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Thermal Stability of Whey Protein Concentrate Mixtures: Aggregate Formation[1]

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ABSTRACT

Solutions (17% TS) of whey protein concentrate (65% protein) were dialyzed against simulated milk ultrafiltrate containing 0 to 9 m*M* total Ca^{2+}. The dialyzed solutions were heated at 66 or 71°C for 120 min to study the effect of Ca^{2+} on the heat denaturation and aggregation of whey proteins. As Ca^{2+} decreased, the whey protein concentrate solutions formed more soluble aggregates and fewer insoluble precipitates; the amount of α-LA relative to the β-LG associated with the soluble aggregates also increased. Protein aggregates, as shown by electron microscopy of the Ca^{2+}-adjusted solutions, became smaller and less densely packed as Ca^{2+} decreased. The effects of addition of low heat NDM or a mixture of Ca^{2+} and Na caseinate to the whey protein concentrate solution (17% TS) and heat treatment at 71°C for 120 min on whey protein denaturation and aggregate formation were also investigated. Compared with the whey protein concentrate solution (17% TS), whey protein denaturation was much lower when low heat NDM was added to the solution, but not when a mixture of Ca^{2+} and Na caseinate was added. Electron micrographs showed that the whey protein aggregates that formed upon heating the mixture of whey protein concentrate and low heat NDM at 71°C were smaller and less dense, and the micellar appendages were more compact, than those in the whey protein (17% TS) or low heat NDM (27% TS) solutions. The micrographs of the mixture of whey protein concentrate and caseinate were not comparable with either that of low heat NDM or of the mixture of whey protein concentrate and NDM.

(**Key words**: calcium, casein, electron microscopy, whey protein concentrate)

Abbreviation key: **Ca-Na caseinate** = Na caseinate to which Ca^{2+} had been added, **SA** = soluble aggregate, **SEC** = size exclusion chromatography, **SMUF** = simulated milk ultrafiltrate, **TEM** = transmission electron microscopy, **WPC** = whey protein concentrate, **WPCa** = WPC65 (17% TS) dialyzed against SMUF (used with number indicating 0, 3, 6, or 9 m*M* total Ca^{2+}), **WPC65** = WPC containing 65% protein.

INTRODUCTION

The role of Ca^{2+} in the stabilization of systems based on milk protein has been studied extensively. Both the concentration and equilibrium state of Ca^{2+} (i.e., ionic, soluble, or colloidal) are important factors (6, 7, 11, 18, 27, 30, 31, 33). Protein concentration, heating temperature, and the type and concentration of salts present in milk protein systems are also important parameters in aggregate and gel formation (4, 5, 8, 9, 11, 13, 19, 28, 32). Whey protein aggregation begins with the initial swelling of the protein structure when it is first exposed to heat; as the intensity of the heat treatment increases, the whey proteins unfold and aggregate (16). The structure and textural properties of whey protein isolate gels depend, in part, on whether the Ca^{2+} is present during heating or is added after the heat treatment (1).

Although much has been reported on the role of Ca^{2+} in systems based on milk protein on the formation of gels, the effect of Ca^{2+} on the formation of protein complexes is not fully understood, nor is the effect of addition of NDM, which helps to stabilize heated milk systems.

Our objectives were to determine how the concentration of Ca^{2+} and added low heat NDM or a mixture of Ca and Na caseinate affect whey protein denaturation and protein aggregate formation in solutions of whey protein concentrate (**WPC**).

MATERIALS AND METHODS

Materials

Whey protein concentrate containing 65% protein (**WPC65**) and low heat NDM samples were obtained

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from the Vermont Whey Company (Milton, VT). Proximate analyses of these samples were supplied by the manufacturer (Table 1). Deionized water was purified (Polisher I HPLC Laboratory Reagent Grade Water System; Continental Water Systems Corp., San Antonio, TX) prior to use.

Simulated milk ultrafiltrate (**SMUF**), containing 9 m*M* total Ca^{2+} and 2 to 5 m*M* free Ca^{2+}, was prepared using the procedure of Jenness and Koops (10) as modified by Parris et al. (20). Modified SMUF was also prepared containing 0, 3, or 6 m*M* Ca^{2+}.

The Na caseinate was prepared from low heat NDM (Maryland and Virginia Milk Producers Association, Inc., Laurel, MD). Low heat NDM was dissolved in deionized water (10%, wt/vol), and the caseins were isoelectrically precipitated with 1 *M* HCl. After the casein and whey mixture was filtered (Whatman number 1; Whatman Laboratory Division, Maidstone, Kent, England), the casein was washed twice with deionized water. The acidified casein was solubilized in water at pH 6.7 with 1 *M* NaOH. The Na caseinate solution was lyophilized and stored at –20°C.

Sample Preparation

The WPC65 was dissolved in deionized water (25°C) to obtain 17% TS (wt/wt). To alter the soluble Ca^{2+} content of the WPC65 solution (17% TS), a 25-ml aliquot was dialyzed against 500 ml of SMUF containing 0, 3, 6, or 9 m*M* total Ca^{2+} at pH 6.6 and 5°C with buffer changes after 5 h and an additional 15 h, for a total of 24 h. The WPC65 solutions dialyzed against SMUF containing 0, 3, 6, and 9 m*M* Ca^{2+} are identified as **WPCa** 0, 3, 6, and 9, respectively. Dialysis lowered the TS from 17% to approximately 12% for all amounts of Ca^{2+}.

