Aggregation of Whey Proteins in Heated Sweet Whey

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Aggregation of whey protein after heat treatment was examined for sweet whey prepared from bulk whole milk and milk containing the A, the B, or both variants of β-lactoglobulin by size exclusion chromatography. Both soluble and insoluble protein aggregates formed after heat treatments, the proportions of each being dependent on pH. Changes in phosphate and lactose concentrations had no significant effect, whereas the amount of insoluble protein aggregate was found to increase with increasing concentrations of calcium. The quantity of soluble protein aggregate was greatest in the whey containing the B variant of β-lactoglobulin, while the quantity of insoluble protein aggregate was greatest in the whey containing the A variant.

Treatment of milk and rennet enzymes, a process commonly used in cheese-making, causes the casein micelles to form a gel. Over time, this gel shrinks to exude whey.

When sweet whey is heated at temperatures above 70° C, a series of reversible and irreversible reactions occur, resulting in a flocculent precipitate of whey proteins.

Heat stability of whey proteins decreases with increasing protein concentration (Hillier et al., 1979; Modler and Jones, 1987). Although milk composition does not greatly affect the structure of the whey proteins directly, changes in the amounts of lactose and milk salts may alter the rates and pathways of denaturation during processing (de Wit, 1981). Whey proteins can be destabilized to varying degrees by duration and temperature of heat treatment (Kessler and Beyer, 1991), pH (Creamer et al., 1978; Harwalkar, 1980; Hill, 1988; Singh and Creamer, 1991), and ionic strength and ionic composition (de Wit, 1981; Farrell, 1988). Aggregate formation is aided by ionic calcium and the formation of intermolecular disulfide bonds (Kinsella, 1984; Mangino, 1984).

β-Lactoglobulin, which is the whey protein in highest concentration, is stabilized against heat denaturation by increasing amounts of lactose (Garrett et al., 1988) and total solids (Schmidt et al., 1984) and destabilized by increasing amounts of ionic calcium and higher pH (de Wit and Klarenbeek, 1984; Dumay and Cheftel, 1989).

In this study some factors affecting thermally induced aggregation and precipitation of whey proteins in sweet whey were investigated.

MATERIALS AND METHODS

Sweet Whey. Bulk whole milk and milks containing only the A or B variant of β-lactoglobulin were obtained from selected groups of Friesian cows on the Udy Farm, Bunnythorpe, New Zealand. Milkfat was removed by skimming at 35 °C, and sweet whey was prepared by adding 0.96 mL of standard strength (60 RU/mL) calf rennet (New Zealand Rennet Co., Eltham, New Zealand)/3 L of skim milk at 35 °C. After 1 h, the curd was broken into pieces and the whey was separated by filtration with a fine mesh nylon cloth.

Sweet Whey with Altered Levels of Calcium, Phosphate, and Lactose. Simulated milk ultrafiltrate (SMUF) was prepared as follows [cf. Jenness and Koops (1962)]: 1.58 g (12 mM) of KH_2PO_4, 1.73 g of sodium citrate·$2H_2O$, 0.60 g of KCl, 0.65 g of $MgCl_2 \cdot 6H_2O$, 55.67 g (155 mM) of lactose, and 0.10 g of NaN_3 were dissolved in 0.8 L of water. A solution of 1.31 g (9 mM) of $CaCl_2 \cdot 2H_2O$ in 50 mL of water was added to the mixture. The pH was adjusted to 6.60 with 1 M NaOH; the mixture was diluted to 1 L and stored at 5 °C until needed. Modified SMUF, in which the phosphate and lactose concentrations were altered, was also prepared.

Samples (25 mL) of sweet whey were dialyzed against 500 mL of SMUF or modified SMUF at pH 6.60 at 18 °C with two buffer changes for a total of 24 h.

Heat Treatment. Vials containing 5-mL samples of whey or dialyzed whey were heated in a water bath at 35, 65, 75, and 85 °C for 10, 20, or 30 min and immediately immersed in ice. The samples were centrifuged at 12000*g* for 30 min at 4 °C, and the supernatants and precipitates were collected for analysis. In some cases 25-mL samples of sweet whey were adjusted to pH 5.80 or 6.20 with 1 M HCl or to pH 7.00 with 1 M NaOH prior to the heat treatment.

Chromatography. The supernatants from the skimmed or heated whey were analyzed by size exclusion chromatography (SEC) at room temperature on a TSK 3000 SW or 4000 SW column (Toya Soda) in a buffer consisting of 0.02 M NaH_2PO_4 and 0.05 M Na_2SO_4, pH 6.80, at a flow rate of 0.5 mL/min with a Waters (Milford, MA) Model 4000 A pumping system, a WISP Model 710B sample processor, a Model 440 detector set at 280 nm, and a Model 820 Maxima chromatography workstation.

