Properties of acid whey as a function of pH and temperature

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ABSTRACT

Compositional differences of acid whey (AW) in comparison with other whey types limit its processability and application of conventional membrane processing. Hence, the present study aimed to identify chemical and physical properties of AW solutions as a function of pH (3 to 10.5) at 4 different temperatures (15, 25, 40, or 90°C) to propose appropriate membrane-processing conditions for efficient use of AW streams. The concentration of minerals, mainly calcium and phosphate, and proteins in centrifuged supernatants was significantly lowered with increase in either pH or temperature. Lactic acid content decreased with pH decline and rose at higher temperatures. Calcium appeared to form complexes with phosphates and lactates mainly, which in turn may have induced molecular attractions with the proteins. An increase in pH led to more soluble protein aggregates with large particle sizes. Surface hydrophobicity of these particles increased significantly with temperature up to 40°C and decreased with further heating to 90°C. Surface charge was clearly pH dependent. High lactic acid concentrations appeared to hinder protein aggregation by hydrophobic interactions and may also indirectly influence protein denaturation. Processing conditions such as pH and temperature need to be optimized to manipulate composition, state, and surface characteristics of components of AW systems to achieve an efficient separation and concentration of lactic acid and lactose.

Key words: acid whey, minerals, lactic acid, pH

INTRODUCTION

“Whey” is usually defined as the liquid portion of milk devoid of caseins and fat (de Wit, 2001). However, it has become customary for the dairy industry to classify it into 2 groups depending on the manufacturing process, namely, sweet whey, which is obtained during

manufacturing of rennet type of hard cheeses, and acid whey, generated during production of acid-coagulated dairy products such as fresh cheeses, cream cheese, or strained (Greek-style) yogurt (Schmidt et al., 1984). The dairy industry produces ~180 to 190 million tonnes per year of whey from these 2 streams, accounting for more than a half of the total solids present in the original whole milk, including whey proteins (20% of the total protein) and most of the lactose, minerals, and water-soluble vitamins (de Wit, 2001). Nowadays, concentrated, demineralized sweet whey is successfully used in a variety of products in the food industry such as beverage powders, nutrition bars, soups, bakery items, confectionery coatings, and ice cream and frozen dairy dessert products (Young et al., 1980). Furthermore, whey-protein concentrates and isolates are also used as food ingredients in several different food products including hams, custards, confectionery, crab sticks, cakes, infant formula, sports drinks, and formulated stock foods (Morr and Ha, 1993).

The dairy industry now faces a similar challenge in relation to acid whey (AW). Because of current consumer trends and market demands, production of acid-coagulated products has tripled over the last 5 yr, and the predictions are that this trend will continue (Elliott, 2013). In turn, the industry is facing an increasing problem associated with 1.6 billion liters per year of AW, generated as a waste stream. Generally, AW has a lower pH and lower protein and lactose contents, but more calcium, phosphorus, and lactic acid (LA), in comparison with sweet whey (Schmidt et al., 1984). This, in turn, may cause differences in the behavior of lactose and other acid-whey components during concentration, rendering the stream very hard to process (Dec and Chojnowski, 2006). The science supporting the understanding of compositional influence on different processing applications of AW is not well established, which limits solutions to the current and growing disposal problem.

Membrane technology plays a great role in valorization of whey waste streams and has been used by the dairy industry to recover a range of valuable components from sweet whey (Prazeres et al., 2012).

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However, the industry has not been successful in applying the same approach to the treatment of AW, mainly because of compositional differences, which in turn influence the physicochemical characteristics of separated compounds including whey proteins, lactose, and minerals. Lactose concentration and consequent crystallization are also governed by environmental conditions including presence of minerals, organic acids, and pH (Gernigon et al., 2013). Thus, a likely strategy toward improving separation processes would be to manipulate the concentration of minerals, organic acids, or both by process control during AW processing. For example, nanofiltration (NF) membranes have already been used in the demineralization and separation of lactose and LA in fermented-cheese whey broths (Li and Shahbazi, 2006; Li et al., 2008). Performance and selectivity of these membrane systems were clearly dependent on pH (Alkhatim et al., 1998) and temperature (Kelly and Kelly, 1995), because of effects on mineral solubility and electrostatic protein interactions in the feed (Gonzalez et al., 2008; Luo and Ding, 2011; Rice et al., 2011a,b).

