Factors Affecting the Denaturation and Aggregation of Whey Proteins in Heated Whey Protein Concentrate Mixtures[1]

C. M. HOLLAR and N. PARRIS
USDA, Agricultural Research Service
Eastern Regional Research Center
Philadelphia, PA 19118

A. HSIEH
Wyeth Nutritionals Inc.
Milton, VT 05468

K. D. COCKLEY
Wyeth-Ayerst International Inc.
Philadelphia, PA 19101

ABSTRACT

Whey protein concentrate (65% protein) mixtures, alone or in the presence of NDM, were heated at 66 and 71°C for up to 120 min to yield 16, 25, and 35% TS to simulate the formulation of a nutritional product. Addition of low heat NDM to a whey protein concentrate mixture to yield 35% TS resulted in 6 and 20% denaturation after 120 min and 27 and 83% in the whey protein concentrate mixture (16% TS) at 66 and 71°C, respectively. The effects of Ca and pH on whey protein denaturation and aggregation (66°C) were also studied. A whey protein concentrate mixture (16% TS) dialyzed against simulated milk ultrafiltrate containing 0 to 9 m*M* Ca and heated (66°C); the mixture was progressively more denatured and formed less soluble aggregate and more insoluble precipitate as the Ca increased. When the whey protein concentrate mixture (16% TS) was heated (66°C) at increasing pH (5.8 to 7.0), whey protein denaturation and insoluble precipitate increased. The α-LA denatured more extensively than β-LG at 66 and 71°C.

(**Key words**: denaturation, whey protein concentrate, nonfat dry milk)

Abbreviation key: **SMUF** = simulated milk ultrafiltrate, **WPC** = whey protein concentrate powder, **WPC65** = WPC containing 65% protein.

INTRODUCTION

The advent of improved and more cost-effective processing technologies and production procedures (i.e., ultrafiltration, reverse osmosis, and electrodialysis) have made possible a dramatic increase in production of whey protein concentrate (**WPC**). However, WPC produced using different procedures may not demonstrate the same functionality or stability in standard formulations (6, 20, 31).

Of the whey proteins, α-LA has the lowest denaturation temperature, 62°C, followed by BSA at 64°C, Ig at 72°C, and β-LG at 78°C (2). β-Lactoglobulin denatures more quickly than α-LA at 85°C and at other temperatures in heated milk and whey systems, and the overall effect of heating is greater on β-LG than on α-LA (8, 9, 11, 18). Because α-LA can renature upon cooling, it is considered to be the most thermostable whey protein (30). However, de Wit and Klarenbeek (6) found that, although purified α-LA renatured upon cooling, the α-LA present in heated WPC did not.

Whey protein denaturation 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, aggregate, and sediment through a

Received April 4, 1994.
Accepted October 18, 1994.

[1]Mention of brand or firm names does not constitute an endorsement by the USDA, Wyeth Nutritionals Inc., or Wyeth-Ayerst International Inc. over others of a similar nature not mentioned.

multiple-reaction process (12, 21). In various whey systems, Ca, TS, and pH affect whey protein denaturation (4, 5, 6, 7, 13, 14, 17, 24, 25, 26, 28, 32, 33). By helping to protect globular proteins from thermal denaturation, lactose also affects whey protein solubility following severe heat treatment (7).

Newstead et al. (23) found that recombined concentrated milks made from heat-treated milk were more heat stable than milks made from milk that was not heat treated. Forewarming to denature the whey proteins partially improves the heat stability of milk and whey systems. Addition of sweet WPC or dried WPC to milk during the manufacture of cultured dairy products to alter the casein:whey protein ratio was not detrimental to the heat stability of the milk (3, 15).

Although denaturation of whey protein, α-LA, and β-LG has been investigated in various milk and whey systems, the effect of heat on whey protein denaturation has not been determined when low heat NDM is added to WPC powder containing 65% protein (**WPC65**) to simulate the formulation of a nutritional product. We investigated the effects of heating WPC65 in the presence of low heat NDM and simulated NDM (unadjusted for mineral or soluble salt contents) at 66 and 71°C and at 16, 25, and 35% TS. The effects of Ca and pH on whey protein denaturation and aggregate formation at 66°C are also reported.