Deionized water (66 or 71°C) was used to prepare low heat NDM (27% TS) solutions prior to heat treatment. In addition, low heat NDM was added to a WPC65 solution (17% TS) to yield 36% TS; the NDM:WPC65 ratio in the mixture was 1.88:1 (vol/vol).

Casein micelles were prepared from 7.6% (wt/wt) Na caseinate in 25 m*M* pH 6.75 PIPES (piperazine-N,N′-bis-(2-ethanesulfonic acid) buffer (19) by addition of 0.8 mmol of Ca^{2+}/g of casein (29) and termed **Ca-Na caseinate**. After the Ca^{2+} addition, the pH was adjusted to 6.75 with 1 *M* NaOH. The mixture was allowed to equilibrate at room temperature (23°C) for at least 2 h prior to heat treatment and addition of WPC65. Following addition of WPC65 to the solution (7.6% TS) of Ca-Na caseinate to yield 20.5% TS, the ratio of WPC65 to caseinate mixture was 2.12:1.

The stability of the casein micelles that were formed from Na caseinate was determined using a modification of the method of Mora-Gutièrrez et al. (15). As described in their report, the supernatant protein concentration was determined at 280 nm using an absorption of 0.85 ml/mg·cm for whole casein (24). The range of Ca^{2+} (molar) producing a stable colloid was determined by plotting milligrams of casein per milliliter of supernatant against $CaCl_2$ (molar).

TABLE 1. Proximate composition of solutions containing whey protein concentrate with 65% protein (WPC65), low heat NDM, and caseinate solutions to which Ca^{2+} had been added (Ca-Na caseinate).

Solution or mixture	TS	Casein	Whey protein	CHO[1]	Fat	Ash
	(%)					
WPC65[2]	17.0	. . .[3]	11.4	3.8	1.0	0.8
WPC65, NDM[4]	36.2	5.6[5]	12.8[5]	14.0	1.1	2.4
NDM[2]	27.3	7.9[5]	2.0[5]	14.3	0.2	2.2
WPC65, Ca-Na caseinate[6]	20.5	7.6	8.6	2.9	0.8	0.6
Ca-Na caseinate	7.6	7.6	. . .[3]	. . .[7]	. . .[7]	. . .[7]

[1]Carbohydrate.

[2]Proximate composition of WPC65 and low heat NDM supplied by manufacturer.

[3]Results of SDS-PAGE showed no detectable amount of this protein in the sample.

[4]Proximate analysis calculated from that supplied by the manufacturer for WPC65% and NDM; WPC65 contained 17.0% TS, and NDM contained 19.2% TS.

[5]Low heat NDM protein estimated to be 80% casein and 20% whey protein.

[6]Estimated proximate analysis calculated from that of WPC65% supplied by manufacturer and of the Ca-Na caseinate, WPC65 contained 12.9% TS, and Ca-Na caseinate contained 7.6% TS.

[7]Not determined.

Heat Treatment

Approximately 25 ml of the WPCa 0, 3, 6, and 9 solutions were each heated at 66°C with continuous stirring in a covered flask for 120 min for analysis using size exclusion chromatography (**SEC**) and SDS-PAGE. The solutions were heated at 71°C for analysis by SEC and transmission electron microscopy (**TEM**). The appearance of the solution (increased viscosity or visible thickening) and the presence of visible particulates were noted when aliquots were removed at 0, 30, 60, 90, and 120 min. At the conclusion of the heating regimen, a 1.09-g sample was removed for analysis of the soluble aggregate (**SA**) fraction.

Approximately 25 ml of the WPC65 and NDM solutions and the WPC65-NDM mixture were heated at 71°C with continuous stirring in a covered flask for up to 120 min or until they became too viscous to stir for analysis using SEC and TEM. Aliquots (1.09 g) were removed at 0, 30, 60, 90, and 120 min for analysis using SEC and at 0 and 60 min for analysis using TEM.

Approximately 25 ml of the Ca-Na caseinate solution and the WPC65-caseinate mixture were heated at 71°C with continuous stirring in a covered flask for up to 120 min or until they became too viscous to stir for analysis using SEC and TEM. Aliquots (1.09 g) were removed at 0, 30, 60, 90, and 120 min for analysis using SEC and at 0 and 60 min for analysis using TEM.

SEC

To study the effect of Ca^{2+} on heat denaturation and aggregation of whey proteins, 1.09-g aliquots of the heated WPC samples described earlier were diluted to 10 g with buffer (0.25 *M* Na phosphate, pH 6.7) and centrifuged with a Sorvall® RC-5B centrifuge (DuPont Company, Wilmington, DE) at 13,000 × *g* at 4°C for 30 min. The supernatant was filtered (0.45 μm) and analyzed at room temperature (23°C) on a Zorbax GF-250 column (Rockland Technologies, Inc., Newport, DE) using the procedure of Parris et al. (21) and a Spectra-Physics (San Jose, CA) model 8700XR pumping system, model 8750 injection system containing a 10-μl sampling loop, model 4240 data system, and an ISCO (Lincoln, NE) model V^4 absorbance detector set at 280 nm with a detector gain of 0.1 AUFS (absorbance units full scale). Using the time of initiation (time 0) as the control, the change in the sum of the standardized peak areas of BSA, β-LG, and α-LA was used to monitor denaturation over time. The extinction coefficients used for BSA, β-LG, and α-LA were 6.6, 9.3, and 20.6, respectively (14). The elution volumes were 7.34, 8.93, 9.92, and 10.78 ml for SA, BSA, β-LG, and α-LA, respectively. The molecular mass of the SA fraction was >400,000.