Protein Determination. Whey proteins in the supernatant and precipitate were quantified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a method based on that of Laemmli (1970) with mini Protean II equipment (Bio-Rad Laboratories, Richmond, CA).

The supernatants and precipitates were dispersed in 1% (w/v) SDS solution in chamber buffer to give 1:10 or 1:20 dilution of the original whey, and 20-μL samples were used.

A standard procedure for running, staining, and destaining the gels was adopted to ensure reproducibility and accuracy for quantitative measurement of the relative band intensities. The gels were run using the Bio-Rad 1000/500 power supply with the voltage controlled at ≤210 V, current ≤70 mA, power ≤3.25 W/gel, and time ≤1.75 h, until the bromophenol blue tracer dye seeped from the bottom of the gel (approximately 1.5 h depending on the gel system) and then stained for 1 h in 50 mL of Coomassie blue dye solution (0.1% Coomassie blue R-250, 25% 2-propanol, 10% acetic acid in water) in a closed 500-mL container with continuous agitation. This was followed by two destaining steps of 1 and 19 h, respectively, with 100 mL of destaining solution (10% 2-propanol, 10% acetic acid). Band intensities were determined using a Molecular Dynamics computing densitometer

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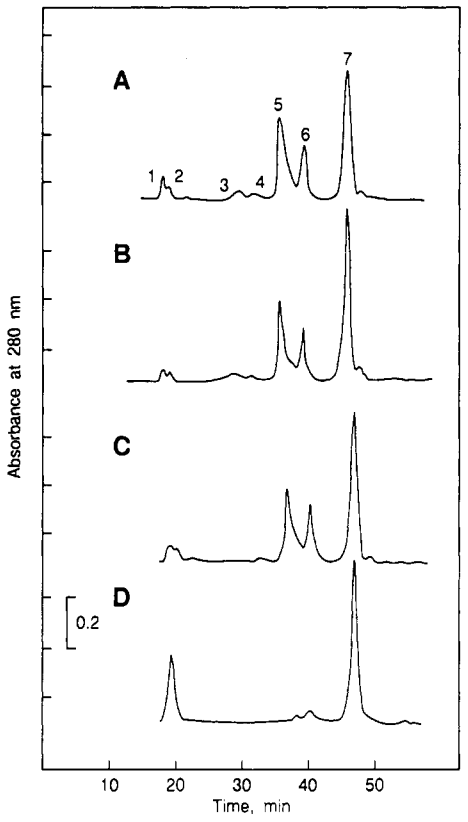

Figure 1. SEC separation of the major components in bulk whey after heat treatment. (A) No heat treatment. Identification of proteins in control sample: 1, caseins or lipoproteins; 2, soluble whey protein aggregates; 3, immunoglobulins; 4, bovine serum albumin; 5, β-lactoglobulin A and B; 6, α-lactalbumin; 7, low molecular weight peptides, etc. (B) Heated at 65 °C for 30 min. (C) Heated at 75 °C for 30 min. (D) Heated at 85 °C for 30 min.

(Model 300A) and the associated ImageQuant software. The results were adjusted to the standard casein samples that were included on every gel to ensure accuracy and reproducibility. Results with standard materials and following the above procedure were accurate to within 5%.

Chemical Analysis. Samples were analyzed for non-casein nitrogen (by Kjeldahl), calcium (by EDTA titration), phosphate (by a colorimetric method), and lactose concentrations in the Analytical Section of the New Zealand Dairy Research Institute and for minerals at the Department of Scientific and Industrial Research (DSIR), Palmerston North, New Zealand.

Chemicals. All chemicals were of analytical grade and obtained from BDH (NZ) Ltd. or Sigma Chemical Co. Water was prepared by reverse osmosis of well water followed by purification using a Milli Q system (Millipore Corp., Waltham, MA).

RESULTS

A typical SEC profile of the supernatant of the control sample of sweet whey prepared from bulk skim milk is shown in Figure 1A. The protein peaks were identified (see Figure 1 caption) as described by Morr and Foegeding (1990). The error associated with the method was low for the proteins at a relatively high concentration; e.g., β-lactoglobulin had a CV of <2%, while the immunoglobulin was about 3% and bovine serum albumin was 11%. Heating whey at 65 °C for 30 min caused few changes to the profile (Figure 1B). After heating at 75 °C for 30 min (Figure 1C), the soluble aggregate peak had increased slightly in size and the immunoglobulin and bovine serum albumin peaks were no longer present. After heating at 85 °C for 30 min, there was more soluble aggregate but very little undenatured whey protein present (Figure 1D).