MATERIALS AND METHODS

Materials

Three different manufacturing lots of sweet whey WPC65, designated as samples A, B, and C, and one low heat NDM sample were obtained from the Vermont Whey Company (Milton, VT). Proximate analyses of these samples were supplied by the manufacturer (Table 1). Approximately 15% (wt/wt) of each of the three samples remained insoluble following isoelectric precipitation and centrifugation (1000 × *g* at 20°C for 30 min). Deionized water was purified (Polisher I HPLC Laboratory Reagent Grade Water System; Continental Water Systems Corp., San Antonio, TX) prior to use. Simulated NDM (29% casein, 7% whey protein, and 52% lactose) was prepared using WPC65 (sample A), sodium caseinate (Sigma Chemical Co., St. Louis, MO), and β-D-lactose (Eastman Fine Chemicals, Rochester, NY) to determine which components of low heat NDM affected the heat stability of the whey proteins. In this study, the mineral and soluble salt composition was not adjusted. Simulated whey protein (67.9% β-LG, 25.6% α-LA, and 6.5% BSA) was prepared using purified whey proteins (Sigma Chemical Co.). Simulated milk ultrafiltrate (**SMUF**) was prepared using the procedure of Jenness and Koops (16) as described by Parris et al. (25). Modified SMUF, containing 0, 3, or 6 m*M* Ca, was also prepared. A .25 *M* sodium phosphate buffer (pH 6.7) was prepared for sample dilution and size exclusion chromatography (26).

TABLE 1. Proximate composition of low heat NDM and whey protein concentrate (WPC, 65% protein) powder samples.

	NDM	WPC Samples A	B	C
	(%)			
Protein	36.40	67.30	66.64	69.54
Fat	.77	5.81	5.66	2.29
Ash	7.97	4.52	5.07	4.88
Moisture	4.10	3.95	4.05	4.38
Lactose	52.32	22.40	21.30	20.10

Sample Preparation

The WPC65 was dissolved in deionized water (71°C) to obtain 16, 25, or 35% TS prior to heating treatment. Low heat or simulated NDM was added to the WPC65 mixture (16% TS) to yield 35% TS. To alter the soluble Ca content of the mixture, a 25-ml aliquot of the WPC65 sample A mixture (16% TS) was dialyzed against 500 ml of SMUF containing 0, 3, 6, or 9 m*M* Ca at pH 6.6 and 5°C with buffer changes after 5 h and after an additional 15 h, for a total of 24 h. During dialysis, TS were reduced from 16% to approximately 13% for all amounts of Ca. The pH of the WPC65 mixture (16% TS) was adjusted at 20°C from pH 6.4 to 7.0, 6.8, or 6.6 with 1 *M* NaOH or pH 6.2 or 5.8 with 1 *M* HCl prior to heating.

Heat Treatment

With continuous stirring, 25 to 50 ml of each WPC65 mixture were heated at 66 or

71°C for up to 120 min in a covered flask to simulate processing conditions. The samples adjusted for Ca or pH were heated at 66°C. Aliquots (1.1 g) were removed at 0, 30, 60, 90, and 120 min and cooled to 20°C in an ice bath. The appearance of the sample, i.e., increasing viscosity (visible thickening) and floc formation (visible particulates), was noted when aliquots were removed. The heat treatment proceeded for 120 min or until the mixture became too viscous to stir.