It appears that no systematic study has thus far been performed to thoroughly characterize the chemical and physical properties of an AW system. This information would allow appropriate strategies to be designed to manipulate compositional behavior and to perform efficient membrane processing of AW. Although a few studies (Colbert and Decker, 1991; Roman et al., 2009) have dealt with the role of pH in treatment of AW on permeate quality and filtration performance, we are not aware of a study that has investigated the combined effect of temperature and pH on chemical and physical properties of AW, especially on its main constituents—minerals, LA, and proteins. Thus, the present study aimed to establish the chemical and physical properties of AW obtained from cream-cheese manufacturing as a function of pH (pH 3 to 10.5) and temperature (15, 25, 40, or 90°C). Characterization of the minerals and LA speciation among soluble and insoluble fractions was undertaken to establish the influence and importance of the behavior of these components on filtration performance under different theoretical processing conditions.

MATERIALS AND METHODS

Materials

Commercial AW was obtained from cream-cheese manufacturing having a pH of ~4.54 (Tatura Milk Industries, Victoria, Australia). All other chemicals were analytical grade, and they were obtained from BDH Chemicals (Kilsyth, VIC, Australia) and Sigma-Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Ultrapure wa-

ter (Milli-Q water; Merck Millipore, Bayswater, VIC, Australia) was used at all times.

Experimental Design

The experimental design used in the present study is depicted in Figure 1. The AW solution obtained from the factory was centrifuged at $5,000 \times g$ for 20 min at 10°C using an Avanti J-26XP centrifuge (Beckman Instruments Australia Pty Ltd., Gladesville, NSW, Australia) to remove all coarse materials. A full factorial randomized design with 2 main factors (pH and temperature) at 6 and 4 levels, respectively, including their interactions was applied. The pH adjustments of the AW solutions were carried out by drop-wise addition of 0.1 M NaOH or 0.1 M HCl to achieve pH of 3.0, 4.5 (natural pH of AW), 5.8, 7.3, 8.8, or 10.5. Higher pH levels were chosen to clearly identify pH effects despite any practical viability. After pH adjustment, the solutions were kept overnight at 4°C for equilibration. On the following day, the solution was equilibrated at 25°C for 1 h. Minor pH adjustments were carried out as necessary. The solutions were then placed at 4 different temperatures (15, 25, 40, or 90°C) for 20 min in a temperature-controlled water bath. The first 3 temperatures were chosen to imitate typical membrane-processing conditions, and 90°C was used to accelerate any changes that may take place and observe clear effects induced by this change. After heating, the solutions were immediately centrifuged at $22,000 \times g$ for 20 min using a Beckman L8 80M ultracentrifuge with a type 80 rotor.

Chemical Analyses

Total solids content was determined using the International Standard FIL-IDF method 26 (International Dairy Federation, 1964), where 1 g of sample was dried by heating at $102 \pm 2^\circ\text{C}$ in an oven to a constant weight. The Australian Standard method 2300.1.5 (Standards Association of Australia, 1988) was used to assess the mineral content by ashing. An aliquot (10 g) of solution was dried and the residue charred. Oxidation was completed in a furnace at 500°C overnight. The ash and TS contents were expressed as a percentage of the original weight of the solution.

For the determination of minerals, the samples were prepared by dissolving ash in 10 mL of 1 M HNO₃ acid and water to achieve 0.1% TS content. Five standard solutions containing concentrations of Ca at 0.02 to 1% wt/wt and K at 0.1 to 1% wt/wt, Na at 0.02 to 2% wt/wt, and Mg at 0.002 to 0.02% wt/wt, respectively, were prepared. The samples and standards were analyzed by inductively coupled plasma atomic emission

Figure 1. Summary of the experimental design applied in the study. AW = acid whey.

spectrometry using an ICP E 9000 (Shimadzu Scientific Instruments, Rydalmere, New South Wales, Australia). The wavelengths used for Ca, Na, K, and Mg were 220, 589, 766, and 383 nm, respectively.

Proteins were separated by diluting an aliquot (5 g) of solution with 20 g of Milli-Q water and 25 g of 24% wt/wt TCA followed by suction filtration through a Whatman No. 40 filter paper (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) in to a preweighed Buchner flask. The precipitated proteins were washed with 10 g of 12% wt/wt TCA solution. The total mass of the TCA filtrate was then determined prior further analyses.

Total inorganic phosphate was determined colorimetrically by IDF method 42B (International Dairy Federation, 1990). An aliquot (1 g) of the TCA filtrate was mixed with ammonium molybdate and potassium antimony tartrate to form phosphomolybdic acid, which was then reduced by the addition of ascorbic acid to form a blue complex. The absorbance was measured at 820 nm by UV-visible spectroscopy (Biochrom

LibraS11, Cambridge, UK) within 15 to 30 min after mixing. Standard solutions in the range 0 to 0.8 mg of P/g of solution were prepared as described in IDF method 42B.

