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Heat-Induced Aggregation of Whey Proteins: Comparison of Cheese WPC with Acid WPC and Relevance of Mineral Composition

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Heat-induced aggregation of whey proteins in solutions made from two commercial whey protein concentrates (WPCs), one derived from mineral acid whey (acid WPC) and the other from cheese whey (cheese WPC), was studied using polyacrylamide gel electrophoresis (PAGE), size exclusion chromatography (SEC), and transmission electron microscopy (TEM). Heat treatment (75 °C) of acid WPC solutions (12.0%, w/w, pH 6.9) resulted in formation of relatively small "soluble" aggregates that were predominantly disulfide-linked. By contrast, heat treatment of the cheese WPC solutions (under the same conditions) caused formation of relatively large aggregates, containing high proportions of aggregates linked by noncovalent associations. The rate of aggregation of both β-lactoglobulin and α-lactalbumin at 75 °C, measured as the loss of native proteins by PAGE, was higher in the cheese WPC solution than in the acid WPC solution. Cross dialysis of the two WPC solutions resulted in alteration of the mineral composition of each WPC solution and reversing their heat-induced aggregation behavior. The results demonstrated that the mineral composition is very important in controlling the aggregation behavior of WPC products.

KEYWORDS: Heat-induced aggregation; aggregation behavior; acid WPC; cheese WPC; WPC mineral environment; GMP; α-lactalbumin; β-lactoglobulin; rate constant; polyacrylamide gel electrophoresis; size exclusion chromatography; transmission electron microscopy; divalent cations; monovalent cations

INTRODUCTION

Whey protein concentrates (WPCs) are used as functional ingredients in many foods such as processed meat, and bakery and dairy products (*1*). However, commercial uses of WPCs are limited because of large variations in their functional properties. These variations are due mainly to differences in their compositions and processing treatments (*2*). Morr and Foegeding (*3*) demonstrated that WPCs obtained from different commercial sources had considerably different mineral contents, including amounts of calcium, sodium, and phosphorus. Hence, better control of the mineral concentrations in WPC products may increase their commercial value as functional ingredients.

The properties of the heat-induced protein gel network depend on the type of interactions between the denatured protein molecules. When WPC solutions (e.g., >8%, w/w, pH 6.9) are heated at a sufficiently high temperature (e.g., 75 °C), the protein molecules unfold and interact to form intermediate aggregates prior to the formation of a gel network (*4*, *5*). The formation of intermediate aggregates involves inter- and intramolecular disulfide bonds (*6*) formed via sulfhydryl−disulfide interchange or sulfhydryl oxidation reactions (*7*). In addition, other noncovalent interactions, such as hydrophobic and ionic interactions, also contribute to the formation of aggregates and a gel network (*8*−*10*).

The presence of mineral ions can alter the net charge on the protein molecules and hence the types of interaction and aggregation that are involved in the formation of the overall gel network. Most of the studies on the effects of minerals on WPC aggregation and gelation have considered only one or two mineral ions at a time. For example, many workers have studied the effect of calcium concentration on WPC gel strength and texture (*11*−*15*). Other workers have studied the effects of sodium (*2*, *15*, *16*) on WPC gel properties. Limited information is available on the possible combined effects of these ions present in the WPC solutions, which are likely to influence the protein interactions that lead to aggregation and gel formation. Consequently, we investigated how variations in the overall mineral composition of WPC solutions affect their aggregation behavior.

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MATERIALS AND METHODS

Materials. Two commercial spray-dried WPC powders were obtained from the Fonterra Co-operative Group (formerly New Zealand Dairy Board), Wellington, New Zealand. One was derived from mineral acid whey (acid WPC) and the other was derived from cheese whey (cheese WPC). These WPCs were typical standard commercial products with no additives. Analysis showed that the acid and cheese WPC powders had similar total protein contents, (85% and 84%, w/w, respectively). The cheese WPC contained a considerable amount (17%, w/w) of glycomacropeptide (GMP), also measured as part of the total protein, compared with that of the acid WPC.

The chemicals used for the preparation of the electrophoresis buffers (obtained from Bio-Rad Laboratories, Richmond, CA) were of analytical grade.

WPC Composition. The total protein content of the WPC powders was determined using the Kjeldahl method (*17*), with a nitrogen conversion factor of 6.38. The fat content was determined using the Soxhlet extraction method, as described by Russell et al. (*18*). The moisture content was determined by oven drying of preweighed duplicate samples at 105 °C for 24 h, cooling them in a desiccator for 2 h, and reweighing the samples. The glycomacropeptide (GMP) content was determined by cation-exchange fast liquid chromatography, as described by Leonil and Mollé (*19*). The mineral analyses were carried out at the New Zealand Pastoral Agricultural Research Laboratory, Palmerston North, by inductively coupled argon-plasma emission spectrometry using the method described by Lee et al. (*20*).

