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Identification of Altered Proteins in Nonfat Dry Milk Powder Prepared from Heat-Treated Skim Milk

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Reversed-phase high-performance liquid chromatography (RP-HPLC) and gel electrophoresis (SDS-PAGE) were used to monitor changes in the protein profiles of pooled herd (Holstein, Ayrshire, and Brown Swiss) nonfat milk (NFM) preheated to 63, 74, and 85 °C before spray-drying and storage for 8 months. Elution profiles of rehydrated nonfat dry milk (NFDM) changed with preheat temperature and storage time. A whey–casein complex comprised of BSA, β-lactoglobulin, α-lactalbumin, and κ- and α_{s2}-caseins formed in NFDM samples preheated to 74 and 85 °C. Some renaturation of whey proteins occurred as a result of storage time. The whey–casein complex is stabilized through disulfide linkage. Examination of isolated insoluble material (2–6% of total protein) indicated that it is composed almost entirely of caseins that appear to be aggregated with lactose.

Nonfat dry milk (NFDM) powders prepared from skim milk processed at different temperatures have different functional properties when used for the manufacture of various products. Physicochemical changes occur due to heat treatment, which affect the rehydration of NFDM powders and the functional behavior of the dispersed powder (Pallansch, 1970). Skim milk can be heated before spray-drying to meet certain product specifications on whey protein denaturation. Preheat treatment also affects the water absorption properties of NFDM powders. Low- and medium-heat NFDM (63, 74 °C) are frequently used to make ice cream and dairy beverages, in which some water absorption is necessary. High-heat NFDM (85 °C) absorbs more water, which improves its emulsion stabil-