Each of the five aliquots of heated WPC65-NDM mixtures was diluted to 15 mg of protein/ml of deionized water and isoelectrically precipitated at pH 4.6 with 1 *M* HCl. After 30 min of stirring at 20°C, the isoelectrically precipitated samples were centrifuged (Sorvall® RC-5B centrifuge; DuPont Company) at 1000 × *g* for 30 min at 20°C. The supernatant was filtered (0.45 μm) and analyzed at room temperature (23°C) using SEC as described previously.

Aliquots (1.09 g) of the heated WPC65 and a mixture of Ca-Na caseinate were removed at 0, 30, 60, 90, and 120 min, diluted, isoelectrically precipitated, and centrifuged for analysis using SEC as described previously.

Ultrafiltration

For analysis of the SA fraction by SDS-PAGE, the sample was prepared as for SEC; however, the supernatant was not filtered. The concentration of SA in the supernatant was increased by ultrafiltration (Centriprep-100 concentrator; 100,000 molecular mass cutoff; Amicon, Inc., Beverly, MA). The filtrate was decanted, and the retentate (3.73 ± 0.34% TS) containing the SA fraction was retained for further analysis.

SDS-PAGE

The protein composition of the retentate, containing the SA, was determined using SDS-PAGE, under reducing and nonreducing conditions, with a PhastSystem™ (Pharmacia, Piscataway, NJ) using an 8 to 25% gradient PhastGel® as described by Parris et al. (22). Deionized water replaced β-mercaptoethanol in the protocol for nonreduced samples. The protein bands were stained with Coomassie blue. The stained gels were dried, and the intensities of the bands were scanned (ImageQuaNT™; Molecular Dynamics, Inc., Sunnyvale, CA).

TEM

The proteins in all samples were chemically crosslinked by addition of 10% aqueous solution of glutaraldehyde to a final concentration of 1% (wt/vol) with gentle mixing, followed by storage at 4°C. Next, the crosslinked protein samples were encased in 2%

agarose (Sea Plague, FMC, Rockland, ME), washed in 0.1 *M* Na cacodylate buffer (pH 7.2) for 30 min, and then immersed in a solution of 2% osmium tetroxide in 0.1 *M* cacodylate buffer for 2 h. Samples were washed in distilled water, dehydrated in a graded series of ethanol solutions, and embedded in an epoxy-resin mixture (Embed-812; Electron Microscopy Sciences, Ft. Washington, PA). Thin sections of embedded samples (60 to 70 nm thick; silver and gray interface color) were cut, stained with solutions of 2% uranyl acetate and lead citrate (25), and examined with an electron microscope (Philips CM 12; Philips Electronic Instruments Co., Mahwah, NJ) operated at 60 kV. Brightfield images of selected areas of thin film sections were recorded on photographic film (type 4489; Eastman Kodak Co., Rochester, NY) at instrumental magnifications of 3000× and 28,000×.

At 0 and 60 min, 3-g aliquots were removed from the heated WPC65 and NDM solutions and the WPC65-NDM mixture for analysis using TEM as described previously. The control for each treatment was time 0.

At 0 and 60 min, 3-g aliquots of both the WPC65 and the Ca-Na caseinate solutions were removed for TEM analysis as described previously. The appearance of the sample was noted when aliquots were removed; the control for each treatment was time 0.

RESULTS AND DISCUSSION

Effect of Ca^{2+}

SEC. Initially, the elution volume for the SA peak, using SEC, was approximately 7 ml for all adjusted WPC65 solutions at both 66 and 71°C. Over time and with increasing Ca^{2+} concentration, the elution volume decreased, indicating an increase in size of the SA. Based on molecular mass standards run on the SEC column, the estimated molecular mass of the SA peak was >400,000. At both temperatures, the SA peak on the SEC chromatogram, as a percentage of total area, increased as Ca^{2+} concentration decreased (Table 2). When the WPC65 solutions adjusted for Ca^{2+} were heated at 66°C for up to 120 min, the amount of SA, as a percentage of sample weight, also increased as Ca^{2+} decreased. At 71°C, however, the amount of SA, as a percentage of sample weight, increased for the first 30 min but then decreased over the remainder of the heat treatment. This decrease coincided with an increase in insoluble precipitate.

SDS-PAGE. After the WPCa 0, 3, 6, and 9 solutions were heated at 66°C for 120 min, diluted with buffer, and centrifuged, the supernatant was ultrafiltered; then the composition of the retentate containing SA was determined using SDS-PAGE (Figure 1). The difference in the BSA, β-LG, and α-LA bands between the nonreduced and reduced SA fraction reflects the composition of the SA (Figure 1; compare lanes 2, 4, 6, and 8 with lanes 3, 5, 7, and 9). The presence or absence of β-mercaptoethanol in the sample preparation affected the migration of BSA on the SDS-PAGE gels; reduced BSA did not migrate as far as nonreduced BSA. In the reduced fractions, the aggregate dissociated, and the relative intensities of the BSA, β-LG, and α-LA bands were altered, indicating that the aggregate was held together primarily by disulfide bonds.