Sample Preparation. Appropriate quantities of WPC powder were dissolved in water (purified using a Milli-Q-system, Millipore Corp., Bedford, MA) so that the final solutions contained WPC (dry weight) concentrations of 12.0% (w/w). The protein concentration in these solutions was approximately 9.8% (w/w). The solutions were stirred for 2 h at room temperature using a magnetic stirrer, and the pH was adjusted to 6.9 using 0.1 M NaOH or HCl. Aliquots (1.5 mL) were transferred to a set of preweighed 2-mL round-bottomed Beckman polyallomer centrifuge tubes (0.35-mm wall thickness, 11-mm i.d., and 34-mm ht). The tubes containing the samples were then reweighed. The tubes were closed with appropriately fitted lids, and placed in a thermostatically controlled (75 °C) water bath. The tubes and the samples were removed after heating for 2, 4, 6, 8, 10, 12, or 15 min (the time taken for the samples to attain 74.9 °C was 20 s). The tubes and heated WPC solutions were immediately placed in ice water for 5 min and then held at room temperature (approximately 20 °C) for 2 h before being used for further analyses.

Dialysis of WPC Solutions. Acid and cheese WPC solutions (12.0%, w/w, pH 6.9) were prepared. Aliquots (300 mL) of each solution were placed in dialysis tubing (molecular weight cutoff 1 kDa), and then dialyzed against 10 L of the other solution at approximately 4 °C (i.e., a 300-mL aliquot of acid WPC solution was placed in 10 L of cheese WPC solution, and vice versa). An aliquot was removed after dialyzing for various times (from 5 to 24 h), and the mineral content was determined (by inductively coupled argon-plasma emission spectrometry using the method described by Lee et al. (*20*)). The sample was then heated at 75 °C, as described above, before further analyses using polyacrylamide gel electrophoresis (PAGE), size exclusion chromatography (SEC), and transmission electron microscopy (TEM).

Polyacrylamide Gel Electrophoresis. The heated WPC solutions were analyzed using either native-PAGE or sodium dodecyl sulfate (SDS)-PAGE as described by Havea et al. (*5*). After the appropriate gel was prepared, 10-μL samples of 0.01% (w/w) protein solution, diluted with the appropriate sample buffer, were injected into the sample wells and then electrophoresed to separate the proteins. After the gels had been stained with Coomassie blue and destained, they were photographed, as described by Havea et al. (*5*).

The gels were scanned using an Ultrascan XL laser densitometer and the results were analyzed using an LKB 2400 GelScanXL software program (LKB Produckter AB, S-161 26 Bromma, Sweden) to obtain quantitative results. The peak area of each protein band was reported as a percentage of the corresponding band in the unheated control WPC samples.

Reaction Kinetics Evaluation. The rates of loss of nativelike and SDS-monomeric β-lactoglobulin and α-lactalbumin at 75 °C, as measured by PAGE, were determined using the general kinetic equations described by Dannenberg and Kessler (*21*). Each set of results was fitted to the appropriately derived equation for $n = 1$, 1.5, or 2, and the reaction rate constants were calculated using the FigP statistical program (Biosoft, Ferguson, MO). The coefficients of determination (r^2) were used to indicate how well each data set fitted the equations.

Size Exclusion Chromatography. The heated WPC solutions were diluted with 20 mM imidazole/HCl buffer (pH 6.8, 50 mM NaCl) to give a final protein concentration of 0.6% (w/w) and then filtered using an Alltech cellulose acetate membrane 0.22 μm (Type HA) and a syringe filter. The samples (50 μL) were separated on a Superose 6HR 10/30 column (Pharmacia Biotech), using a GBC HPLC system (GBC Scientific Equipment Pty Ltd., Dandenong, Victoria, Australia), equipped with a LI 1150 HPLC pump, a LI 1200 UV/Vis detector set at 280 nm, and a LI 1440 system organizer.

The protein composition of each peak was determined by collecting peaks separately from the column outlet, freeze-drying them, and then analyzing them by native- and SDS-PAGE under nonreducing and reducing conditions.

The above experiments were repeated at least in triplicates.

Transmission Electron Microscopy (TEM). Some of the WPC solutions were analyzed by TEM. In these cases, the WPC solutions were heated at 75 °C for different times (2−10 min) before preparation and analysis using the TEM method, as described by Langton and Hermansson (*22*).

RESULTS

Heat Treatment of WPC Solutions. *Nomenclature.* The terms "nativelike" and "SDS-monomeric" proteins have been defined in previous work (*5*, *23*) and are used to describe the different forms of proteins in heated systems. Nativelike proteins refer to protein bands on a native-PAGE gel that coincide with the native proteins in the unheated samples. SDS-monomeric proteins refer to protein bands on an SDS-PAGE gel that run as though they are monomeric. These terms are used to discuss the results of the current study.

PAGE Patterns of Heated Acid WPC and Cheese WPC Solutions. The two WPC solutions showed very similar native-PAGE patterns (**Figure 1 I*a*** and **I*b***). The protein band intensities decreased with an increase in heating time at 75 °C, indicating gradual loss of nativelike proteins, accompanied by concomitant accumulation of high-molecular-weight aggregates on top of the stacking and resolving gels. In both WPC solutions, the loss of bovine serum albumin (BSA) (and other minor whey proteins) was faster than the loss of β-lactoglobulin or α-lactalbumin, whereas the loss of α-lactalbumin was faster than that of β-lactoglobulin.