Citrate concentration was determined by the colorimetric method proposed by Marier and Boulet (1958). An aliquot (1 g) of the TCA filtrate was mixed with 1.3 g of pyridine and 5.7 g of acetic anhydride to form a yellow-colored complex. The sample was placed in a water bath at 32°C for 30 min, and the absorbance was measured at 428 nm. The standards were made from trisodium citrate (0–1.5 mg/g).

Chloride content was determined by a titrimetric method (Sheen and Kahler, 1938). An exactly 10-mL aliquot of sample was added to a solution of 50 mL of distilled water and 1 mL of chromate indicator. The diluted sample was titrated with 0.05 M silver nitrate solution until a red-brown color appeared.

The Australian Standard method 2300.4.10 (Standards Association of Australia, 1994) was used to determine the lactose content of the TCA filtrate.

An aliquot (10 g) of the TCA filtrate was mixed with 22.5 g of chloramine T reagent (5.7 g/kg) to oxidize the lactose. This was followed by the addition of 7.5 g of aqueous potassium iodide (100 g/kg). The liberated iodine was determined by titration with ~0.04 *M* prestandardized sodium thiosulfate using starch as the indicator. Chloramine T solution was standardized by carrying out a blank titration. The sodium thiosulfate was standardized against a solution of 0.01 mol/L potassium iodate and 100 g/L potassium iodide with starch as the indicator.

Lactic acid was determined by HPLC using a Varian 9012 system controller (Agilent Technologies Inc., Santa Clara, CA) coupled with a UV-VIS detector (Varian 9050) and an HPLC column (Aminex HPX 87H, 300X7.8 mm, Biorad Instruments, Gladesville, NSW, Australia) as described by Donkor et al. (2007). The mobile phase used was 0.01 *M* sulfuric acid with isocratic elution at a flow rate of 0.6 mL/min. The detection of LA was set at $\lambda = 220$ nm. The calculation of LA was derived from the peak area and quantified using a standard series of LA concentrations (0–2% wt/wt).

Physical Analyses

Surface hydrophobicity of the particles present in supernatants was determined by fluorometric assay as described by Chandrapala et al. (2011). The protein solutions were diluted with 0.1 *M* pH 7 phosphate buffer solutions to typical concentration ranges of 0.005 to 0.025% (wt/wt) using 1-anilinonaphthalene-8-sulphonate (ANS). For hydrophobicity determination using ANS, the excitation and emission slits and wavelengths were set at 5 nm/5 nm and 390 nm/470 nm, respectively. Exactly 20 μ L of ANS solution was added to 3 mL of diluted protein solution, vortexed, and kept in the dark for 15 min. The relative fluorescence intensity (RFI) of each solution was measured starting from buffer blank and then the lowest to highest protein concentration. The RFI of each dilution blank was subtracted from that of the corresponding protein solution with ANS to obtain the net RFI. Surface hydrophobicity was expressed as the initial slope of the plot of standardized net RFI values versus percent protein concentration. Surface hydrophobicity was determined by taking the average of 2 analyses, where R^2 values of >0.95 were noted for the linear regression analyses used to calculate the surface hydrophobicity.

A Malvern Zetasizer (Malvern Instruments Ltd., Malvern, UK) was also used to measure the surface charge of particles present in supernatants. A solution sample (150 μ L) was diluted in 1.25 mL UF of AW

and then placed in the cell to measure the charge. The average was taken using 2 replicates.

To determine the presence and the sizes of soluble aggregates, combined fast protein liquid chromatography-size exclusion chromatography (FPLC-SEC) was conducted using an AKTA micro FPLC (GE Healthcare Pty Ltd., Rydalmere, NSW, Australia) equipped with a UV-VIS detector. Samples (250 μ L) of the filtered supernatants were injected into a Superdex 75 10 300 GL (GE Healthcare Pty Ltd.) column and run at 0.5 mL/min in 100 mM ammonium bicarbonate buffer at pH 7.0. The elution profiles were monitored at 220 nm. The total run time was 45 min.

To determine the types of proteins involved in soluble aggregates, PAGE analyses were performed using a 2100 Bioanalyser (Agilent Technologies Inc., Springvale Road, Mulgrave VIC, Australia) under reducing and nonreducing conditions. First, the ladder and samples were covalently modified with a fluorescent dye as described in the manufacturer's High Sensitivity Protein 250 Kit Guide (Agilent). Upon 1:200 dilution with Milli-Q water, 4 μ L of sample or ladder were mixed with sample buffer with (reducing) and without (non-reducing) dithiothreitol. The solutions were heated at 95°C for 5 min before chip analysis. Agilent 2100 Expert software was used for data analysis.