The reduced and nonreduced lanes were scanned, and the band intensity was analyzed using densitometry; the percentage of total area for the SA, BSA, β-LG, and α-LA bands was calculated (Table 3). Simi-

TABLE 2. Soluble aggregates[1] in heated solutions of whey protein concentrate containing 65% protein and increasing concentrations of Ca^{2+}.

Whey protein solution	66°C, 120 min	71°C, 30 min
(m*M* Ca)	(g/100 g)	
0	9.8	39.4
3	8.3	16.6
6	4.2	8.2
9	2.2	2.4

[1]Percentage of total area of size exclusion chromatogram.

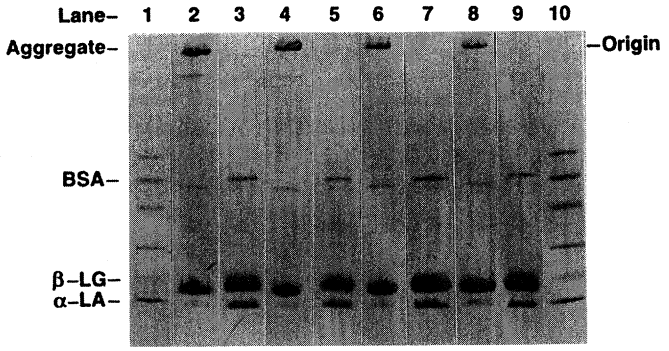

Figure 1. The SDS-PAGE profile of the retentate fraction, reduced or not reduced, following ultrafiltration of the supernatant. The supernatant was obtained from whey protein concentrate (65% protein) solutions dialyzed against simulated milk ultrafiltrate containing 0, 3, 6, or 9 m*M* Ca^{2+} (WPCa 0, 3, 6, and 9, respectively). The solutions were then heated at 66°C for 120 min, diluted, and centrifuged (13,000 × *g* for 30 min at 4°C). Lanes 1 and 10, molecular mass standards (reduced): lanes 2 and 3, WPCa 0; lanes 4 and 5, WPCa 3; lanes 6 and 7, WPCa 6; and lanes 8 and 9, WPCa 9. Samples are reduced in lanes 3, 5, 7, and 9 and not reduced in lanes 2, 4, 6, and 8.

TABLE 3. Composition of the unreduced and reduced retentate[1] following a densitometric scan of the SDS-PAGE gel.

Retentate	Aggregate		BSA		β-LG		α-LA		β-LG:α-LA
	(%)[2]								
	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	$\bar{X}$	SE	
Nonreduced									
WPCa 0	52.2	0.3	4.4	1.1	37.4	1.4	6.0	0.5	6.25:1
WPCa 3	40.8	0.7	4.6	0.9	47.5	1.1	7.1	0.4	6.71:1
WPCa 6	24.2	1.4	6.1	1.0	60.2	2.3	9.5	0.6	6.36:1
WPCa 9	19.5	0.7	7.9	0.1	62.5	0.5	10.1	1.2	6.19:1
WPCa C	13.8	0.8	7.8	0.8	69.3	1.3	9.1	0.9	7.65:1
Reduced									
WPCa 0	0		8.5	1.1	62.7	2.0	28.8	1.8	2.17:1
WPCa 3	0		10.0	0.2	65.2	1.5	24.9	1.9	2.62:1
WPCa 6	0		8.2	0.6	69.6	0.7	22.2	0.8	3.14:1
WPCa 9	0		7.9	0.8	71.3	1.3	20.8	0.6	3.42:1
WPCa C	0		8.3	0.7	72.0	1.2	19.7	0.5	3.65:1

[1]The retentate was obtained following ultrafiltration of the supernatant from whey protein concentrate (WPC; 65% protein) solutions that had been dialyzed against simulated milk ultrafiltrate containing 0, 3, 6, or 9 m*M* Ca^{2+} (WPCa 0, 3, 6, or 9, respectively). The control, WPCa C, contained 13.56 m*M* Ca^{2+}. The solutions were heated at 66°C for 120 min, diluted, and centrifuged (13,000 × *g* for 30 min at 4°C).

[2]Percentage of the total area scanned for a given sample; n = 3.

lar to findings using SEC, the amount of SA increased as Ca^{2+} concentration decreased; however, much less SA was detected by SEC than by SDS-PAGE (Tables 2 and 3). This result can probably be attributed to the nonspecific binding of the dye to the aggregated proteins, which would have enhanced the amount of SA. In addition, large aggregates tend to scatter light when detected by UV absorbance, which, however, would make the aggregate peak appear larger. Because not all low molecular mass protein is removed by ultrafiltration, bands for nonaggregated BSA, β-LG, and α-LA were present in the nonreduced retentate fractions. The percentage of total area for BSA, β-LG, and α-LA increased as Ca^{2+} increased in the unreduced fractions. However, no trend was evident for the β-LG:α-LA ratio.

The increase in band intensity between the nonreduced and reduced lanes results from the dissociation of the SA to its constituent proteins. The Ca^{2+} concentration affected the amount and composition of the SA formed. As a percentage of total area, the amount of β-LG decreased, and the amount of α-LA increased, as Ca^{2+} decreased; the β-LG:α-LA ratio decreased. Therefore, both electrostatic and disulfide interactions were apparently involved in the formation of SA.