There were two main differences between the native-PAGE patterns of the heated acid WPC and cheese WPC solutions: (1) the loss of β-lactoglobulin and α-lactalbumin appeared to be faster from the cheese WPC solutions (**Figure 1 I*b***) than from the acid WPC solutions (**Figure 1 I*a***), and (2) the accumulation of high-molecular-weight material on top of the stacking and resolving gels appeared to be more prominent in the heated acid WPC solutions than in the heated cheese WPC solutions.

The SDS-PAGE patterns of the two WPC solutions (**Figure 1 II*a*** and **II*b***) showed some of the features observed on the native-PAGE gels. The loss of SDS-monomeric proteins from the cheese WPC solutions was faster than that from the acid WPC solutions. The accumulation of high-molecular-weight material on top of the stacking and resolving gels was more prominent in the acid WPC solutions (**Figure 1 II*a***) than in the cheese WPC solutions (**Figure 1 II*b***). In the acid WPC

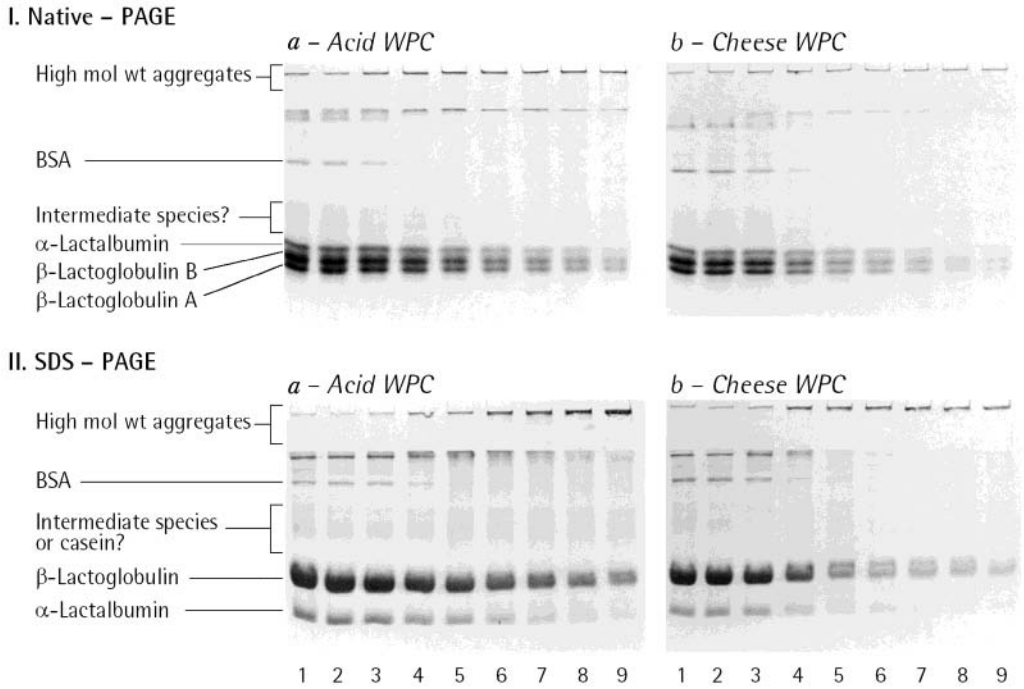

Figure 1. Native-PAGE (I) and SDS-PAGE (II) patterns of acid WPC (*a*) and cheese WPC (*b*) solutions (12.0%, w/w, pH 6.9), heated at 75 °C for 0 (slots 1 and 2), 2 (slot 3), 4 (slot 4), 6 (slot 5), 8 (slot 6), 10 (slot 7), 12 (slot 8), and 15 (slot 9) min.

solutions (**Figure 1 II*a***), some faint bands in the region between BSA and β-lactoglobulin appeared to increase in intensity with heating time; the samples heated beyond 4 min showed several faint distinct bands, which were probably intermediate protein species (e.g., β-lactoglobulin dimers, trimers) that were formed during heating. However, these bands were not seen in the SDS gel of the heated cheese WPC solutions (**Figure 1 II*b***).

Quantitative analysis of the native- and SDS-PAGE gels (**Figure 1**) of the heated WPC solutions showed that the loss of proteins from the cheese WPC solutions (**Figure 2*b***, ***d***, and ***f***) was considerably faster than that from the acid WPC solutions. For the cheese WPC solutions, the loss of nativelike proteins was also considerably faster than the loss of SDS-monomeric proteins. However, for the acid WPC solutions (**Figure 2*a***, ***c***, and ***e***), the loss of nativelike proteins was only slightly faster than the loss of SDS-monomeric proteins. These considerable differences between the losses of nativelike proteins and SDS-monomeric proteins from the cheese WPC solutions indicate that some noncovalently linked aggregates were formed during heating. The small differences between the losses of nativelike and SDS-monomeric proteins from the acid WPC solutions indicate that smaller proportions of noncovalently linked aggregates were formed in these solutions during heating and that the protein aggregates were predominantly disufide-linked (*5*).