Statistical Analysis

The SAS software (SAS Institute Inc., Cary, NC) was used to perform data analysis. All results were analyzed as a randomized full factorial design using the general linear model (GLM). Significance was considered at $P \leq 0.05$ for all analyses. The statistical model included effects of pH (3, 4.5, 5.8, 7.3, 8.8, or 10.5) and temperature (15, 25, 40, or 90°C) as the main independent variables. The whole design was replicated on 2 separate occasions with at least one subsampling of the samples, resulting in at least 4 independent ($n \geq 4$) observations.

RESULTS AND DISCUSSION

Composition of AW

Processability of dairy streams depends on their chemical and physical properties. Limited information is available regarding the properties of AW streams originating from production of fresh soft cheese, cream cheese, or Greek-style yogurt, thus hindering attempts to resolve the issues associated with its handling. The composition of the AW assessed in our study is presented in Table 1. The data are in fair agreement with a report on cottage-cheese whey composition by

Table 1. Compositional analysis of the acid whey solution obtained from commercial cream cheese manufacturing

Component	Composition (% wt/wt)
TS	5.5 ± 0.13
Ash	0.61 ± 0.02
Lactose	4.41 ± 0.34
Protein	0.52 ± 0.003
Fat	0.32 ± 0.02
Calcium	0.092 ± 0.001
Potassium	0.135 ± 0.002
Sodium	0.045 ± 0.004
Magnesium	0.013 ± 0.002
Citrate	0.097 ± 0.002
Total phosphates	0.064 ± 0.001
Lactic acid	0.58 ± 0.007
Chloride	0.11 ± 0.012

Barrantes and Morr (1997). Similar to cottage-cheese whey, our cream-cheese whey samples contained less proteins, citrate, and inorganic phosphorus and had higher concentration of calcium and LA than previously reported for sweet whey (Schmidt et al., 1984; Blaschek et al., 2007). These differences are not surprising, given the variety of production conditions, such as different starter cultures or ultimate pH used in the manufacture of different products, which would give rise to variations in the citrate, lactose, and protein concentrations in their respective whey (Josephson et al., 1975).

Changes in Minerals as a Function of pH and Temperature

Varying processing conditions such as pH and temperature during membrane processing of whey streams affect the separation efficiency greatly (Vasiljevic and Jelen, 1999). Most processing parameters influencing efficiency are linked to one another, which complicates any analysis of these effects. As an example, in the dairy industry, the maximum operating temperature for membrane processing is 50 to 55°C (Adams, 2012). However, calcium phosphate is less soluble at these temperatures, thus limiting permeation through the membranes resulting in scaling and associated loss of throughput (i.e., flux). In addition, these mineral changes can affect the pH of dairy systems (Chandrapala et al., 2010). Furthermore, there are incentives to lower operating temperatures to <7°C to retard bacterial growth that may occur because of prolonged processing (Adams, 2012). Hence, it is of utmost importance that the chemical changes of a dairy system with regards to varying processing conditions such as pH and temperature are well understood. To assist in this interpretation, the mineral environment, especially the presence of calcium and phosphate, in the corresponding AW supernatants are presented as a function of

pH and temperature in Figure 2a and 2b, respectively. Both minerals showed the general trends of declining concentrations in the supernatant with increase in temperature and pH. The presence of these minerals and their interactions with each other in large proportions might result in deposition on the membrane surface and a barrier to filtration.

A more thorough analysis of proportional decline of calcium and phosphates with temperature and pH was conducted from the original data present in Figure 2. Calcium concentration in supernatants decreased from

Figure 2. Supernatant concentrations of (a) calcium and (b) phosphates as a function of pH and temperature. Error bars are SD from duplicates.

pH 3 to 10.5 by 13 and 20% at 15 and 90°C, respectively, whereas phosphates decreased by 40 and 22%, respectively. Interestingly, at lower temperatures the decline in phosphate concentration was 3 times higher than that of calcium. At high temperature this decrease was proportional and resulted in calcium-to-phosphates ratio of 1:1. The calculation of phosphate species present at 25°C as a function of pH was thus performed. It showed that ~75% of $\text{H}_2\text{PO}_4^{1-}$ was present at pH 7.2, whereas decreasing pH to 6.2 led to an increase in $\text{H}_2\text{PO}_4^{1-}$ to about 83%. It highlights the importance of pH in the speciation of phosphates. However, temperature also plays a major role in influencing the equilibrium of the phosphate species. With increase in temperature, the activity coefficients of these species decrease, thereby leading to decreased $\text{H}_2\text{PO}_4^{1-}$ form. Hence, the decrease in phosphates with increase in pH at low temperatures may probably be due to the presence of high levels of $\text{H}_2\text{PO}_4^{1-}$. However, as the decline in phosphate concentration was 3 times, it raised a question whether phosphates were also involved in interactions with proteins or whether there was involvement of other cations in formation of salts, which may precipitate into the colloidal phase. However, sodium, potassium, and magnesium did not exhibit significant ($P > 0.05$) concentration changes with increase in pH and temperature, and complexation of other ions with phosphates was considered unlikely. Hence, some fraction of the phosphates may likely get attached to the proteins and pelleted with the colloidal phase, as well as being involved in calcium phosphate precipitation, which is further explained with evidence in later sections. At high temperatures the concentration decline of calcium and phosphate was proportional, which most probably indicates CaHPO_4 precipitation.