Denaturation, Soluble Aggregate, and Insoluble Precipitate

Whey protein denaturation was determined by size exclusion chromatography. After cooling, aliquots of WPC65 mixtures were diluted to 15 mg of protein/ml of deionized water. The diluted aliquots were isoelectrically precipitated at pH 4.6 with 1 *M* HCl and centrifuged (1000 × *g* at 20°C for 30 min). Aliquots of samples adjusted for pH or Ca were diluted to 15 mg of protein/ml of buffer for size exclusion chromatography and centrifuged (13,000 × *g* at 4°C for 30 min), but not isoelectrically precipitated. The supernatant was diluted 1:2 (vol/vol) with buffer, filtered (.45 μm), and analyzed at 25°C with size exclusion chromatography on a Zorbax GF-250 column (DuPont, Wilmington, DE) using the procedure of Parris et al. (26) 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 a ISCO (Lincoln, NE) model V^4 absorbance detector set at 280 nm with a detector gain of .1 absorbance units full scale. The percentage of denaturation (% D) was calculated from the sum of the standardized peak areas (SPA) for the heated (H) whey proteins (BSA, β-LG, and α-LA) and compared with those of the control (C). The control for each treatment was time 0 (when powder ingredients dissolved or samples reached 66 or 71°C).

$$\% \text{ D} = \left[\frac{(\Sigma \text{SPA}_\text{C} - \Sigma \text{SPA}_\text{H})}{\Sigma \text{SPA}_\text{C}}\right] \times 100.$$

The extinction coefficients used for BSA, β-LG, and α-LA were 6.6, 9.3, and 20.6, respectively (19).

The amount of soluble protein in the sample was calculated as the difference between the total protein and insoluble precipitate. The amount of soluble aggregate in the soluble protein was calculated from the percent area of the aggregate peak (estimated molecular weight >400,000) relative to the total area of the chromatogram.

The insoluble precipitate was weighed following centrifugation (13,000 × *g* at 4°C for 30 min) and lyophilization. Insoluble precipitate, as a percentage of sample weight, was monitored over time in samples adjusted for pH and Ca.

Each analysis was repeated three times. The percentage of whey protein denaturation and the amount of soluble aggregate and insoluble precipitate as a percentage of sample weight for each sample treatment were accurate to within 4.9, 23.4, and 12.7%, respectively. Variations in whey protein denaturation and insoluble precipitate formation both contributed to the variation in soluble aggregate formation.

Gel Electrophoresis

The WPC6 sample A and the supernatant, following isoelectric precipitation of WPC65 sample A (16% TS) heated at 71°C for 0, 30, 60, 90, or 120 min, were analyzed using SDS-PAGE, reduced, with a PhastSystem™ (Pharmacia, Piscataway, NJ) using an 8 to 25% gradient PhastGel as described by Parris et al. (27).

RESULTS AND DISCUSSION

Denaturation of 16% TS WPC65 Mixtures

The effects of temperature on whey protein denaturation for WPC65 samples A, B, and C (16% TS) are shown in Figure 1A; denaturation occurred more rapidly and completely at 71°C than at 66°C. For example, after 120 min, WPC65 samples A and B were 27 and 43% denatured at 66°C, and were 83 and 74% denatured at 71°C. Samples A and B did not flocculate during the 66°C heat treatment; sample C flocculated between 60 and 90 min. At 71°C, all three samples flocculated between 0 and 30 min; sample C became too viscous to stir after 60 min, and the heat treatment was aborted. According to the proximate analyses of these samples (Table 1), sample C contained approximately 2.3% fat and 70% protein, and

samples A and B contained approximately 5.7% fat and 67% protein. The higher fat content and lower protein content in samples A and B appeared to inhibit flocculant formation. Mulvihill and Kinsella (22) reported that protein:fat ratios might account for similar differences in gelation characteristics of WPC. Researchers have found that increases in protein concentration also promote the aggregation and subsequent gelation of whey proteins (20, 22, 23).