Analysis of Heated WPC Solutions Using SEC. The elution profile of the unheated WPC solutions showed four peaks (**Figure 3**). The molecular weights of the material eluted in peaks 1, 2, 3, and 4 were found to be $>1 \times 10^6$, $\approx 7 \times 10^4$, $\approx 3 \times 10^4$, and 1.5×10^4 Da, respectively. Native- and SDS-PAGE results (not shown) showed that peak 1 contained all the whey proteins (probably aggregated), whereas peak 2 contained mainly BSA and immunoglobulin, peak 3 contained mainly β-lactoglobulin, and peak 4 contained mainly α-lactalbumin. Peaks 2−4 decreased progressively with heating time at 75 °C, indicating continuous loss of nativelike proteins. The decrease in size of peak 2 was faster than that of peaks 3 and 4, indicating that the loss of BSA and other minor whey proteins was faster than the loss of either β-lactoglobulin or α-lactalbumin. The decreases in the size of peaks 3−4 were faster for the cheese WPC solutions than for the acid WPC solutions, supporting the PAGE results (**Figures 1** and **2**).

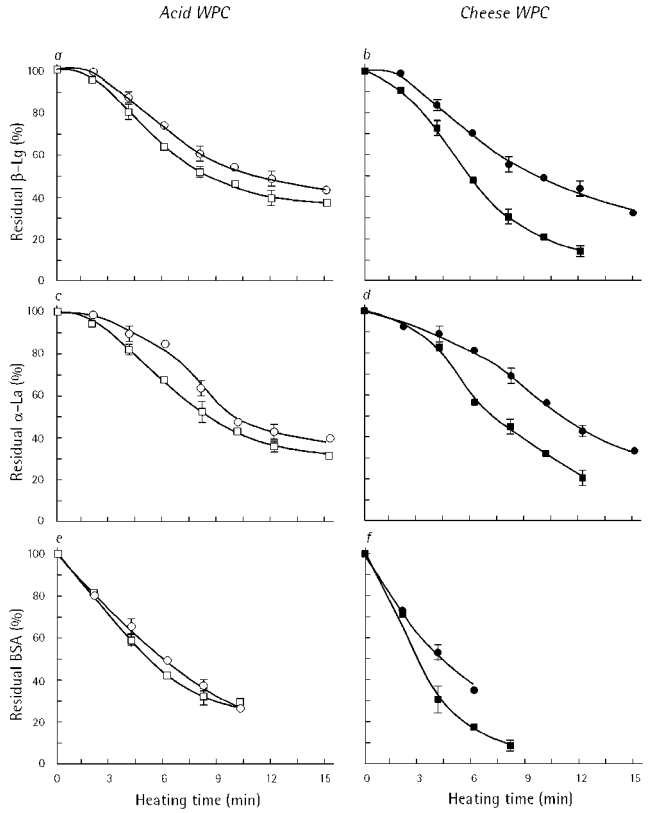

Figure 2. Loss of nativelike (●, ○) and SDS-monomeric (■, □) β-lactoglobulin (β-Lg, *a*, *b*), α-lactalbumin (α-La, *c*, *d*), and BSA (*e*, *f*) from 12.0% (w/w) acid WPC (○, □) and cheese WPC (●, ■) solutions during heating at 75 °C.

The main difference between the heated acid and cheese WPC solutions was that peak 1 increased with heating time up to 6

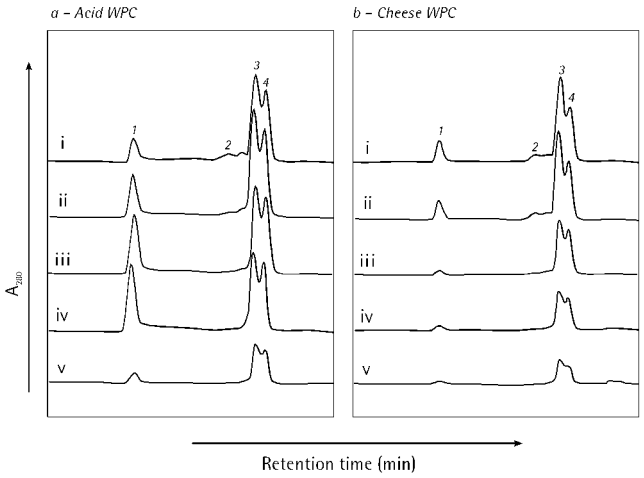

Figure 3. SEC elution profiles of acid WPC (*a*) and cheese WPC (*b*) solutions. Superose 6HR 10/30 column, 50 μL sample, flow rate 0.4 mL/min, and eluent 20 mM imidazole buffer (pH 6.9, 50 mM NaCl). 12.0% (w/w) WPC solutions heated at 75 °C for 0 (i), 2 (ii), 4 (iii), 6 (iv), and 10 (v) min. The samples were diluted with the buffer so that the final WPC concentration was 0.6% (w/w), and were filtered through a 0.22-μm filter before analysis.

min in the acid WPC solutions, but decreased with heating time in the cheese WPC solutions. For SEC analysis, all WPC solutions were filtered through a 0.22-μm filter before injection into the column. This filtration step would have removed particles >0.22 μm. The results (**Figure 3**), therefore, suggest that when the acid WPC solutions were heated, small "soluble" protein aggregates were formed (<0.22 μm) with heating time up to 6 min. After heating for 10 min, these aggregates interacted to form larger aggregates, which were subsequently removed by the filtration step, resulting in a decrease in the size of peak 1 (**Figure 3*a***, **v**). In contrast, in the heated cheese WPC solutions, the aggregates formed were probably larger than 0.22 μm and consequently were removed by the filtration step, and hence peak 1 decreased continuously with heating time.