These mineral salts may have a tendency to absorb onto the membrane surface, which can reduce permeation rates. However, as shown above, formation of these complexes involves multiple factors including processing conditions and compositional variations of the original solutions, which may as well affect membrane performance. Barrantes and Morr (1997) for example showed that NF was most effective for removing minerals from cottage-cheese whey adjusted to pH >4.35. However, the membrane flux appeared to be the lowest under these conditions, leading to prolonged processing times. In addition, AW is considered a difficult process stream because of failure of lactose to crystallize, which thus remains in its amorphous form during concentration and hinders further processing, including spray drying (Dec and Chojnowski, 2006). It appears that the role of LA and minerals, especially calcium, are crucial in governing properties of AW and thus its processability. In this context, demineralization and

deacidification of AW appears as an attractive strategy with membrane processing and selection of appropriate conditions playing a major role. In the present study, increasing pH would not be considered a feasible one-step approach because of precipitation of salts or formation of salt-protein aggregates discussed earlier, which in turn decreases the membrane's efficiency. A 2-step process involving precipitating or removing these aggregates before a NF membrane process may lead to an efficient separation of LA from lactose at elevated pH. However, proteins can also be removed by this process if involved in complexation, which would not be ideal because proteins are preferred to be retained along with lactose. Low pH would be more effective because formation of aggregates or precipitates would be minimized. This would in turn prevent changes on the membrane surface because of diminished particle-membrane interactions and blocking of the membrane pores. As the results indicated, pH and temperature have a significant effect on the physicochemical nature of the systems where minerals can be manipulated in applying a processing technique.

Changes in LA as a Function of pH and Temperature

Another charged compound that appears at a higher concentration in AW than in sweet whey is LA (Table 1). As a weak acid, its state is pH dependent, and its dissociation behavior as a function of pH may be used to improve efficiency of membrane processing during the LA removal (Ecker et al., 2012). It is related to the pKa value of lactate (3.86). The amount of LA in the supernatant as a function of pH and temperature is presented in Figure 3. Interestingly, increasing pH from 3 to 10.5 resulted in a decrease in LA concentration in supernatants ($P < 0.05$). At high pH values the lactate form predominates, and hence, calcium lactate formation may be facilitated. This is also supported by high LA concentration in supernatants at high temperatures because of the dissolution of calcium lactates at high temperatures. As an example, 42% LA was reduced at 15°C, whereas only 14% has been reduced at 90°C with increase in pH from 3 to 10.5. Although a direct correlation of the presence of LA on the protein or mineral precipitation with increase in pH and temperature could not be established, involvement of LA in mineral complexation or protein precipitation may be considered as concentration of LA declined in the supernatants.

Removal of LA by NF appears to be highly dependent on pH of the feed solution (Gonzalez et al., 2008). Ratio of lactate ions to nonionized LA affects the rate at which both of these compounds pass through NF membranes (Ecker et al., 2012). The amount of undissociated LA and lactate anions in solution is based on

Figure 3. Supernatant concentrations of lactic acid as a function of pH and temperature. Error bars are SD from duplicates.

the LA equilibrium at the operating temperature and can be calculated by the Henderson–Hasselbalch equation using pKa for LA at 25°C as 3.86. As an example, at pH 2.7 only 6.47% of the LA is dissociated, whereas 93.25% of the LA is dissociated at pH 5 and 99.28% at pH 6. This equilibrium is also temperature dependent. However, failure of lactose to crystallize in the presence of LA may hinder further processing, i.e., crystallization and drying. Hence, the varying degrees of separation of LA and its forms may be crucial for lactose crystallization and, consequently, further processability. In this context, understanding how the interplay of different forms of LA, its composition, and processing conditions has on lactose crystallization behavior would be of great importance and would further advance knowledge toward solving processing options for AW.