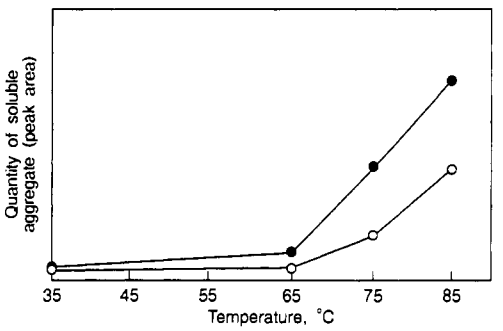

Figure 2. Formation of soluble aggregate in whey samples after heating at various temperatures for 30 min. ○, pH 6.6; ●, pH 7.0.

The peak that eluted at about 47 min was essentially unchanged by the heat treatments (Figure 1B–D) compared with the control (no heat treatment) sample (Figure 1A). SDS–PAGE showed that the soluble aggregate peak was composed of a mixture of all whey proteins. It was likely that the small quantity of casein present in the control sample (Figure 1A) became incorporated into the insoluble aggregates with the heat treatments.

The changes in the quantity of materials in the soluble aggregate peak as a function of temperature are shown in Figure 2. It was apparent that the amount of soluble aggregate increased almost linearly with temperature above 65 °C. Increasing the pH from normal (~6.6) to 7.0 increased the amount of soluble protein aggregate, whereas only insoluble aggregate formed on heating at a pH lower than 6.6.

Soluble Components. The effects of different concentrations of soluble calcium, phosphate, or lactose in samples of sweet whey after heating at 85 °C for 10, 20, or 30 min on the size of the soluble protein aggregate peak are shown in Figure 3. Generally, the amount of soluble aggregate increased with increasing heating time. The formation of soluble aggregates was not significantly affected by changes in the concentrations of soluble phosphate and lactose but decreased with increasing soluble calcium concentration (Figure 3C). Because of these insignificant changes, these data were used to estimate the variability of the method and the CV for the soluble aggregate peak was close to 4%. At low levels of calcium, heated whey samples formed two similarly sized aggregate peaks which were partially resolved by chromatography on the TSK 4000 SW column (data not shown).

SDS–PAGE of (nonreduced) samples indicated the presence of a new component in most of the samples on heating which was not detected by SEC. The band was present regardless of the concentrations of soluble phosphate and lactose but was not present at pH values below that of bulk skim milk. Formation of the band was affected, however, by the soluble calcium concentration (Figure 4), and the band was absent when the $CaCl_2$ concentration was increased to 6 or 9 mM in the SMUF. The band may have been caused by intermolecular disulfide aggregation of β-lactoglobulin and/or other whey protein as this band was not present when samples were reduced with mercaptoethanol prior to electrophoresis.

Genetic Variants. When samples of sweet whey (I–III) prepared from skim milk containing the A (II) or B (III) variant or equal quantities of the A and B (I) variants of β-lactoglobulin were analyzed, it was found that the non-casein nitrogen (NCN) and calcium contents for II were higher than those of either I or III (Table I), suggesting that II contained a higher whey protein content. The

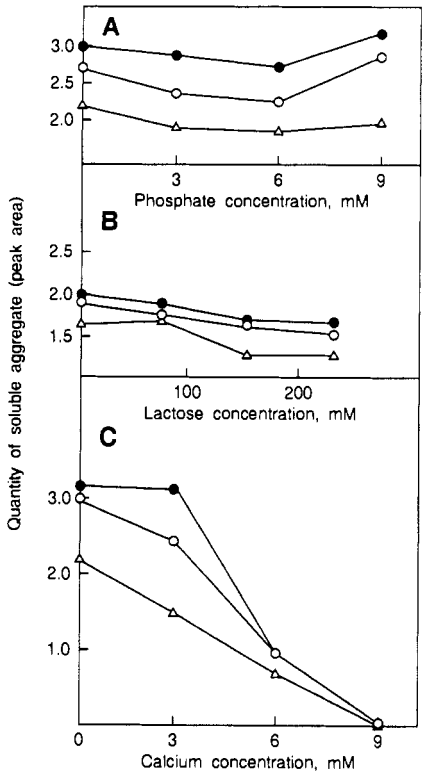

Figure 3. Effect of (A) phosphate, (B) lactose, and (C) calcium concentration and heating time on soluble aggregate formation in dialyzed bulk whey heated at 85 °C. △, 10 min; ○, 20 min; ●, 30 min.