Analysis of Heated WPC Solutions using TEM. The electron micrograph of the acid WPC solution heated at 75 °C for 2 min (**Figure 4*a***) showed a fine mesh of tiny homogeneous protein aggregates. The micrograph of the sample heated for 6 min (**Figure 4*b***) showed a similar particle size distribution but a higher degree of "hairiness". These results suggest that when a solution of acid WPC was heated, fine aggregates, $\ll 0.5$ μm, were formed. Further heating (e.g., for 6 min) did not seem to change the structures significantly (**Figure 4*b***). These are similar to the "fine-stranded" structures of heat-induced β-lactoglobulin gels (12%, w/w, pH 7.5) reported by Stading and Hermansson (*24*).

The micrographs of the heated cheese WPC solutions showed aggregate structures very different from those of the acid WPC solutions. After heating for 2 min (**Figure 5*a***), relatively large (>10 μm) irregularly shaped clusters with large spaces between them were formed. It appeared that these clusters consisted of conglomerates that were linked together by intertwined fibrils or hair-like structures. The hairy structures also appeared to extend from the surfaces of the clusters. The micrograph of the sample heated for 4 min (**Figure 5*b***) showed that clusters of similar size were present but they appeared to be joined to each other by the hairy structures. The heat-induced aggregate structures of the proteins in the cheese WPC solutions are similar to the "particulate" structures of heated whey proteins reported by other workers (*22*, *24*, *25*).

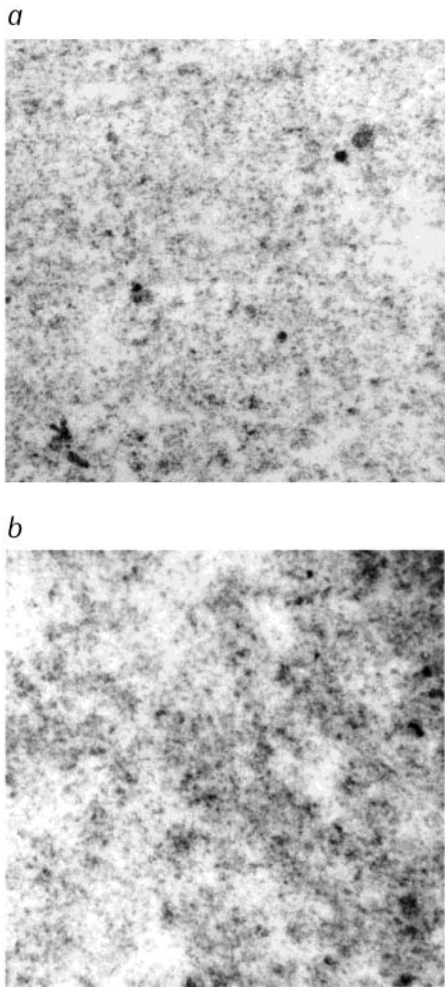

Figure 4. TEM micrographs of acid WPC aggregates. 12.0% (w/w) WPC solutions (pH 6.9) were heated at 75 °C for 2 (*a*) or 6 (*b*) min before analysis. Magnification: 72000×.

Effect of Dialysis on the Mineral Content of WPC Solutions. During dialysis for up to 24 h at 4 °C, the sodium content of the cheese WPC solution decreased by about 40% (**Table 1**), whereas the potassium content increased to more than double its initial level after dialysis for 6 h and then increased gradually thereafter. Both the calcium and magnesium contents decreased by approximately 50% after dialysis for 24 h.

The sodium content of the acid WPC solution increased significantly during the first 5 h of dialysis and then increased gradually thereafter (**Table 1**). The potassium content decreased considerably (approximately 50%) after 5 h of dialysis and then decreased gradually thereafter. The calcium content increased gradually during the 24 h of dialysis.

It appears that dialysis changed the concentrations of monovalent cations relatively quickly so that there was an equilibrium between the two WPC solutions (**Figure 6*a***). When a sample of cheese WPC solution was dialyzed against excess acid WPC solution, the monovalent ion concentration of the cheese WPC sample increased, within 6 h, to a level that was comparable with that of the acid WPC solution. An opposite trend was observed when a sample of the acid WPC solution was dialyzed against excess cheese WPC solution; the monovalent ion concentration of the acid WPC sample decreased, within 6 h, to a level that was comparable with that of the cheese WPC solution. These results suggest that the monovalent ions were present in a fully diffusible state in the WPC solutions.