α-Lactalbumin denatured more extensively than β-LG at 66 and 71°C in WPC65 (Figure 1B), supporting previous findings that α-LA does not renature in complex food systems (6). At 66°C, α-LA and β-LG were 52 and 16% denatured, respectively, after heating for 120 min. After heat treatment for 60 min at 71°C, the α-LA in sample A appeared to be completely denatured; β-LG in the same sample

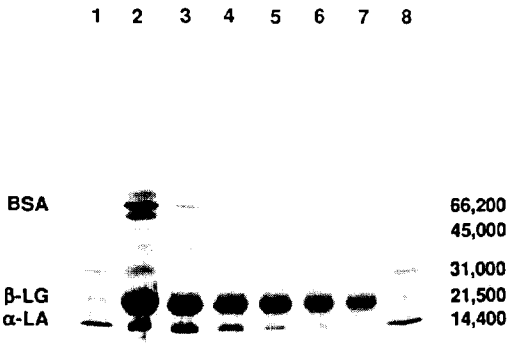

Figure 2. Electrophoretogram of SDS-PAGE (reduced) whey protein concentrate powder containing 65% protein (WPC65) sample A and WPC65 sample A supernatant. Lanes 1 and 8, molecular weight standards; lane 2, WPC65 sample A powder; and lanes 3 to 7, the supernatant of an isoelectrically precipitated WPC65 sample A mixture heated at 71°C for 0, 30, 60, 90, and 120 min, respectively.

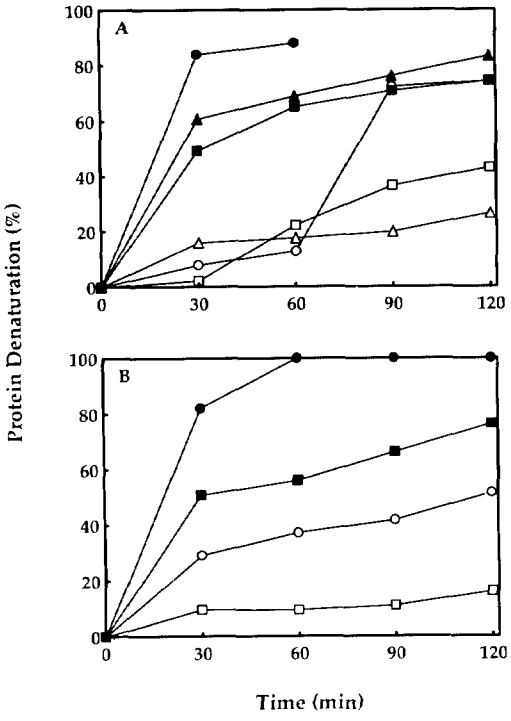

Figure 1. Denaturation of A) whey protein in whey protein concentrate powder (65% protein) mixtures (16% TS), samples A (▲), B (■), and C (●); and B) α-LA (●) and β-LG (■) in the sample A mixture (pH 6.4) at 66°C (open symbols) and 71°C (solid symbols).

was 76% denatured after heating for 120 min. Although flocculant formation coincided with increased β-LG denaturation, which is consistent with the idea that the free thiol group in β-LG is important in gel formation (7) and that the thermal behavior of WPC is controlled by the β-LG fraction (1), electrostatic and hydrophobic forces were also involved in whey protein denaturation (4, 8, 12, 21, 22, 25, 29, 32, 33).

Further confirmation that α-LA denatured more extensively than β-LG was demonstrated by SDS-PAGE, reduced, of the supernatant (isoelectrically precipitated) for WPC65 sample A (16% TS) heated at 71°C (Figure 2). Lane 2 is WPC65 sample A; the protein concentration is similar to that of the supernatant. In addition to BSA, β-LG, and α-LA, the WPC65 contained protein aggregates, lactoferrin, and heavy and light chain Ig, which were removed by isoelectric precipitation and centrifugation. The electrophoretic separation of the supernatant indicated that BSA was completely denatured after heat treatment for 30 min (lane 4), and α-LA steadily denatured with heating and was no longer present after 120 min (lane 7). The finding, in the present study, that β-LG denatured more slowly than α-LA during the heat treatment was contrary to the findings of a

number of other researchers (9, 10, 17, 18, 25, 26, 29) who investigated the thermal denaturation of β-LG and α-LA in a variety of milk and whey systems.