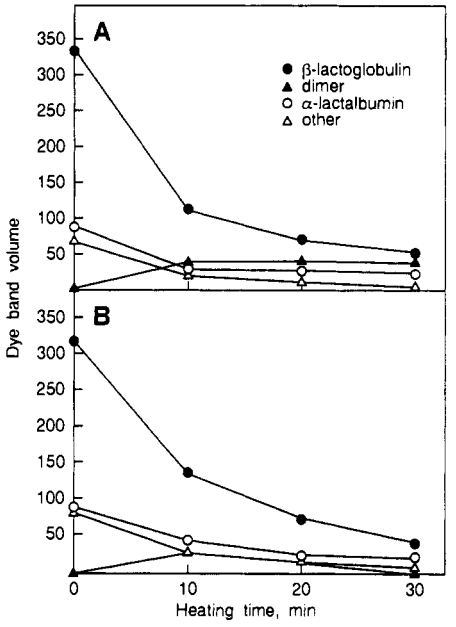

Figure 4. Relative quantities of the major components seen in heated whey using SDS–PAGE on samples that had not been reduced. Calcium ion concentration: (A) 0 mM; (B) 3 mM. ○, α-Lactalbumin; ●, β-lactoglobulin; ▲, dimer; △, other.

phosphate content was lowest in II, and the lactose content was highest in III. The pH values for I–III were similar.

The distribution of protein components in the control samples of I–III indicated that II contained much more immunoglobulin and β-lactoglobulin than I or III and that the concentrations of protein components in I and III were similar (Table II). Heat treatment at 65, 75, or 85 °C for 30 min produced more soluble aggregate in II, even though almost all of the whey proteins were denatured after heat treatment at 85 °C for 30 min (cf. Figure 1D). Adjustment of the pH to 7.00 before heating increased the quantity of heat-induced soluble aggregates for all three samples. Alterations in the levels of soluble components in I–III by dialysis showed that increasing the soluble calcium concentration decreased the amounts of soluble aggregate in I and III to similar extents. The amount of soluble aggregate found in II, however, was more dramatically affected by changes in soluble calcium concentration than that in either I or III (Figure 5).

Formation of insoluble aggregate (precipitate) was determined indirectly by measuring the loss of whey protein area (WPA) from the chromatogram of the supernatant of the heated whey sample compared with that of the control (Table III). In general, between pH 5.80 and 7.00 more precipitate formed with heating in II than in either I or III. Reducing the pH of sweet whey to 5.80 or 6.20 before heating at 85 °C for 30 min increased the amount of precipitate formed in I and III with no significant change in II. Less precipitate formed in all cases after similar heat treatment of sweet whey at pH 7.00 compared with lower pH values. Similar heat treatment of sweet whey at pH 7.00 significantly reduced the amount of precipitate formed in all cases. The greater amount of precipitate found in II compared with I or III on heating was not due to its higher protein concentration (see Table II), as spiking III with β-lactoglobulin B to the same protein concentrations as II did not increase the amount of precipitate formed.

DISCUSSION

The results of this study demonstrated that calcium level and the genetic variant type of β-lactoglobulin had significant effects on thermally induced aggregation and complex formation of whey proteins. The extent of formation of soluble aggregates increased with a concomitant decrease in large insoluble aggregates, as the calcium ion concentration of sweet whey was reduced. This indicates that calcium ion-mediated aggregation reactions are involved in the formation of large insoluble aggregates. These results are consistent with those of de Rham and Chanton (1984), who found that protein solubility in heated demineralized whey decreased gradually with the addition of calcium chloride. The effect of calcium on protein solubility was partly reversed by adding calcium sequestering agents, e.g., citrate or EDTA. Morr and Josephson (1968) suggested that heat aggregation of whey proteins is a multireaction process, the third stage of which involves the formation of large-sized aggregates (sedimentable at 1000*g*), and that this latter reaction is dependent on calcium concentration. The changes observed in the levels of soluble and insoluble aggregates with variation in the calcium concentration are in agreement with the mechanism of aggregation proposed by Morr and Josephson (1968).

The different thermal behaviors of the genetic variants of β-lactoglobulin A and β-lactoglobulin B have been reported previously (Gough and Jenness, 1962; Sawyer, 1968; Hillier, 1979; Dannenberg and Kessler, 1988). At temperatures up to 90 °C the B variant denatured more rapidly than the A variant. However, Imafidon et al. (1991), using differential scanning calorimetry, found that β-lactoglobulin B had a higher denaturation temperature than β-lactoglobulin A.