Changes in Proteins as a Function of pH and Temperature

Earlier sections presented findings related to a possible protein aggregation within the system as a function of pH and temperature; the present section focuses on the physical and surface characteristics of these protein aggregates. Presence of protein aggregates in whey systems plays an important role during membrane processing (Koh et al., 2014). Membrane fouling due to protein aggregation reduces filtration performances, resulting in a sharp decline in permeate flux and a transmembrane pressure drop. Costly cleaning cycles and in some cases replacement of the membrane modules are required to restore the original

flux. Ultrafiltration and NF membranes have pore sizes of 10 to 100 nm and 1 to 10 nm, respectively (Sagle and Freeman, 2006). Therefore, a thorough understanding of protein behavior in AW under various membrane-processing conditions may provide invaluable input for designing an efficient separation process.

The proportion of supernatant proteins to total proteins as a function of pH and temperature is presented in Figure 4. Interestingly, increasing pH from 3 to 10.5 with concomitant rise in temperature from 15 to 90°C resulted in a decrease in protein content in the supernatant ($P < 0.05$). For example, protein concentration in supernatants decreased from pH 3 to 10.5 by 7 and 20% at 15 and 90°C, respectively. Temperature increase to 90°C led to greater protein aggregation, reducing their concentration in the supernatant, than that at 15°C. The extent of reduction was also proportional to pH increase. The present protein data clearly indicate the involvement of proteins in these precipitates or aggregates as discussed earlier.

Rate and extent of aggregation is governed by behavior of major whey proteins, which are also influenced by their environment. For example, NPN components retard irreversible denaturation of β -LG and α -LA, leading to a slower aggregation rate (Anema et al., 2006). The NPN content of the AW solutions was found to be $\sim 0.09\%$, representing about 16% of the total nitrogen content. The NPN constituents appeared unaffected by pH change. However, a slight decrease, $\sim 7\%$, with

Figure 4. Supernatant (Super) concentrations of proteins with respect to total proteins as a function of pH and temperature. Error bars are SD from duplicates.

temperature rise from 40°C (0.085%) to 90°C (0.079%) was observed. These findings infer that NPN played a minor role during protein aggregation and might have been even directly involved in this process as indicated by their disappearance from the supernatant.

The complexity of interactions and their effects on behavior of whey proteins is evident from our results, thus further determination was conducted to elucidate the nature of these aggregates using FPLC-SEC chromatography. The FPLC-SEC chromatograms after the treatments are presented in Figure 5A and 5B. The first peak up to 12 min was assigned as the soluble aggregate peak, whereas the remaining peaks were assigned as native whey proteins and some free caseins (Ryan et al., 2011). Increasing pH to 10.5 induced formation of large soluble aggregates as indicated by a size decrease of the peak at 11 to 12 min and the appearance of a peak at <10 min (Figure 5A). The sample at pH 7.3 showed slight reductions in native protein peaks but no observable formation of large soluble aggregates at 90°C. On the other hand, the small size soluble aggregate peak (11–12 min) increased. In contrast, heating the pH-10.5 sample at 90°C resulted in no observable changes within the soluble aggregate profile in comparison with that at 25°C. This may be due to some pH-induced conformational changes within the protein molecules that have already occurred at 25°C and no increase in soluble aggregates is experienced if a heat treatment is given for such a system. In contrast, a reduction in total supernatant content decrease was evident due to formation of large aggregates, which may pellet during centrifugation (Figure 4). High pH such as 10.5 increase the tendency of deamidation with an exposure of a carboxylic group. This in turn increases the repulsion forces and thereby increases protein unfolding, which may lead to formation of large aggregates through hydrophobic associations. The size of these soluble aggregates was estimated by reducing and nonreducing PAGE as a function of pH at 25 and 90°C. Figures 6A and 6B represent the nonreducing gel electrophoresis patterns obtained from AW solutions of pH 4.5 and 10.5 at 25 and 90°C, respectively. Increasing pH at 25°C apparently increased the size of particles, evident by the shifts of the curves toward higher molecular weight range (Figure 6C). Heating to 90°C led to further increase in particle size for both pH levels, with pH 10.5 resulting in the greatest change. Holding AW at 90°C resulted in the appearance of protein species around 50 kDa at both pH, which could be attributed to the presence of oligomers of β -LG or caseins in the aggregates. These 50-kDa particles were even more pronounced at pH 10.5.