Increased TS

At 66°C, the whey protein denaturation profiles for 16 and 25% TS were similar (Figure 3A). At 35% TS, however, the whey proteins were >50% denatured after 30 min, the mixture became too viscous to stir, and the heat treatment was aborted. Whey protein denaturation for all three WPC65 samples (16% TS) was >26 and 74% at 66 and 71°C, respectively (compare Figures 1A and 3). At 71°C, an increase in WPC65 to 25% TS with additional WPC65 resulted in a mixture too viscous to stir after 15 min (Figure 3B). The WPC65 (35% TS) could not be dissolved without the mixture becoming too viscous to stir at 71°C.

Low Heat NDM and Simulated NDM

Addition of low heat NDM to the WPC65 inhibited whey protein denaturation at 66 and 71°C (Figures 3, A and B). After 120 min of heating, whey protein denaturation in all three samples was <10 and 25% at 66 and 71°C, respectively (sample A, Figure 3; samples B and C, not shown). Similarly, Jelen et al. (15) found that milks containing additional sweet WPC were stable upon heating, regardless of the casein:whey ratio, and Patocka et al. (28) found that skim milk blends containing sweet whey UF retentates were more stable than those containing acid whey UF retentates. The addition of low heat NDM to WPC65 decreased whey protein denaturation at 66 and especially at 71°C significantly more than did simulated NDM. Although whey protein denaturation was greater with the addition of simulated NDM, it was less than in the WPC65 mixtures (25 and 35% TS), suggesting that casein was involved in stabilizing the whey proteins. In addition, the same degree of whey protein denaturation did not occur in WPC65 mixtures containing low heat and simulated NDM. Differences in the Ca and soluble salt contents of these two mixtures, which were not adjusted in this study, could also have contributed to the heat stability of the whey proteins (Figure 3). During the manufacture of sodium caseinate, the Ca:P ratio, which helps to stabilize the casein micelle, is compromised. This result suggests and further supports the idea that the reduced casein micelle stability combined with the altered Ca content in the simulated NDM are important factors in stabilizing the mixtures of WPC65 and NDM. These data are consistent with the idea that whey proteins are stabilized against aggregation in skim milk by complexing with casein micelles through Ca linkages (21).

Ca

Alteration of the Ca content of the WPC65 sample A with SMUF of 0, 3, or 6 m*M* Ca resulted in <19% denaturation after heating at 66°C for 120 min and in 26% denaturation for the sample with 9 m*M* Ca. At lower Ca concentrations, more soluble aggregate (Figure 4A) and less insoluble precipitate (Figure 4B)

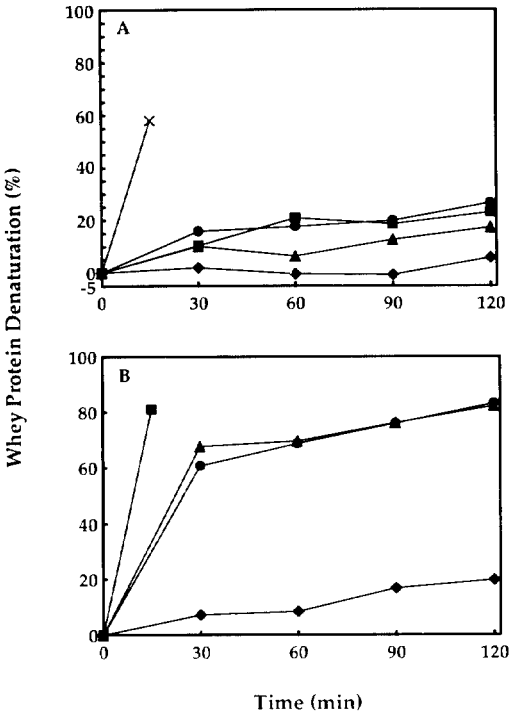

Figure 3. Effect of TS on whey protein denaturation of whey protein concentrate powder (65% protein) sample A mixtures heated at A) 66°C and B) 71°C. Whey protein denaturation at TS of 16 (●), 25 (■), 35 (×), 35 (simulated NDM) (▲), and 35% (low heat NDM) (♦).