The results obtained in this study with sweet whey prepared from cows homozygous for the A or B variant showed that, under similar conditions of temperature, the B variant formed soluble smaller-sized aggregates, whereas the A variant formed mainly large insoluble aggregates.

Table I. Chemical Analysis of Samples of Sweet Whey Containing the A Variant (II), B Variant (III), and Equal Quantities of the A and B Variant (I) of β-Lactoglobulin

sweet whey	NCN,[a,b] % w/w	calcium,[a,c] mM	phosphate,[a,c] mM	lactose,[a] mM	pH
I	0.151, 0.155	6.8, 7.4, *8.1*	10.9, 9.9, *13.9*	127, 126	6.61
II	0.188, 0.210	8.0, 7.9, *8.9*	6.7, 5.4, *10.0*	126, 113	6.64
III	0.152, 0.163	7.5, 8.0, *8.3*	12.3, 11.4, *13.4*	140, 138	6.61

[a] Duplicate values by chemical analysis. [b] NCN, non-casein nitrogen. [c] Values in *italics* was obtained by plasma emission spectroscopy (DSIR).

Table II. Effect of β-Lactoglobulin Variant Type on the Distribution of Protein Components in Sweet Whey after Heat Treatment

heating temp, °C	casein	soluble aggregate	immunoglobulins	bovine serum albumin	β-lactoglobulin	α-lactalbumin
both A and B variants (I)						
35	0.31[a]	0.08	0.56	0.14	3.05	1.38
65	0.27	0.14	0.35	0.08	2.70	1.34
75		0.59			2.19	1.14
85		1.58			0.11	0.26
A variant only (II)						
35	0.26	0.18	1.19	0.19	4.11	1.44
65	0.35	0.49	1.12	0.13	3.81	1.44
75	0.40	0.12			2.23	0.97
85	0.19	0.18				0.29
B variant only (III)						
35	0.53	0.10	0.34	0.16	2.96	1.51
65	0.52	0.22	0.34	0.08	2.82	1.48
75		1.30			1.92	1.14
85		2.17				0.25

[a] Detector response based on area.

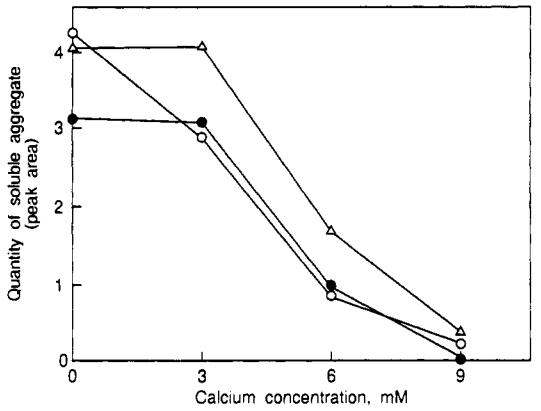

Figure 5. Effect of calcium ion concentration on soluble aggregate formation for I–III heated at 85 °C for 30 min. ●, I; ○, II; △, III.

Table III. Effect of β-Lactoglobulin Variant Type on Formation of Precipitate with Heat Treatment

sweet whey	treatment temp, °C	% precipitate[a] at pH 5.80	pH 6.00	pH ~6.6	pH 7.00
both A and B variants (I)	65	15	0	12	7
	75	21	31	29	34
	85	71	69	65	40
A variant (II)	65	10	3	0	8
	75	42	49	49	42
	85	90	87	91	73
B variant (III)	65	5	2	2	9
	75	23	32	22	19
	85	80	74	57	21

[a] % precipitate = [[WPA (control) − WPA (heated)]/WPA (control)] × 100.

There appears to be little comparative work reported in the literature, although observations have been made by early workers that β-lactoglobulin B does not appear to form aggregates under heating, whereas such aggregates do arise with β-lactoglobulin A solutions (Timasheff and Townend, 1958; Townend et al., 1960).

At neutral pH values, β-lactoglobulin B possesses a lower net charge than β-lactoglobulin A due to the replacement of an aspartic acid residue by glycine (Gordon et al., 1961; Kalan et al., 1962). It is possible that the differences in the aggregation behavior of the A and B variants are related to the lower charge on the B variant, thus binding less calcium and forming smaller aggregates. Sawyer (1968) observed that thiol-dependent reactions are more important in the denaturation of the A variant than in the B variant. Thus, it is also likely that the differences in the reactivity of thiol groups between the variants were responsible for the observed differences in the aggregation behavior.

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