Moreover, the separation mechanism of salts through NF membranes is a function of both charge interac-

tions and solute size (Gonzalez et al., 2008; Rice et al., 2011a). Hence, it is of importance to evaluate the surface characteristics of these aggregates as function of pH and temperature, to find appropriate membranes for further processing of this stream efficiently. In general, protein physical behavior and functionality is dependent on intrinsic hydrophobic, electrostatic, and steric interactions, which are all essential for governing protein conformation and extrinsic interactions.

Charged membranes may reject ions much smaller than the membrane pore size, whereas the uncharged solutes are rejected by steric effects. Surface potential of protein aggregates is strongly pH dependent as also confirmed in the present study. However, temperature also plays a role in governing the charge of these particles, which could be related to mineral equilibrium and the state of proteins. For example, Donato et al. (2009) showed that zeta potential of soluble protein

Figure 5. Fast protein liquid chromatography-size exclusion chromatograms of treated acid whey as a function of pH (A) at 25°C and (B) at 90°C. Absorbance is in arbitrary units.

Figure 6. (A) Nonreducing and (B) reducing gel patterns of acid-whey supernatants at pH 4.5 (samples 1 and 3 and samples 5 and 7) and 10.5 (samples 2 and 4 and samples 6 and 8) under 25 and 90°C conditions. MW = molecular weight. (C) Concentrations of different particle sizes present and obtained through scanning of the gel electropherograms obtained under nonreducing conditions (represented in panel A): pH 4.5 at 25°C (solid black line, sample 1), pH 10.5 at 25°C (solid gray line, sample 2), pH 4.5 at 90°C (dashed black line, sample 3), pH 10.5 at 90°C (dashed gray line, sample 4).

particles decreased when heated at 70°C for 24 h and was independent of associated pH decline. Table 2 shows the surface potential of protein particles present in AW solutions as a function of pH at different temperatures. Notably, the present study showed significant differences of surface charges with respect to pH only showing a significant increase in the negative charge of the particles with increase in pH. This effect can also be due to deamidation of proteins at high pH such as 10.5. Deamidation is a basic hydrolysis reaction where a molecule of water is added to an amide releasing ammonia or more complex β -shift mechanisms (Wakankar

and Borchardt, 2006). This in turn may affect the protein structure, where a polar amide is converted into a potentially charged carboxyl group, which thus results in more negative surface potential. Temperature had no apparent effect on zeta potential at all pH levels studied, which likely was influenced by combination of salt and structural changes of proteins that compensated the overall surface charge. Therefore, hydrophobic attraction (see below) or other types of weak interactions may be more responsible for aggregate formation rather than electrostatic attraction and covalent bonding with respect to temperature.

Table 2. Surface hydrophobicity and potential expressed as ζ -potential of particles present in acid-whey supernatants as a function of temperature and pH

pH	Temperature (°C)	Surface hydrophobicity	Surface charge (mV)
3	15	21.4	-19.4
	25	42.1	-19.5
	40	116.3	-19.6
	90	56.7	-20.3
4.5	15	69.7	-23.6
	25	103.8	-24.3
	40	189.7	-25.2
	90	114.0	-26.0
5.8	15		-26.7
	25		-27.4
	40		-27.9
	90		-28.1
7.3	15	132.9	-28.4
	25	172.7	-39.8
	40	273.7	-29.9
	90	154.4	-30.0
8.8	15		-33.9
	25		-34.7
	40		-35.8
	90		-35.9
10.5	15	187.6	-35.3
	25	215.3	-36.5
	40	432.4	-37.0
	90	324.7	-39.2
SEM		2.3	0.4

Quantitation of protein hydrophobicity is considered an essential step for accurate prediction of protein functionality (Cardamone and Puri, 1992). Changes in surface hydrophobicity are mainly influenced by conformational changes of whey proteins (Dissanayake et al., 2013). Although covalent interactions are apparent during heating of the whey proteins above their isoelectric point, the rate and extent of the whey protein aggregation is predominantly mediated through noncovalent associations below the isoelectric point (Dissanayake et al., 2013). As shown in Table 2, the surface hydrophobicity was substantially affected by temperature. This is in agreement with previous reports (Zhu and Damodaran, 1994; Monahan et al., 1995). The lowest surface hydrophobicity in our study was recorded at 15 and 25°C. By increasing the temperature, hydrophobicity was enhanced, peaking at 40°C, and subsequently declined at 90°C. Conformational changes of the whey proteins thus appeared to start above 25°C, with the hydrophobic motifs still exposed at 40°C, which contributed to the protein denaturation (Monahan et al., 1995). Dissanayake et al. (2013) showed that whey proteins were denatured mainly by disruption of hydrophobic interactions. However, at 90°C the soluble aggregates started to bury their hydrophobic regions, which led to aggregate formation and overall decline in surface hydrophobicity. This is in alignment with several reports. For example, Zhu and Damodaran (1994) found that