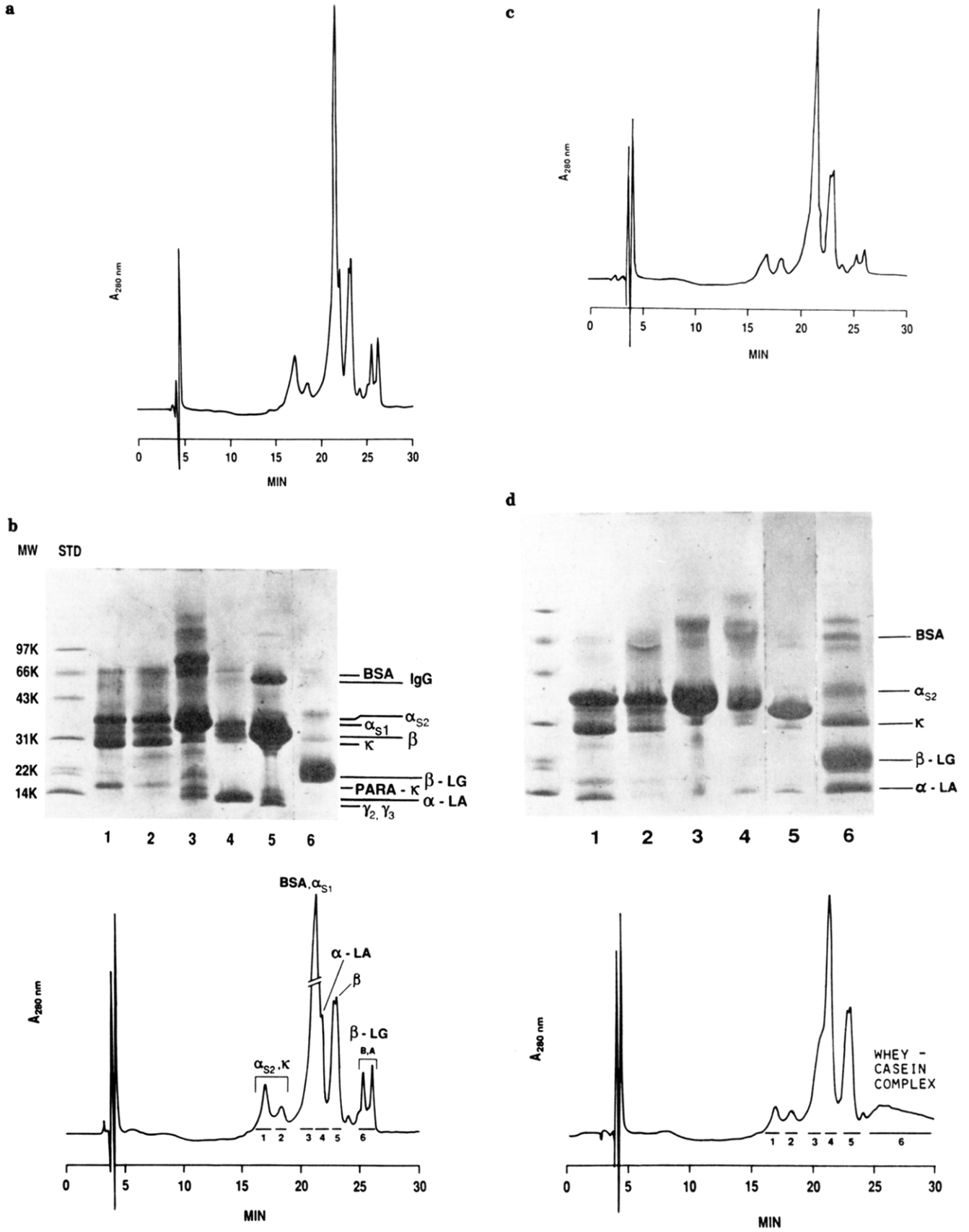

Figure 1. RP-HPLC elution profile and electrophoretic (SDS-PAGE) patterns of dialyzed soluble proteins from rehydrated NFDM stored for 10 days. Preheat treatment: (a) control, 38 °C; (b) 63 °C; (c) 74 °C; (d) 85 °C. Milk proteins: caseins, α_{s2}, α_{s1}, β, κ, para-κ, γ_2, γ_3; whey, BSA, Ig G, β-LG A, B, α-LA.

ity and gelation properties. These functional properties are important in comminuted meat and confectionary and baked products. Heat treatment above 65 °C, however, denatures whey proteins, leading to aggregation and precipitation (Kinsella, 1984). At about 70 °C, β-lactoglobulin (β-LG) forms complexes with κ- and α_{s2}-caseins (Sawyer, 1969). Thus, excessive heat treatment of skim milk may render powders made from these milks resistant to

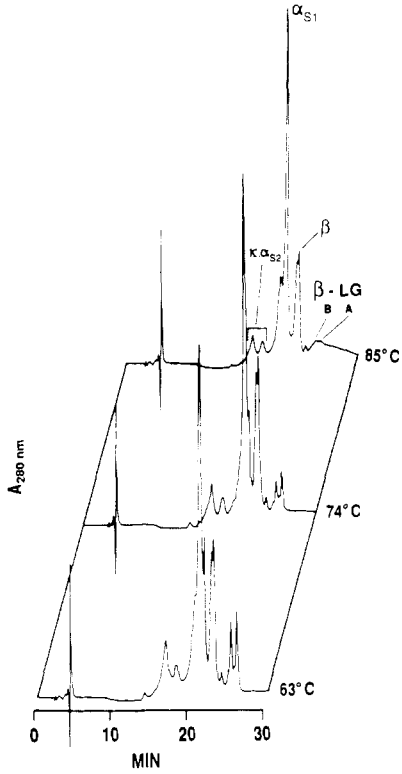

Figure 2. RP-HPLC elution profile of dialyzed soluble proteins from rehydrated NFDM stored for 120 days. Preheat treatment and identification of milk proteins same as in Figure 1.

rennet action and unsuitable for cheese processing. Whey protein aggregation in response to heat has also been explained by the formation of disulfide bonds among whey proteins (α-LA-β-LG-BSA) (Garrett et al., 1988) or through hydrophobic interactions and/or calcium-dependent linkages with casein micelles (Morr and Josephson, 1968). Model systems have been studied to explain whey protein–casein interactions (Singh and Fox, 1987; Euber and Brunner, 1982; Doi et al.,1983; Haque et al., 1987; Haque and Kinsella, 1988), the inhibitory effects of lactose on thermal coagulation of whey proteins (Garrett et al., 1988), and complexation of whey proteins with each other (Hiller and Lyster, 1979).