Whey Protein Denaturation

Using time 0 as the control for each solution, the change in the sum of the standardized peak area of BSA, β-LG, and α-LA over time was used to determine whey protein denaturation over time. All of the samples that had been adjusted for Ca^{2+} were >65% denatured after 30 min and >89% denatured after 120 min at 71°C. After 120 min at 66°C, however, they were all <27% denatured. The denaturation temperatures were 64°C for BSA, 62°C for α-LA, and 78°C for β-LG (2); however, the temperature at which the individual whey proteins denature can vary with system conditions (2, 32). Because both temperatures used in this study were between the denaturation temperatures of BSA and β-LG, a similar amount of denaturation was expected at the two temperatures; however, much more whey protein denaturation occurred at 71°C than at 66°C.

Denaturation of the heated WPC65 solution occurred more rapidly and completely at 71°C than at 66°C (Table 4). The addition of low heat NDM to the WPC65 solution to obtain 36% TS reduced the amount of whey protein denaturation after heat treatment at 66 and 71°C for 120 min by approximately 75%. Because it was not clear how NDM protected whey proteins in WPC against thermal denaturation, the effect of adding a mixture of Ca-Na caseinate to WPC solution was investigated. Unlike the addition of low heat NDM, however, the addition of the caseinate mixture to the WPC65 solution did not reduce the amount of whey protein denaturation over time. After 120 min at 71°C, the whey proteins in the WPC65 solution (17% TS) were 9% less denatured than in the WPC65-caseinate mixture.

TABLE 4. Effects of temperature and time on whey protein denaturation for whey protein concentrate containing 65% protein (WPC65; 17% TS), WPC65-low heat NDM[1] (36% TS), or a mixture of WPC65, Ca caseinate, and Na caseinate (20.5% TS).[2]

Time	66°C	71°C
(min)	(%)	
WPC65 (17% TS)		
0	. . .	. . .
30	15.9	59.3
60	17.6	68.0
90	19.8	76.8
120	26.5	83.2
WPC65-NDM (36% TS)		
0	. . .	. . .
30	0.3	7.2
60	1.2	8.5
90	6.7	16.8
120	6.9	19.8
WPC65-caseinate (20.5% TS)		
0	. . .	. . .
30	12.3	81.2
60	26.0	85.0
90	33.6	87.7
120	46.1	90.4

[1]Addition of NDM to WPC (17%) solution to give 27% TS.

[2]The Ca-Na caseinate was prepared by adding 8 mmol of Ca^{2+}/g of 7.6% Na caseinate; WPC65 was added to give 20.5% TS.

Similar to the findings in this study, Donovan and Mulvihill (4) and Parris et al. (20) found that protein aggregation increased as Ca^{2+} in rennet whey increased. However, Hillier et al. (8) found, at much lower whey protein concentrations, that the denaturation of α-LA and β-LG in heated (70 to 133°C) cheese whey slowed when the Ca^{2+} concentration was increased to 0.4 mg/ml (~10 m*M*), which could indicate that Ca^{2+} prevents unfolding but promotes aggregation.

Formation of Insoluble Precipitate

The decrease in SA (Table 2) coincided with the increased formation of insoluble precipitate. The amount of insoluble precipitate that formed at 71°C over 120 min more than tripled; however, addition of low heat NDM to the WPC65 solution significantly reduced the amount of insoluble precipitate at both temperatures (Table 5). Addition of the Ca-Na caseinate to the WPC65 solution also reduced the formation of insoluble precipitate at 71°C but not to the same extent.

TEM

Studies were undertaken using TEM to understand further the role of Ca^{2+} in the formation of SA and insoluble precipitate. To analyze the crosslinked structure of the entire samples, aliquots of the heated mixtures were not centrifuged prior to fixation with glutaraldehyde. Because we fixed the entire sample, rather than a pellet from sample centrifugation of ultracentrifugation as is commonly done (12, 23), both the soluble and insoluble protein complexes were crosslinked. The native whey proteins were too small to be seen by TEM (12). Numerical values for those particles that were identical or similar to those in the figures were obtained from photographic prints at a scaled magnification of ca. 70,000, where 0.7 mm on a micrometer ruler measured 10 nm.

After the WPCa 0 solution was heated at 71°C for 60 min, the aggregates formed were smaller and less dense than those formed when WPCa 3 or 6 was heated, as shown in Figure 2 (A, B, and C). Images of the WPCa 9 solution showed the formation of large, densely packed, multi-aggregate complexes that ranged from about 500 to 1200 nm in size (Figure 2D). Although the visual appearance of the solutions and the range in aggregate size on images of the WPCa 9 solution and the WPC65 solution (17% TS) without Ca^{2+} adjustment were similar, the aggregates formed in the solution adjusted for Ca^{2+} were more densely packed (Figure 2D and 3A).