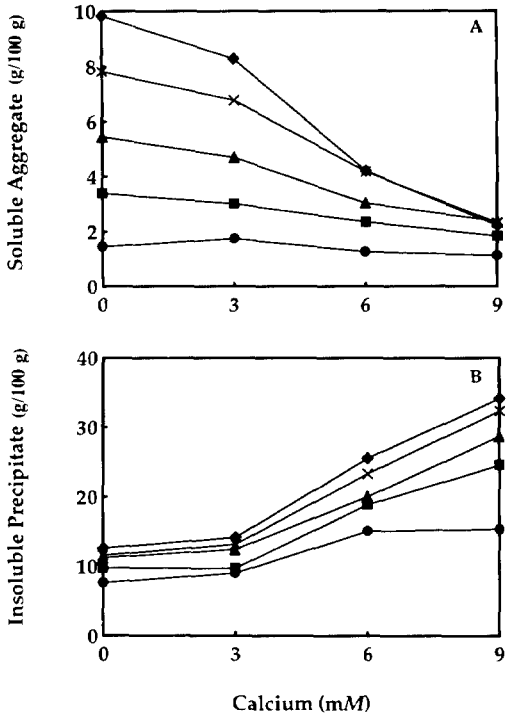

Figure 4. Formation of A) soluble aggregate and B) insoluble precipitate in whey protein concentrate powder (65% protein) sample A mixtures following dialysis with simulated milk ultrafiltrate containing 9, 6, 3, and 0 m*M* Ca and heat treatment at 66°C for 0 (●), 30 (■), 60 (▲), 90 (×), and 120 (♦) min.

were formed. The formation of soluble aggregate suggested a role for partial whey protein denaturation in stabilizing protein systems.

pH

Increasing the pH of the WPC65 sample A mixture (16% TS) above pH 6.6 significantly increased whey protein denaturation after heat treatment at 66°C (Figure 5A). Similar findings were reported by Donovan and Mulvihill (8). More soluble aggregate formed in WPC65 mixtures with pH ≥6.6 when heated 30 min (Figure 5B) as found previously (25). Heating of these mixtures (pH >6.6) for >60 min, however, decreased soluble aggregate but increased insoluble precipitate (Figures 5B and C), which suggested that a critical number or concentration of soluble aggregates is needed before larger, insoluble aggregates are formed. For mixtures adjusted to pH 6.8 and 7.0, flocculant was observed after 60 min; flocculant was not observed in the samples at pH 5.8 to 6.6. Harwalkar and Modler (13) and Li-Chan (17) also found that protein solubility of WPC decreased with increasing pH. However, de Rham and Chanton (4) found that, although