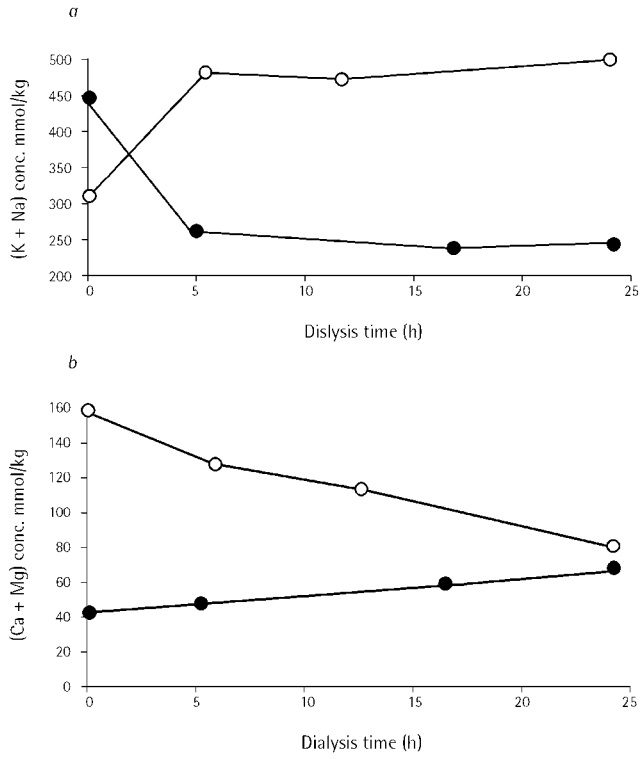

Figure 5. TEM micrographs of cheese WPC aggregates. 12.0% (w/w) WPC solutions (pH 6.9) were heated at 75 °C for 2 (*a*) or 4 (*b*) min before analysis. Magnification: 72000×.

Table 1. Changes in Mineral Contents of the WPC Solutions with Dialysis Time

	mmol/kg dry weight			
cheese WPC solution	0 h	6 h	12 h	24 h
calcium	136	109	96	70
magnesium	21	20	17	12
potassium	193	404	415	438
sodium	91	85	67	57
acid WPC solution	0 h	5 h	17 h	24 h
calcium	40	49	57	67
magnesium	3	4	5	6
potassium	424	212	192	201
sodium	30	47	50	53

The concentration of divalent cations was higher in the cheese WPC solution than in the acid WPC solution (**Figure 6*b***). After dialysis for 24 h, the divalent cation concentrations in either WPC solution reached levels that were intermediate between the initial concentrations in the acid WPC and cheese WPC solutions. These results suggest that the interchange of the divalent cations between the two WPC solutions was limited, probably due to binding of a fraction of the divalent cations to the proteins in the cheese WPC.

Effect of Dialysis on Heat-Induced Aggregation of Whey Proteins Analyzed Using SEC. The SEC profiles of the acid WPC solutions that were dialyzed against excess cheese WPC solution for 24 h (**Figure 7*a***) were quite different from those of the control acid WPC solutions (**Figure 3*a***). There was an accumulation of small (<0.22 μm) aggregates after heat treatment for 2 min, indicated by the increased size of peak 1. These small aggregates interacted quickly to form larger ones (>0.22 μm) that were removed by the preanalysis filtration step, indicated by the decreasing size of peak 1 after heat treatment for ≥4 min. The heated samples of cheese WPC solutions that

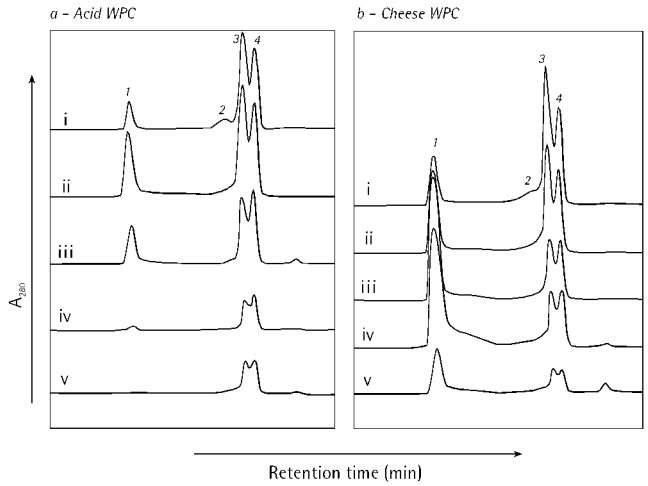

Figure 6. Effect of dialysis on the concentrations of monovalent cations (sodium and potassium) (*a*) and divalent cations (calcium and magnesium) (*b*) in cheese WPC (○) and acid WPC (●) solutions.