Classically, milk proteins have been fractionated by low-pressure column techniques using DEAE–cellulose or size exclusion materials in order to study whey protein and casein interactions. More recently, whey proteins (Pearce, 1983) and caseins (Visser et al., 1983; Carles, 1986) have been separated by reversed-phase high-performance liquid chromatography (RP-HPLC). In this study we examined the effects of preheat treatment and storage time of rehydrated NFDM powders on protein interactions and complex formation. Milk protein profiles were obtained with the aid of RP-HPLC; collected fractions were further characterized by gel electrophoresis (SDS-PAGE) because of the complex nature of milk protein systems due to the presence of genetic variants and varying degrees of phosphorylation and/or glycosylation.

EXPERIMENTAL SECTION

Nonfat Dry Milk. Raw pooled milk from Holstein, Ayrshire, and Brown Swiss cows was skimmed at 38 °C and used as the control. The skim milk was preheated at 63, 74, and 85 °C for 30 min, concentrated, and then spray-dried to yield low-, medium-, and high-heat nonfat milk powders (NFDM) according to a previously published procedure (Parris et al., 1989).

Sample Preparation. NFDM powders, 20 g, were dispersed in 180 mL of water at 25 °C and stirred until all the powder was suspended in solution. Insoluble material was isolated as a pellet by centrifugation (1000*g*, 4 °C, 30 min). The supernatant material was dialyzed at 4 °C against 6 L of water, changed four times over 48 h, and termed soluble protein. Insoluble material and soluble protein from samples taken at 10-, 120- and 240-day intervals after manufacture were lyophilized, weighed, and stored at −4 °C before further analysis.

Chromatography. Chromatographic separations were carried out on a Waters Associates (Milford, MA) HPLC system, which included the following components: Model 6000 A solvent delivery system, Model 660 solvent programmer, Model U6K injector, and Model 450 spectrophotometer, detector gain 0.1 AUFS. The analytical column used was a 0.46 × 25 cm, butyl-substituted reversed-phase, 10-μm particle size, 300-Å pore size column, which was preceded by a guard column containing the same packing material, VYDAC (Hesperia, CA). Aqueous acetonitrile containing 0.1% (w/v) trifluoroacetic acid (TFA) was used as the mobile phase. A flow of 0.8 mL/min was maintained for 30 min with use of two solutions to produce a gradient. Solution A was 0.1% TFA in water, and solution B was 10% (v/v) water and 0.1% TFA in acetonitrile. The volumetric proportion of B was increased linearly with time from 33 to 56%. Purified κ-, α_{s1}-, α_{s2}-, and β-caseins were isolated from milk according to the procedure of Basch et al. (1985). α-LA, β-LG, and BSA were purchased from Sigma (St. Louis, MO). NFDM samples (10 mg) were dissolved in 2 mL of a 50% mixture of solvents A and B and then passed through a 0.2-μm pore size filter, and 50 μL was injected onto the column. NFDM samples, reduced with 2-mercaptoethanol, were prepared as described by Visser et al. (1986) except that incubation was carried out at 37 °C and 200-μL sample volumes were applied. Volatile solvents were removed from collected fractions with a stream of N_2 at 40 °C on a Silla-Therm (Pierce Chemical Co., Rockford, IL).

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins collected from the analytical column was carried out on a Phast System Pharmacia (Piscataway, NJ) with a phast gel gradient of 8–25% acrylamide. Samples were prepared according to the method of Basch et al. (1985). Dried samples were solubilized in 3.5 μL of protein solvent system (0.166 M Tris and 1 mM EDTA, pH 8.0), 3.5 μL of 7% SDS, and 1.0 μL of 2-mercaptoethanol and the mixtures heated at 100 °C for 2 min. After cooling, 1.0 μL of bromophenol blue dye solution (0.1%, w/v) was added to the sample. Gels were stained with 0.2% (w/v) Coomassie R350 dye. Molecular weight standards (Bio-Rad, Richmond, CA) and their corresponding molecular weights were as follows: phosphorylase *b*, 97 400; bovine serum albumin (BSA), 66 200; ovalbumin, 42 699; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 400.