surface hydrophobicity of particles present in 9% wt/wt whey protein isolate (WPI) solutions decreased when heated from 70 to 90°C. Monahan et al. (1995) also reported decline in surface hydrophobicity at temperatures above 65°C for WPI solutions. The fluorescence probe is only capable of binding to proteins via well-defined hydrophobic cavities formed by grouping of nonpolar residues on the protein surface (Damodaran, 1989). Furthermore, individual hydrophobic residues randomly exposed at the protein surface do not have the ability to act as strong binding sites. On the other hand, the distribution of these hydrophobic residues on the protein surface that comes into contact with water would greatly enhance the hydrophobic character of the protein surface and would be significantly affected by change in protein and salts concentrations of the system. Zhu and Damodaran (1994) found that the hydrophobic character of the protein surface was actually enhanced, although the surface hydrophobic cavities of whey proteins were destroyed during heating, highlighting the importance of presence of salts.

A type of acid present in the medium may also exert some influence. Acid-whey systems contain higher proportions of LA. Greater hindering of hydrophobic interactions was observed in the presence of LA, with the aggregation being driven by other molecular interactions such as electrostatic, covalent, and van der Waals forces (Dissanayake et al., 2013), which might have also been the case in our current study. Decreased surface hydrophobicity may also be associated with a spherical shape and denser aggregates in which hydrophobic patches were buried. Although temperature obviously plays an important role in these structural changes, the hydrophobicity can also be manipulated by pH. For example, Schmitt et al. (2007) showed that the surface hydrophobicity of WPI particles at pH 7 was double that at pH 6, apparently contributing to a more spherical shape at lower pH, which in turn lowered the surface area and, consequently, several exposed hydrophobic groups. The changes in surface hydrophobicity detected in whey proteins adjusted to different proteins reflect only irreversible changes (Creamer et al., 1982). Hence, adjusting pH to 7 may have reverted the conformation and thereby did not reflect the actual confirmation (Monahan et al., 1995). However, the present study showed an increase in surface hydrophobicity with increase in pH, which was likely because of intensification in overall negative charge by the particles as was evident from charge data. This consequently led to increased repulsion between charged groups and enhanced unfolding. Changing the quality of the protein environment via pH manipulation may also induce protein unfolding and thus redistribute hydrophobic pockets (Zhu and Damodaran, 1994). However, overall surface character

is substantially modified upon heating, resulting in soluble aggregate morphology influenced by the balance of pH and salt concentration because of their effect of altering electrostatic interactions between proteins (Alizadeh-Pasdar and Li-Chan, 2000). This complex behavior was noticed previously by Lee et al. (1992). In their study on 6% WPI dispersion, a decrease in the surface hydrophobicity was observed when the sample was heated from 25 to 65°C at pH 6; however, this was reversed at pH 7 and consequently surface hydrophobicity increased.

The current findings suggested that calcium-phosphate or calcium-lactate complexes formed initially could be responsible for inducing further attraction with proteins and hence facilitating protein aggregation with increasing pH and temperature. Furthermore, free calcium ions, measured by calcium activity (data not shown), exhibited significant ($P < 0.05$) pH dependence, especially at the extremes, decreasing slightly at low pH and greatly at high pH. The observed decline in calcium activity with increase in temperature further indicated the involvement of free calcium ions in complexation. Importantly, surface characteristics of the pelleted particles showed that surface hydrophobicity, which can be an important factor influencing some membrane-separation processes, can vary significantly with changes in pH and temperature.

CONCLUSIONS

Membrane processes that are currently used in processing of sweet whey have not been successfully applied in AW valorization because of apparent compositional differences affecting process performance. In considering some of the compositional factors that might contribute to these effects, this study showed that concentration of calcium and phosphates significantly ($P < 0.05$) declined in AW supernatants with increase in pH and temperature. Lactic-acid content decreased with pH increase at lower temperatures but then increased as the temperature was raised. Calcium appeared to form complexes with phosphates and lactates in AW. The extent of calcium complexation in AW appeared to be correlated with protein aggregation and precipitation, which increased with increasing pH and temperature. The results of this compositional study suggest that it may be feasible to use careful selection of pH and temperature to manipulate the state of minerals, proteins, and LA and thereby influence separation efficiencies in the membrane processing of AW. For example, the trends observed in the present study indicate that lower pH levels may be more suitable for an efficient NF membrane processing of AW solutions using charged or hydrophobic membranes. The next stage in this investi-

gation will be to test how significant these effects might be within the more complex and dynamic environment of an actual membrane-separation process.

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