Patocka et al. (23) studied the microstructure of acid permeates, decalcified acid permeates, and ren-

TABLE 5. Effect of temperature and time on formation of insoluble precipitates in whey protein concentrate containing 65% protein (WPC65) (17% TS), or WPC65 and low heat NDM (36% TS),[1] or a mixture of WPC65 and Na caseinate to which Ca^{2+} had been added (20.5% TS).[2]

Time	66°C	71°C
(min)	(g/100 g)	
WPC65 (17% TS)		
0	19.6	20.5
30	25.6	54.1
60	29.2	58.7
90	30.7	61.0
120	33.6	63.9
WPC65-NDM (36% TS)		
0	27.4	30.7
30	30.4	32.9
60	32.2	35.1
90	31.8	37.6
120	32.1	39.3
WPC65-caseinates (20.5% TS)		
0	34.1	49.7
30	43.6	71.5
60	51.0	68.3
90	55.8	78.5
120	57.4	81.2

[1]Addition of NDM and WPC (17%) to give 27% TS.

[2]The caseinate was prepared by adding 8 mmol of Ca^{2+}/g of 7.6% Na caseinate; WPC65 was added to give 20.5% TS.

net whey ultrafiltration retentates heated at 93°C for 30 min following ultracentrifugation (95,000 × g for 2 h). In all cases, images of the pellet showed the protein aggregates to be more spherical and more densely packed than those in the WPC65 solutions adjusted for Ca^{2+} in this study. The Ca-binding interaction of β-LG can lead to increased hydrophobicity (11). Therefore, changes that were induced by Ca^{2+} that affect the thermal stability of the system could have affected whey protein denaturation and aggregation in our system.

Unlike the WPC65 solution, the low heat NDM solution remained liquid and did not thicken during heat treatment. Images showed that the casein micelles ranged in size from about 50 to 350 nm and had filamentous appendages after heating (Figure 3B). The WPC and NDM mixture thickened after 60 min (71°C), but did not form visible particulates. Although whey protein aggregates were present, they were smaller (50 to 1000 nm) and were loosely associated with one another (Figure 3C). The image of the WPC65-NDM mixture was more similar to that of low heat NDM than to WPC65.

Schmidt (26) has observed threadlike material in micrographs of thin sections of plastic-embedded samples of casein submicelles and cautioned about the use of organic solvents for dehydration. According to Tessier and Rose (29), both heating and concentration of skim milk can cause Ca phosphate precipitation and therefore alter the Ca^{2+} equilibrium. These

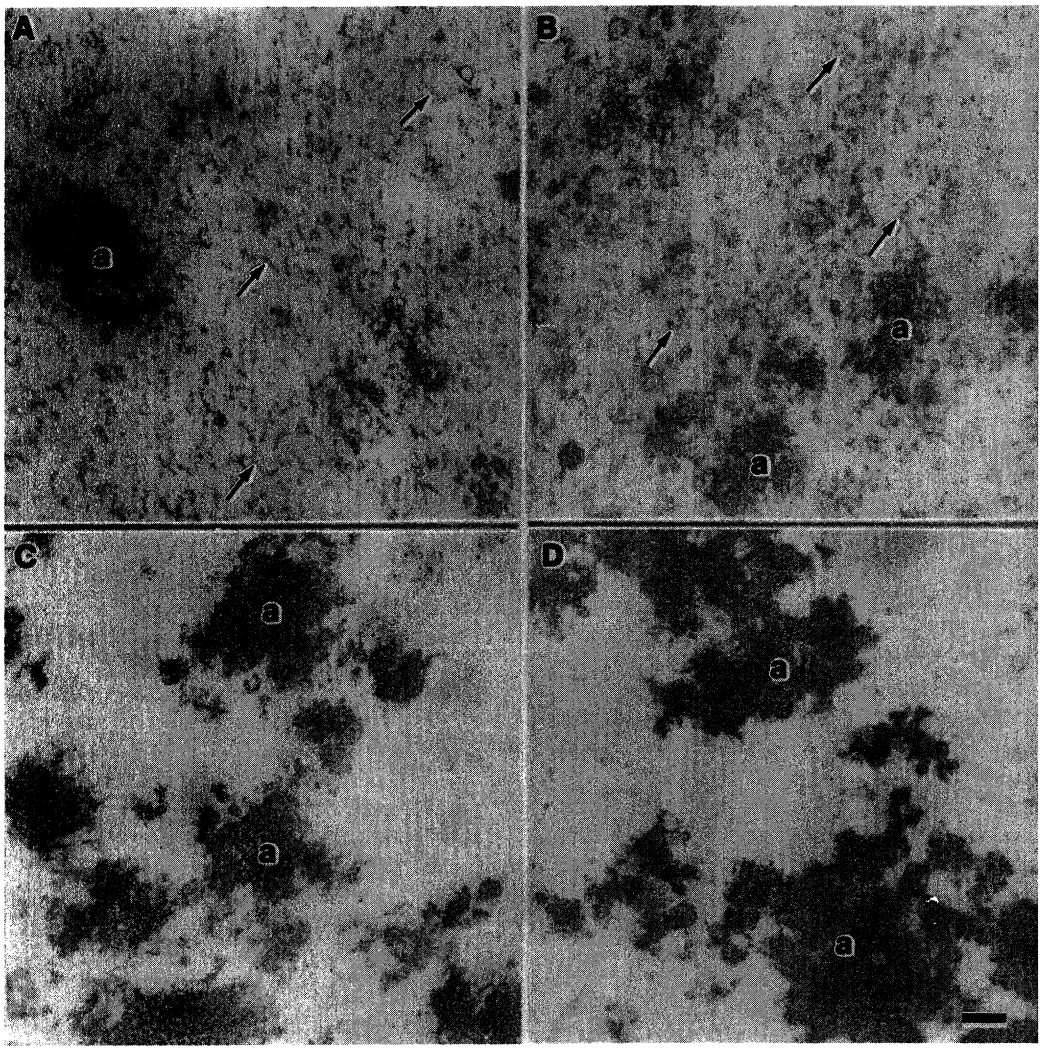

Figure 2. Transmission electron micrographs of whey protein concentrate (65% protein) solutions dialyzed against simulated milk ultrafiltrate containing 0 (A), 3 (B), 6 (C), and 9 (D) mM Ca^{2+} heated at 71°C for 60 min. Arrow indicates filamentous aggregate; letter a indicates nonfilamentous aggregates. Scale bar is 100 nm.