RESULTS AND DISCUSSION

Effects of Heat Treatment. HPLC elution profiles for dialyzed soluble material from NFDM powders prepared with preheat treatment at three different temperatures and stored for 10 days were compared and characterized by SDS-PAGE. The profiles for the control (38 °C) and the low-heat (63 °C) dialyzed soluble material are depicted in parts a and b of Figure 1, respectively; little difference in the elution profiles was observed. The SDS-PAGE patterns of the proteins isolated from corresponding peaks from the analytical column (Figure 1b) are given for the low-heat-treated sample. The positions of whey proteins and caseins in all the elution patterns were checked by separately running purified components on the column under identical conditions. All caseins, except κ-casein, were retained according to their average hydrophobicities: β-casein, > κ-caseins > α_{s1}-casein > α_{s2}-casein (Ekstrand, 1983). α_{s2}- and κ-caseins eluted together from the column and then α_{s1}-casein along with whey proteins BSA and α-LA. β-Casein eluted in

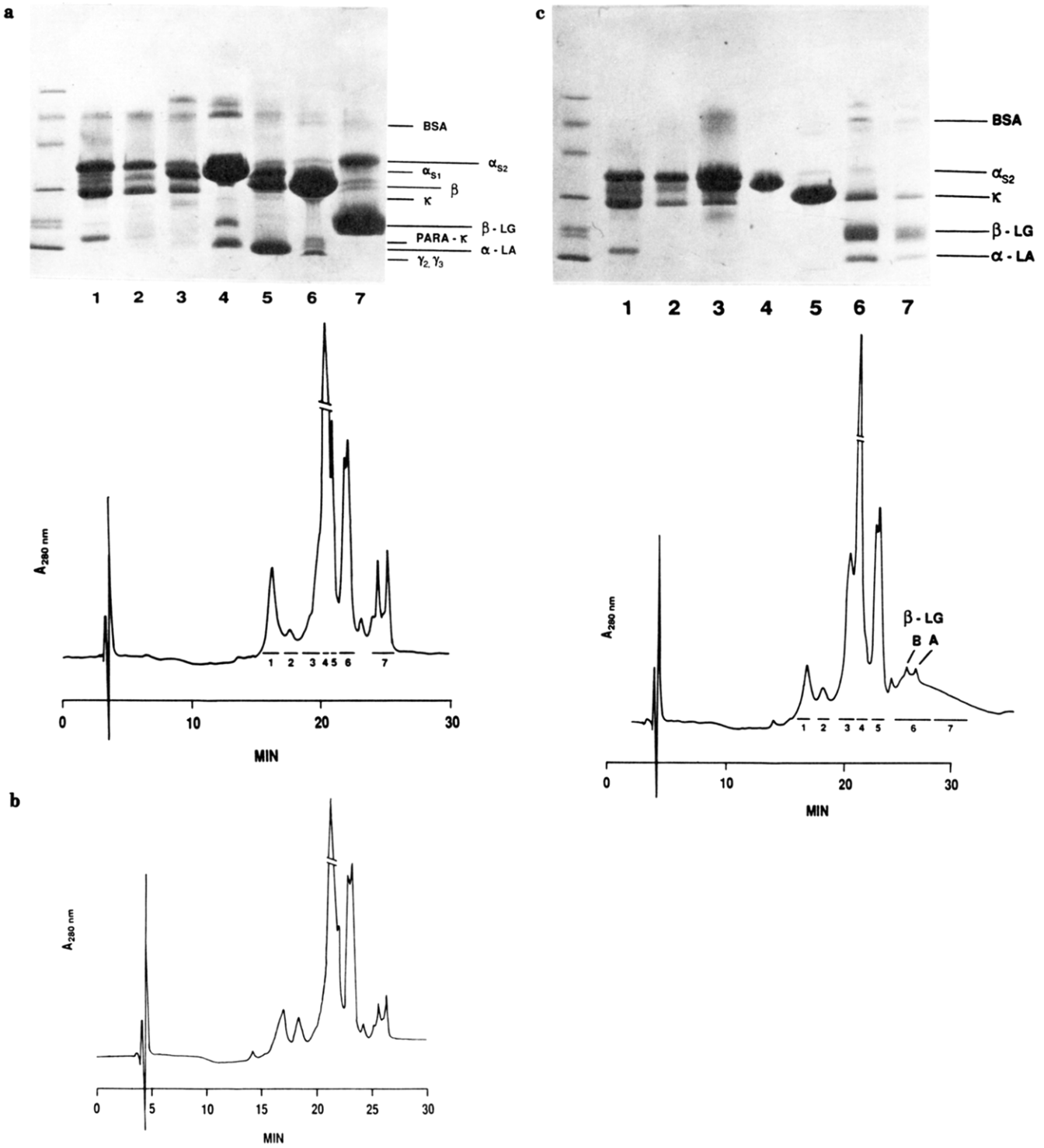

Figure 3. RP-HPLC elution profile and electrophoretic (SDS-PAGE) patterns of dialyzed soluble proteins from rehydrated NFDM stored for 240 days. Preheat treatment: (a) 63 °C; (b) 74 °C; (c) 85 °C. Identification of milk proteins same as in Figure 1.