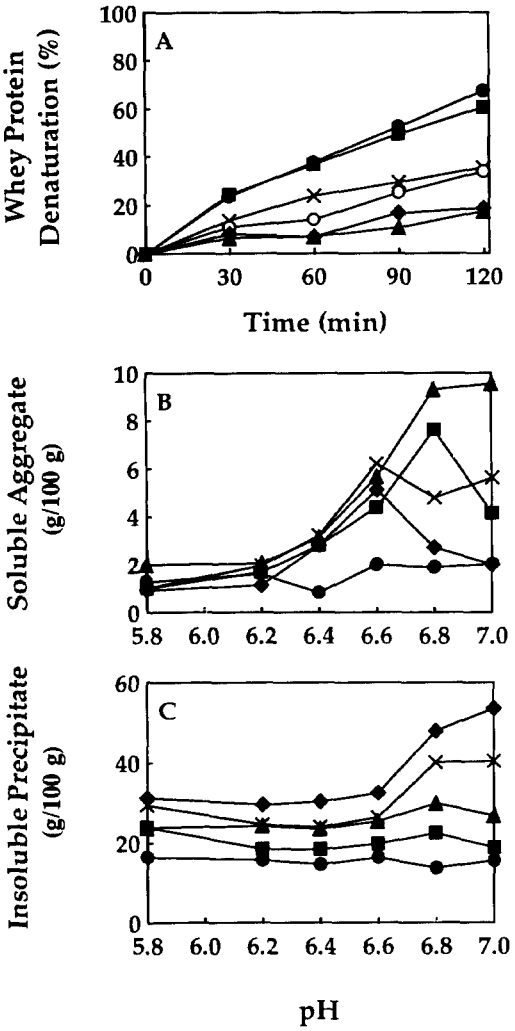

Figure 5. Effect of pH adjustment on whey protein concentrate powder (65% protein) sample A mixtures (16% TS, pH 6.4) adjusted to pH 7.0, 6.8, 6.6, 6.2, and 5.8 and heated at 66°C on A) whey protein denaturation at pH 7.0 (●), 6.8 (■), 6.6 (×), 6.4 (○), 6.2 (▲), and 5.8 (♦) over time and B) soluble aggregate formation and C) insoluble precipitate formation after 0 (●), 30 (■), 60 (▲), 90 (×), and 120 (♦) min.

protein solubility dropped near its isoelectric point, it did not drop near neutral pH unless the "unmasked" Ca concentration exceeded a set threshold that was also pH dependent.

CONCLUSIONS

Addition of low heat NDM limited whey protein denaturation in WPC65 mixtures. As the Ca content of the WPC65 mixtures decreased, more soluble aggregate and less insoluble precipitate formed. More whey protein denaturation occurred, and more soluble aggregate and insoluble precipitate formed, when the pH was increased. Over time, α-LA denatured more extensively than β-LG at 66 and 71°C.

REFERENCES

1 Bernal, V., and P. Jelen. 1985. Thermal stability of whey proteins—a calorimetric study. J. Dairy Sci. 68: 2847.

2 Brown, R. J. 1988. Fundamentals of Dairy Chemistry. 3rd ed. P. Wong, ed. Van Nostrand Reinhold, New York, NY.

3 Buchheim, W. W., K.-H. Peters, and W. Kaufman. 1986. Technologische, physikalisch-chemische und sensorische Aspekte von Milch und Sauermilch mit modifiziertem Casein/Molkenprotein-Verhältnis. Milchwissenschaft 41:139.

4 de Rham, O., and S. Chanton. 1984. Role of ionic environment in insolubilization of whey protein during heat treatment of whey products. J. Dairy Sci. 67:939.

5 de Wit, J. N. 1981. Structure and functional behaviour of whey proteins. Neth. Milk Dairy J. 35:47.

6 de Wit, J. N., and G. Klarenbeek. 1984. Effects of various heat treatments on structure and solubility of whey proteins. J. Dairy Sci. 67:2701.

7 de Wit, J. N., G. Klarenbeek, and M. Adamse. 1986. Evaluation of functional properties of whey protein concentrates and whey protein isolates. 2. Effects of processing history and composition. Neth. Milk Dairy J. 40:41.

8 Donovan, M., and D. M. Mulvihill. 1987. Thermal denaturation and aggregation of whey proteins. Ir. J. Food Sci. Technol. 11:87.

9 Elfagm, A. A., and J. V. Wheelock. 1977. Effect of heat on α-lactalbumin and β-lactoglobulin in bovine milk. J. Dairy Res. 44:367.

10 Elfagm, A. A., and J. V. Wheelock. 1978. Interaction of bovine α-lactalbumin and β-lactoglobulin during heating. J. Dairy Sci. 61:28.

11 Elfagm, A. A., and J. V. Wheelock. 1978. Heat interaction between α-lactalbumin, β-lactoglobulin, and casein in bovine milk. J. Dairy Sci. 61:159.

12 Eynard, L., S. Iametti, P. Relkin, and F. Bonomi. 1992. Surface hydrophobicity changes and heat-induced modifications of α-lactalbumin. J. Agric. Food Chem. 40:1731.