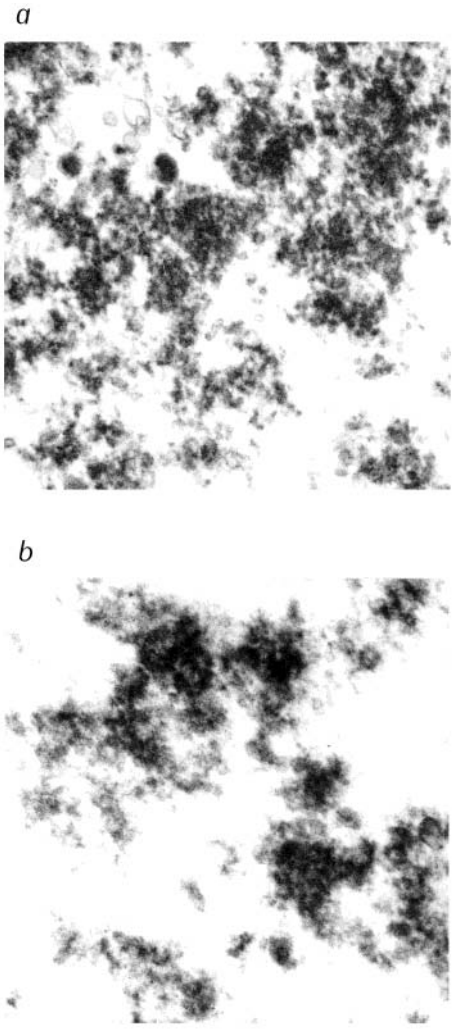

Figure 7. SEC elution profiles of acid WPC (*a*) and cheese WPC (*b*) solutions (12.0%, w/w, pH 6.9) that were dialyzed for 24 h. The WPC solutions were heated at 75 °C for 0 (i), 2 (ii), 4 (iii), 6 (iv), and 10 (v) min. Superose 6HR 10/30 column, 50 μL sample, flow rate 0.4 mL/min, eluent 20 mM imidazole buffer (pH 6.9, 50 mM NaCl).

were dialyzed for 24 h against acid WPC solution showed that considerable quantities of small aggregates (<0.22 μm) were formed (**Figure 7*b***). Even in the sample that was heated for 10

Table 2. Calculated Rate Constants (k_n, min$^{-0.5}$) and Coefficient of Determination (r^2) for the Loss of Nativelike and SDS-Monomeric β-Lactoglobulin and α-Lactalbumin from Dialyzed WPC Solutions during Heating, Using a Reaction Order $n = 1.5$

	β-lactoglobulin		α-lactalbumin	
sample	k_n	r^2	k_n	r^2
loss of nativelike				
acid WPC control	0.0095	0.96	0.0086	0.91
acid WPC dialyzed for 24 h	0.0107	0.96	0.0096	0.99
cheese WPC control	0.0243	0.94	0.0178	0.90
cheese WPC dialyzed for 24 h	0.0172	0.99	0.0121	0.98
loss of SDS-monomeric				
acid WPC control	0.0024	0.98	0.0084	0.92
acid WPC dialyzed for 24 h	0.0117	0.97	0.0127	0.90
cheese WPC control	0.0089	0.97	0.0110	0.98
cheese WPC dialyzed for 24 h	0.0111	0.96	0.0122	0.93

min, there was still a considerable quantity of small aggregates, indicated by the size of peak 1 (**Figure 7*b***, **v**). It is clear that dialysis caused a change in the aggregation behavior of proteins in the cheese WPC solutions, from the formation of relatively large aggregates that were removed by the preanalysis filtration (**Figure 3*b***) to the formation of considerable quantities of small (<0.22 μm) aggregates, as indicated by the size of peak 1 (**Figure 7*b***). Overall, dialysis resulted in the heat-induced aggregation behavior of proteins in the acid WPC solutions being more like that in the cheese WPC solutions and vice versa.

Effect of Dialysis on the Kinetics of the Loss of Proteins from the Heated WPC Solutions. Table 2 shows the kinetic parameters obtained for the loss of proteins during heat treatment of WPC solutions, using quantitative PAGE (e.g., **Figures 1** and **2**). In agreement with the results of Havea et al. (*5*), the loss of nativelike or SDS-monomeric β-lactoglobulin or α-lactalbumin from the heated cheese or acid WPC solutions could be adequately described by $n = 1.5$ (**Table 2**). The values of the rate constant (k_n) for the loss of nativelike β-lactoglobulin and α-lactalbumin from the heated cheese WPC solutions were much higher (approximately 2-fold) than those for the loss of the same proteins from the heated acid WPC solutions. A similar result was found for the loss of SDS-monomeric β-lactoglobulin. Interestingly, the k_n value for the loss of SDS-monomeric α-lactalbumin from the heated cheese WPC solutions was only slightly higher than that for the loss of the same protein from the heated acid WPC solutions.

Dialysis of acid WPC against cheese WPC increased the loss of nativelike and SDS-monomeric proteins (i.e., increase in k_n values) during heating (**Table 2**). In contrast, dialysis of cheese WPC against acid WPC resulted in a decrease in the rate of loss of the nativelike proteins (i.e., decrease in k_n values). The k_n values for the loss of SDS-monomeric proteins from the dialyzed cheese WPC solutions were slightly higher than those for the loss of the same proteins from the control WPC solutions (**Table 2**).

DISCUSSION

The results of this study demonstrate that the heat-induced aggregation behavior of the commercial acid WPC was considerably different from that of cheese WPC. Cross dialysis of the two WPC solutions caused changes in the heat-induced aggregation behavior so that the acid WPC behaved more like the cheese WPC, and vice versa. These changes were shown to be related to the differences in the levels and types of cations in the WPC solutions, rather than protein composition.