23 min followed by genetic varients B and A of β-LG. Gel patterns show that the first two HPLC peaks (Figure 1b, lanes 1 and 2) contain κ-casein, as well as components of the α_{s2} family. Para-κ-casein, which is more mobile than κ-casein, is also present in both peaks. The peak eluting in 21 min contains α_{s1}-casein, BSA, and partially resolved α-LA (lanes 3 and 4). The β-casein fraction (lane 5) contains heavy-chain IgG as well as the plasmin-induced β-casein fragments γ_2 (f106–209) and γ_3 (f108–209), which have the greatest electrophoretic mobilities. β-LG (lane 6) contains a small amount of α_{s2}-casein.

HPLC profiles for the medium- and high-heat material (74 and 85 °C; Figure 1c,d) show a progressive decrease in relative intensity for those peaks containing κ- and α_{s2}-caseins and whey proteins (α-LA, β-LG). The distinctive peaks for β-LG B and A are no longer present in the high-heat-treated material (85 °C, Figure 1d) but are replaced with one broad peak eluting in 26 min. In addition, the peak eluting in 21 min has changed shape from a tailing shoulder to a leading one (Figure 1b,d). Gel patterns of this peak (Figure 1d, lanes 3 and 4) contain reduced amounts BSA and α-LA but are composed primarily of α_{s1}-casein. The mobilities of the former have shifted; this last broad peak is a complex comprised of many proteins including BSA, κ- and α_{s2}-caseins, β-LG, and α-LA (lane 6).