13 Harwalkar, V. R., and H. W. Modler. 1981. Whey protein concentrate by heating under acidic conditions. 2. Physico-chemical evaluation of proteins. Milchwissenschaft 36:593.

14 Hillier, R. M., R.L.J. Lyster, and G. C. Cheeseman. 1979. Thermal denaturation of α-lactalbumin and β-lactoglobulin in cheese whey: effect of total solids concentration and pH. J. Dairy Res. 46:103.

15 Jelen, P., W. Buchheim, and K.-H. Peters. 1987. Heat stability and use of milk with modified casein:whey protein content in yogurt and cultured milk products. Milchwissenschaft 42:418.

16 Jenness, R., and J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. Neth. Milk Dairy J. 16:153.

17 Li-Chan, E. 1983. Heat-induced changes in the proteins of whey protein concentrates. J. Food Sci. 48:47.

18 Lyster, R.L.J. 1970. The denaturation of α-lactalbumin and β-lactoglobulin in heated milk. J. Dairy Res. 37:233.

19 Manji, B., and Y. Kakuda. 1987. Determination of whey protein denaturation in heat-processed milks: comparison of three methods. J. Dairy Sci. 70:1355.

20 Morr, C. V., and E. A. Foegeding. 1990. Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report. Food Technol. 44:100.

21 Morr, C. V., and R. V. Josephson. 1968. Effect of calcium, N-ethylmaleimide, and casein upon heat-induced whey protein aggregation. J. Dairy Sci. 51: 1349.

22 Mulvihill, D. M., and J. E. Kinsella. 1987. Gelation characteristics of whey proteins and β-lactoglobulin. Food Technol. 41:102.

23 Newstead, D. F., W. B. Sanderson, and E. F. Conaghan. 1977. Effects of whey protein concentrations and heat treatment on the heat stability of concentrated and unconcentrated milk. N.Z. J. Dairy Sci. Technol. 12:29.

24 Nielsen, M. A., S. T. Coulter, C. V. Morr, and J. R. Rosenau. 1973. Four factor response surface experimental design for evaluating the role of processing variables upon protein denaturation in heated whey systems. J. Dairy Sci. 56:76.

25 Parris, N., S. G. Anema, H. Singh, and L. K. Creamer. 1993. Aggregation of whey proteins in heated sweet whey. J. Agric. Food Chem. 41:460.

26 Parris, N., J. M. Purcell, and S. M. Ptashkin. 1991. Thermal denaturation of whey proteins in skim milk. J. Agric. Food Chem. 39:2167.

27 Parris, N., A. E. White, and H. M. Farrell, Jr. 1990. Identification of altered protein in nonfat dry milk powder prepared from heat-treated skim milk. J. Agric. Food Chem. 38:824.

28 Patocka, G., P. Jelen, and M. Kalab. 1993. Thermostability of skimmilk with modified casein/whey protein content. Int. Dairy J. 3:35.

29 Regester, G. O., R. J. Pearce, V.W.K. Lee, and M. E. Mangino. 1992. Heat-related changes to the hydrophobicity of cheese whey correlate with levels of native β-lactoglobulin and α-lactalbumin. J. Dairy Res. 59: 527.

30 Ruegg, M., U. Moor, and B. Blanc. 1977. A calori-

metric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. J. Dairy Res. 44:509.

31 Schmidt, R. H., V. S. Packard, and H. A. Morris. 1984. Effect of processing on whey protein functionality. J. Dairy Sci. 67:2723.

32 Varunsatian, S., K. Watanabe, S. Hayakawa, and R. Nakamura. 1983. Effects of Ca^{++}, Mg^{++} and Na^{+} on heat aggregation of whey protein concentrates. J. Food Sci. 48:42.

33 Xiong, Y. L., K. A. Dawson, and L. Wan. 1993. Thermal aggregation of β-lactoglobulin: effect of pH, ionic environment, and thiol reagent. J. Dairy Sci. 76: 70.