GMP is known to have negligible effect on the heat-induced aggregation and gelation of WPC. Because it is very soluble and contains no cysteine residues, it could act as a diluent; therefore, the effective functional protein concentration in the cheese WPC powder was less than expected on the basis of the total protein as determined by the Kjeldahl method. In our earlier work (*5*) we demonstrated that when WPC solutions with different concentrations were heated (75 °C, pH 6.9), the rate of loss of native proteins increased with increasing protein (WPC) concentration. On the basis of that finding, the loss of proteins from the cheese WPC in the current study would be expected to be slower, because of the effective lower protein concentration. Instead, we observed the opposite in the current work. This indicates that the different aggregation behaviors of the acid and cheese WPCs could not be explained by their protein compositions. This led us to consider the effect of the different mineral environments on the heat-induced aggregation behavior of these WPCs.

The cheese WPC contained higher levels of calcium, magnesium, and sodium than the acid WPC, whereas the acid WPC contained a higher level of potassium. The different mineral environments would certainly have influenced the extent of heat-induced aggregation as well as aggregate sizes in the two WPC solutions during heating. Many workers have studied the effects of calcium ions on the heat-induced aggregation of whey proteins. Hollar et al. (*26*) reported that when a WPC solution (16% total solids) was dialyzed against simulated milk ultrafiltrate containing from 0 to 9 mmol calcium and heated at 66 °C, the mixture became progressively more denatured and formed more "insoluble" aggregates (precipitates) as the calcium content increased. Barbut (*27*) reported that increasing the calcium concentration (10−180 mmol/L) in whey protein isolate solutions (100 g/L, pH 7.0) changed the aggregate structure from fine protein strands to thicker strands. Parris et al. (*28*) reported that the amount of insoluble protein aggregates in heated sweet whey increased with increasing calcium level. Morr and Josephson (*29*) suggested that the heat-induced aggregation of whey proteins in the presence of calcium involves the formation of large aggregates, which is dependent on calcium concentration. More recently, Ju and Kilara (*30*) investigated the effect of added calcium chloride on the aggregation behavior of whey protein isolate at 45 °C. They reported that high calcium chloride concentrations (30 to 50 mM) resulted in quicker initial size increases and higher maximum turbidity values after 5 h of incubation. At 50 mM calcium chloride, large aggregates that sedimented during incubation were formed, whereas at <30 mM calcium chloride, the aggregates formed were stable colloidal particles that did not sediment. The results of these studies support the view that the larger, more particulate aggregate structures formed in the cheese WPC solutions during heating could be attributed to the high calcium content of the WPC powder (**Table 1**).

The divalent cations (calcium and magnesium) induce protein aggregation in three different ways: (1) electrostatic shielding, (2) ion-specific hydrophobic interactions, and (3) cross-linking of adjacent anionic molecules by forming protein−calcium−protein bridges (*30*, *31*). Li et al. (*32*) suggested that calcium binding during the initial steps of polypeptide unfolding stabilizes the unfolded form and that inter- and/or intramolecular cross-links between ionic carboxyl groups are bridged by

divalent ions during the gel formation process. A decrease in calcium concentration would be expected to reduce the occurrence of calcium bridges in the gel, and would favor the formation of fine-stranded gels.

The monovalent cations affect WPC gelation in two different ways: (1) they reduce the electrostatic repulsions between the negatively charged protein molecules by masking the charged residues, hence the reactive groups of the unfolded molecules can come into close proximity with each other; and (2) they reduce the extent of divalent cation bridge formation between molecules. Reducing the repulsions between the protein molecules allows the molecules to come closer to each other, giving rise to the formation of noncovalent associations between the exposed hydrophobic groups or covalent disulfide bonds between molecules. Our results showed that the acid WPC aggregates were formed predominantly by disulfide-linked bonds with little contribution from noncovalent associations (**Figure 2**). High concentrations of potassium in the acid WPC system, through competition for binding to negatively charged groups, probably reduced the binding of divalent cations and the formation of divalent bridges between the molecules. Because of the limited number of reactive sites (−SH and S−S) on the protein molecules, the formation of disulfide bonds between the molecules is a relatively slow reaction. This is probably the main cause of the lower reaction rates (**Table 2**) and the formation of smaller protein aggregates observed for the heated acid WPC solutions (**Figures 3**, **6**, and **7**). Overall, the monovalent cations have a positive effect, to a certain level, on the formation of "fine" aggregate structures.

In conclusion, the mineral environment of the WPC powders has a significant effect on the heat-induced aggregation of the whey proteins. The high calcium content of cheese WPC powder facilitates the loss of native proteins during heating, giving rise to the rapid formation of relatively large protein aggregates. In contrast, the high content of potassium in acid WPC limits the formation of calcium bridges between protein molecules during heating, giving rise to the slow formation of relatively small aggregate structures. Although the GMP content of the cheese WPC was considerably higher, it had negligible effect on the heat-induced aggregation behavior of the product.

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