = lactic acid-acidified control; M-LA = lactic acid-acidified and microfluidized; HTM-LA = lactic acid-acidified, heated, and microfluidized.

non-heat-denatured samples. This observation agrees with thermal analysis as well as our previous findings in Dissanayake et al. (2010). Formation of heat-induced gels from heat-treated WP is highly unlikely, primarily because of the unavailability of the required reactive sites for cross-linking, such as hydrophobic groups, in denatured samples. However, the slightly increased G' of these samples may be a consequence of increased hydration of denatured WP particles or weak particle-particle interactions via possibly modified surface properties due to high-pressure shearing.

Figure 7 presents the development of storage modulus of MWP created at low pH and controls during acid gelation with 2% (wt/wt) glucono- δ -lactone. In contrast to heat-set gels, upon acidification, pre-acidified and heat-denatured samples that were subjected to high-

pressure shearing (HTM-CA, HTM-LA) formed gels. As observed with the heat gels of acidified controls, out of the 2 heat-denatured samples, HTM-LA showed greater gel strength compared with HTM-CA during acid gelation. This would have been due to the effect of an ion-specific interaction of citrate and lactate that were present in the systems. Citrate is a kosmotrope according to Hofmeister or lyotropic series with the ability to stabilize protein structure against solubilization and unfolding (Resch et al., 2005b). Thus, citrate would have caused the weaker gel compared with the lactic acid-acidified samples. The same effect is also confirmed by the solubility (Table 2) of control-CA, control-LA, HTM-CA, and HTM-LA. Citric acid appeared to reduce the solubility of MWP compared with lactic acid except when the samples were subjected to microfluidization. Likewise, as the presence of calcium is known to produce stronger gels (Havea and Sing, 2003), the chelating effect of citrate on calcium would have further reduced the gel strength of citric acid-acidified samples.

Ionic strength will markedly reduce solubility of proteins at pH 3 compared with at higher pH values, where ionic strength seems to have little effect (Ju and Kilara, 1998). Because all our samples were re-adjusted to pH 7 for analysis of gelling properties, different trends in gel strengths were expected for salt-induced gels compared with those observed during acid gelation. However, the

addition of 0.1 *M* NaCl to WP samples did not result in gel formation. The maximum G' value obtained for these samples occurred below 2 Pa and was observed for HTM-CA (data not shown). Although 0.1 *M* NaCl was added to induce salt gelation because the concentration was considered to be optimum (Ju and Kilara, 1998), ions already present in the system would also impart their contribution to the overall effect. Similar results were recorded for whey proteins with concentrations higher than 0.1 *M* NaCl (Ju and Kilara, 1998). One reason for citric acid-acidified samples to have a less negative effect on gelation (as evident from a slightly increased G' value for HTM-CA) could be the chelating effect of citrate on calcium ions (De Rham and Chanton, 1984) in the system, which would redirect the total ionic strength toward the optimum value.

CONCLUSIONS

As shown in this study, WP were completely denatured in heat-treated MWP. Most importantly, the particle size of denatured WP was significantly reduced under the implemented experimental conditions to around 100 nm, approximately 2 orders of magnitude particle size reduction compared with the average particle size of MWP produced at neutral pH. In addition, the particle size distribution of all types of WP was restricted to <1 μm . Therefore, at low pH, a combination of heat and high-pressure shearing produced WP micro-aggregates with improved colloidal stability, which was not achieved by microparticulation of denatured WP at neutral pH. Consequently, the solubility of denatured MWP powders was significantly higher, with the values comparable to that of undenatured acidic controls. The solubility values of denatured MWP created at low pH were higher compared with those of denatured MWP produced at neutral pH. Although EAI was not considerably affected by microparticulation, more stable emulsions were created from denatured MWP with greater adsorbing ability to oil droplet surfaces. In addition, the viscosity of denatured MWP samples was greater than that of undenatured WP samples. The gelling attributes upon heating of undenatured MWP were influenced by the 2 acidulants, depending on their relative thermal stability such as slower gelation of WP prepared with lactic acid compared with that of WP with citric acid, which was also reflected by elevated denaturation temperature of WP with lactic acid. Additionally, denatured MWP produced with lactic acid created gels with considerable firmness despite their inability to form salt gels with considerable strength. Furthermore, the effect of high-pressure shearing on different functional properties of MWP varied with acidulant selection, mirrored by vari-

Figure 7. Changes of storage modulus (G') during acid induced gelation of 12% (wt/wt) whey protein dispersions by addition of 2% glucono- δ -lactone. Control = untreated control; Control-CA = citric acid-acidified control; M-CA = citric acid-acidified and microfluidized; HTM-CA = citric acid-acidified, heated, and microfluidized; Control-LA = lactic acid-acidified control; M-LA = lactic acid-acidified and microfluidized; HTM-LA = lactic acid-acidified, heated, and microfluidized. Measurements were performed at 20°C at constant strain (1%) and frequency (1 Hz).

able functional attributes of MWP samples. Altering pH resulted in modulation of intra- and intermolecular interactions that led to particle size reduction of MWP. Acidification in combination with microparticulation fundamentally modified the physical functionalities of whey protein powder dispersions, which could result in further diversified applicability of this important dairy ingredient.

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