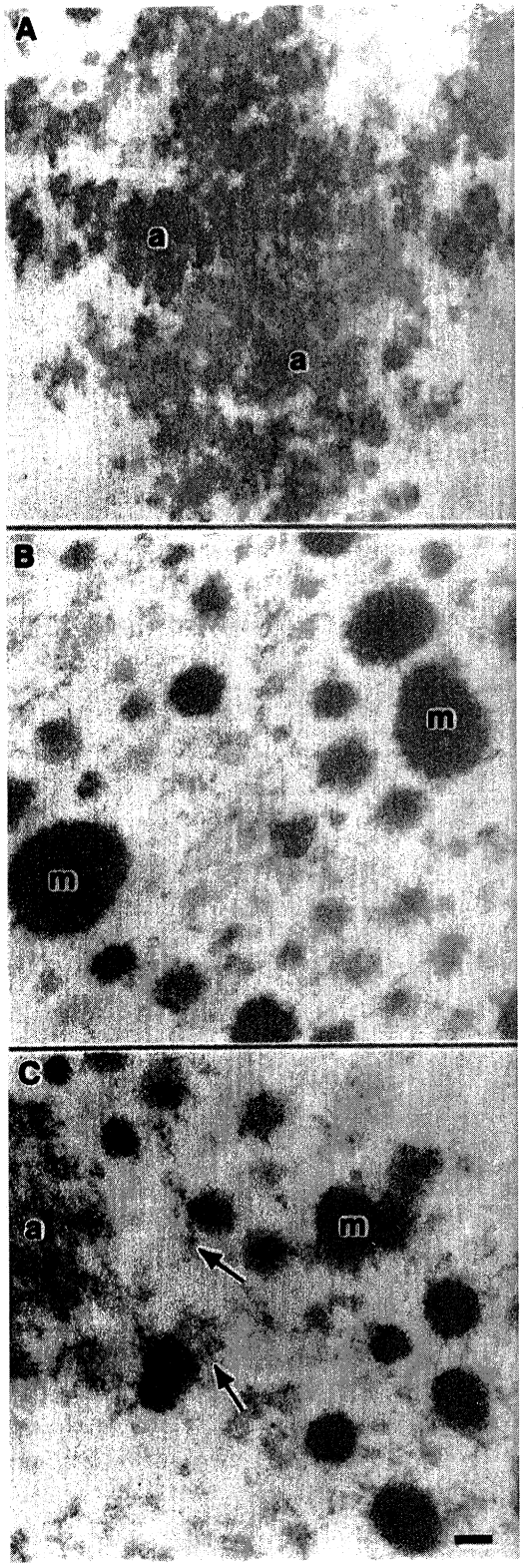

Figure 3. Transmission electron micrographs of 65% protein (WPC65; A), low heat NDM (B), and WPC65 and low heat NDM mixtures (C) heated at 71°C for 60 min. The arrows indicate micellar appendages; a indicates whey protein aggregate, and m indicates casein micelles. Scale bar is 100 nm.

changes, which can occur when low heat NDM is added to the WPC65 solution, could also contribute to the reduction in whey protein denaturation over time (3). Increased lactose concentration can slow the denaturation of α-LA and β-LG (8), and decreased colloidal Ca phosphate can improve the heat stability of milk (6). Morr and Josephson (16) proposed that, in addition to the more specific thiol-disulfide reaction between β-LG and κ-CN, the whey proteins in skim milk were stabilized against heat-induced gross aggregation by forming Ca^{2+}-dependent linkages with the casein micelles.

Large casein micelles, two to three times larger than native casein micelles, were interspersed with smaller, nonmicellar aggregates in the heated Ca-Na caseinate solution (Figure 4A and C). The micrograph of the WPC65-caseinate mixture (Figure 4B and D) was not comparable with that of either low heat NDM or the WPC65-NDM mixture. Although some micelle-like aggregates were present, distinct casein micelles were not. The whey proteins appeared to aggregate with each other as well as with the casein.

During the manufacture of Na caseinate, the Ca:phosphate ratio in the casein micelle is compromised, and the micelles are destroyed. Although PIPES buffer was used and Ca^{2+} was added to facilitate stable casein micelle formation in this study, the individual casein in the reformed casein micelles were less heat stable and might not have behaved as they would as part of the more stable native micelle. According to Singh and Fox (27), changes in soluble Ca^{2+} and phosphate can affect κ-CN dissociation. Because κ-CN, which is known to form complexes with β-LG, is present on the surface of micelles and submicelles, κ-CN could also influence the formation of protein complexes in the WPC65 caseinate mixture.