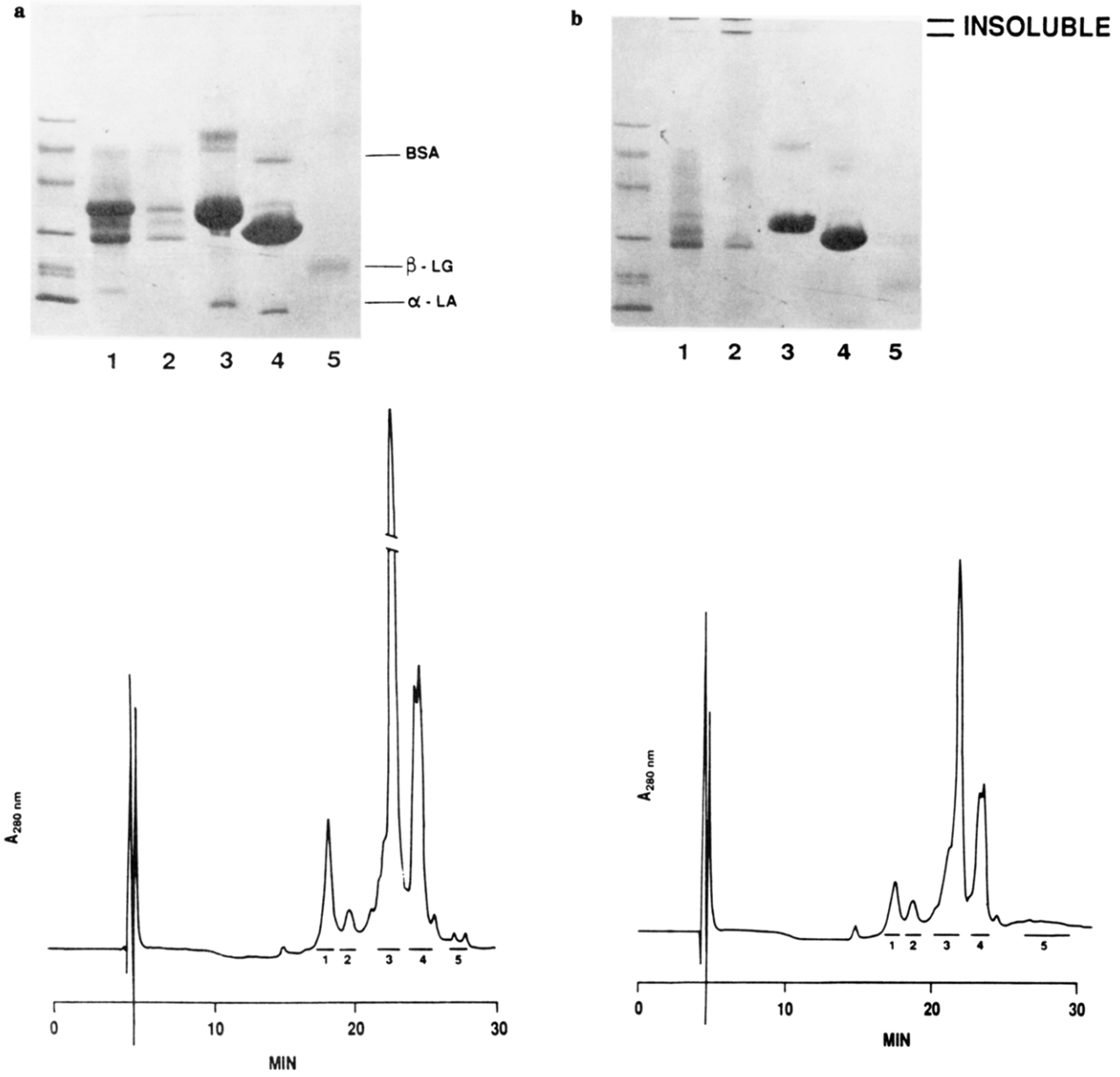

Figure 4. RP-HPLC elution profile and electrophoretic (SDS-PAGE) patterns of insoluble material from rehydrated NFDM stored for 10 days. Preheat treatment: (a) 63 °C; (b) 85 °C. Identification of milk proteins same as in Figure 1.

Effects of Storage. Elution profiles of dialyzed soluble material from NFDM powders prepared from skim milk subjected to different heat treatments and stored for 120 days was essentially the same as the 10-day samples (compare Figures 1 and 2) except that the peaks for β-LG (B and A) are somewhat more visible in the broad whey–casein complex peak centering at 26 min for the 85 °C material (Figure 2). With higher preheat treatment, the first peak became progressively smaller and the α_{s1}-casein peak develops a leading shoulder at 21 min that is partially resolved. β-Casein does not appear to be affected by preheat treatment. HPLC profiles and gel patterns (not identical fractions) for the 63 °C samples (10 and 240 days; Figures 1b and 3a) showed no alteration of the protein profile with storage. In fact, these samples are similar to the 38 °C control (Figure 1a). Elution profiles of dialyzed soluble material from 74 and 85 °C NFDM powders stored for 240 days were essentially unchanged over the 120-day samples (compare Figure 3 with Figure 2) except that the β-LG B and A peaks were even more prominent at 85 °C (Figure 3c). At the high preheat temperature (85 °C), the complex again formed, as indicated by the presence of BSA, κ- and α_{s2}-caseins, β-LG B and A, and α-LA (Figure 3c, lanes 6 and 7). The progressive increase in independently migrating β-LG B and A with time of storage indicates some renaturation of whey proteins has occurred. Gel patterns for the 85 °C sample also indicated that the partially resolved leading peak eluting in 21 min (Figure 3c, lane 3) does not contain a significant amount of whey proteins but is a mixture of α_{s2}-, α_{s1}-, and κ-caseins. This peak, therefore, is not the result of complex formation but appears to emerge because of loss of whey protein to the 26-min broad peak.