The composition and formation of the protein aggregates formed in the WPC65 caseinate mixture need to be elucidated further using more specific techniques, such as immunolabeling. The distribution of the whey proteins and casein as well as the ratio of whey protein to casein in the protein aggregates formed also need to be investigated because they may impart unique functional properties to the mixture.

CONCLUSIONS

As Ca^{2+} concentration decreased, the WPC65 solutions that were adjusted for Ca^{2+} were less viscous and formed more SA and less insoluble precipitate. As the SA decreased in size with decreasing Ca^{2+} concentration, the SA became more soluble and did not

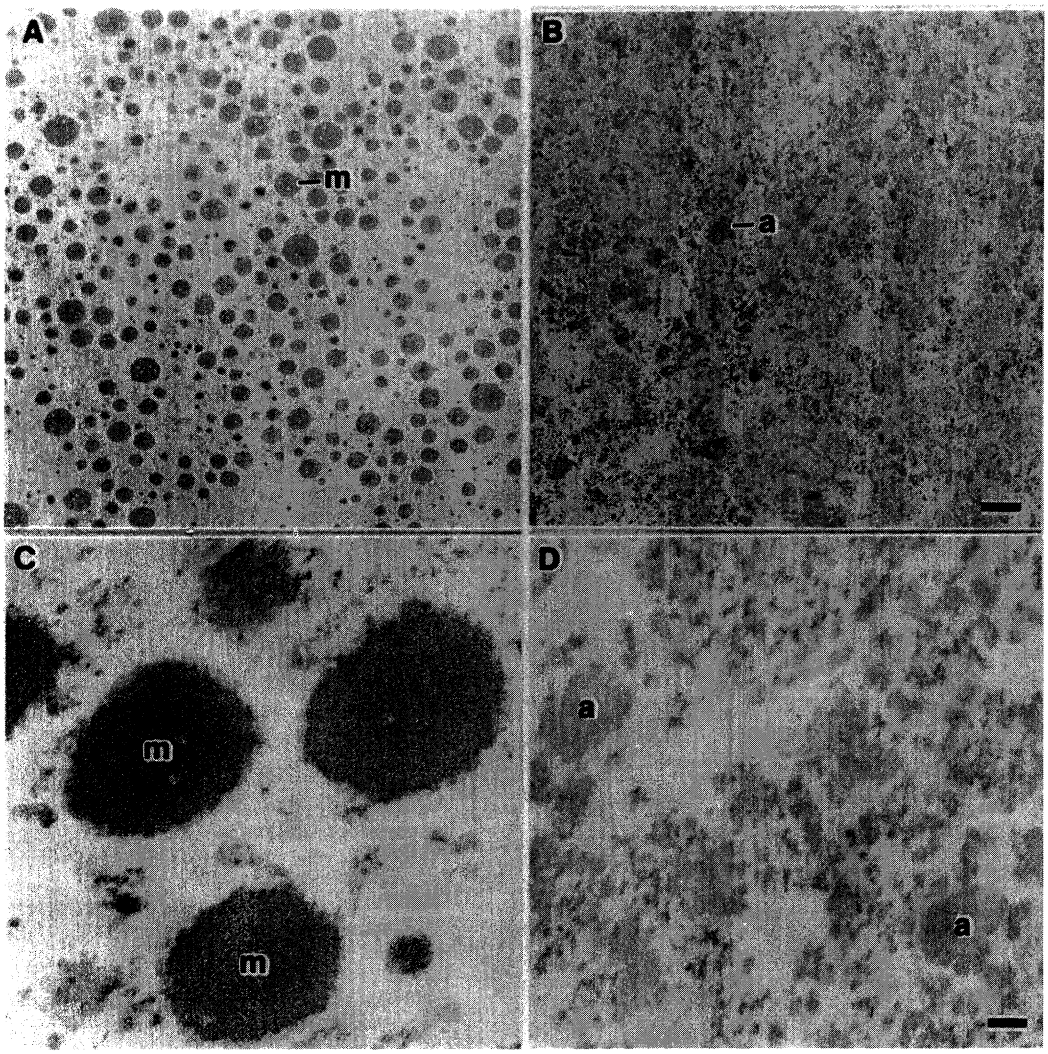

Figure 4. Transmission electron micrographs of Na caseinate to which Ca^{2+} had been added (A and C) and 65% protein: WPC 65-caseinate mixture (B and D) heated at 75°C for 60 min. The m indicates casein micelles, and the a indicates whey protein aggregates. Scale bars are 1 μm (A and B) and 100 nm (C and D).

readily precipitate. The protein aggregates in electron micrographs of the WPC65 solutions that were adjusted for Ca^{2+} were also smaller and less densely packed as the Ca^{2+} concentration decreased. More α-LA relative to β-LG associated with the SA as Ca^{2+} concentration decreased.

Although TS increased, less whey protein denaturation occurred when low heat NDM was added to the WPC65 solution than when the WPC65 (17% TS) solution alone was used; the same was not true when a mixture of Ca-Na caseinate was added to the solution. The electron micrograph of the WPC65-NDM mixture demonstrated that the aggregates formed were smaller and less dense and the micellar appendages were more compact than in the solutions of WPC65 (17% TS) and low heat NDM (27% TS). Electron micrographs of the WPC65-caseinate mixture indicated that the casein micelles dissociated, and the whey proteins and caseins aggregated, when the mixture was heated.

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