The amount of isolated insoluble protein from rehydrated NFDM subjected to three different processing temperatures ranged from 2 to 6% of the total protein in the powders and was greatest at the high preheat temperature. In addition, the protein to lactose ratio decreased with increasing processing temperature, indicating interaction between the protein and lactose (Parris et al., 1989). Elution profiles for the insoluble material isolated after a 10-day storage period indicated the near-absence of whey protein and the presence of very little complex at the

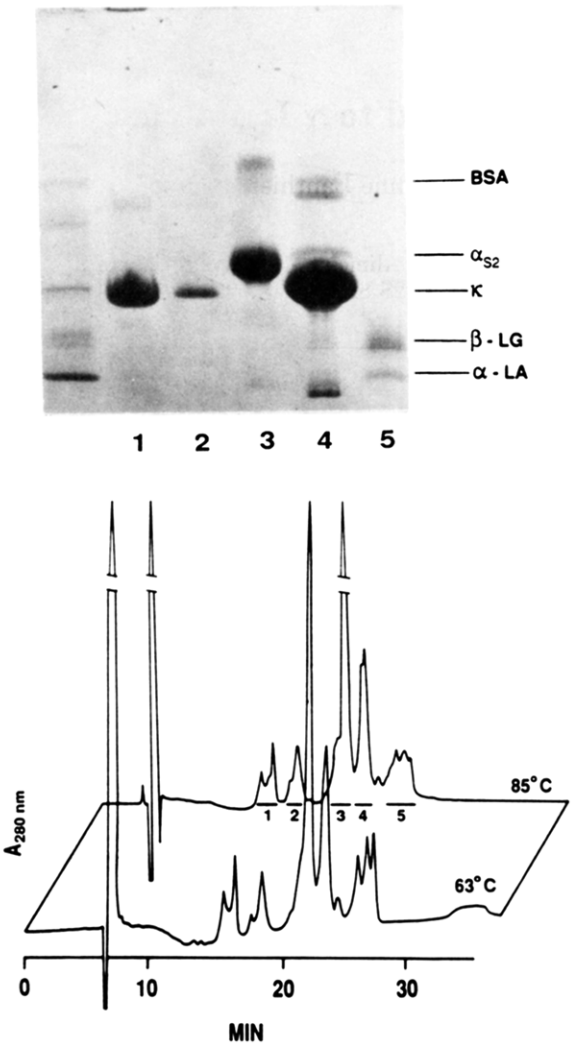

Figure 5. RP-HPLC elution profile and electrophoretic (SDS-PAGE) patterns of reduced dialyzed soluble proteins from rehydrated NFDM stored for 10 days. Identification of milk proteins same as in Figure 1.

high preheat temperature (Figure 4a,b). In addition, the intensities of the UV-absorbing peaks were considerably smaller for the insoluble material from the high-heat powder (Figure 4b), which confirms earlier findings that more lactose interacts with milk proteins subjected to higher preheat temperatures. Profiles were not affected by storage time (not shown). Gel patterns confirm the near-absence of whey proteins BSA, α-LA, and β-LG at the low heat temperature (Figure 4a, lanes 3 and 5). Patterns of insolubles at the high heat temperature (85 °C) indicate that some of the insoluble material did not enter the running gel (Figure 4b, lanes 1 and 2). This material probably resulted from aggregation of protein and lactose.

In order to demonstrate that the whey–casein complex is stabilized through a disulfide linkage, rehydrated NFDM powder stored for 10 days and preheated at 85 °C (Figure 1d) was reduced as described earlier (Experimental Section). The reduced 85 °C sample compared favorably with the reduced 63 °C sample (Figure 5). Gel patterns for the reduced 85 °C sample indicated that the broad whey–casein complex is no longer present and BSA, α-LA, and κ- and α_{s2}-caseins no longer elute in this region (lane 5). Only reduced β-LG B and A are present.

These results demonstrate that separation of milk proteins by RP-HPLC and characterization of collected fractions by gel electrophoresis (SDS-PAGE) is an effective method to identify protein changes in heat-treated NFDM. Whey proteins from rehydrated NFDM are not significantly denatured for low-heat powders (63 °C) but are almost completely denatured for high-heat powders (85 °C). The whey–casein complex found in medium- and high-heat powders was stabilized through disulfide linkages and contained BSA, α-LA, β-LG, and κ- and α_{s2}-caseins. The presence of all the whey proteins in the broad peak can be explained by the formation of a disulfide-stabilized whey complex (α-LA-β-LG-BSA) that coelutes in 26 min with the whey–casein complex. The apparent renaturation of whey proteins during storage probably results from dissociation of whey proteins stabilized through hydrophobic interactions or calcium-dependent linkages with casein micelles and warrants further investigation.

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