Heating Skim Milk Alters the Migration of Immunoreactive Milk Proteins in Acrylamide Gels

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Effects of heat treatment on skim milk proteins were characterized using an immunoblot method. Proteins in heat-treated skim milk were separated by polyacrylamide gel electrophoresis (nonreducing and reducing conditions) and electrophoretically transferred to nitrocellulose, and β-lactoglobulin, α-lactalbumin, and κ-casein were detected by immunoblotting. High molecular weight immunoreactive β-lactoglobulin was apparent in skim milk samples heated at 65 °C and above when electrophoresed under nonreducing conditions. Formation of high molecular weight immunoreactive β-lactoglobulin was time and temperature dependent. Only monomeric β-lactoglobulin was observed in heat-treated samples electrophoresed under reducing conditions. Immunoreactive α-lactalbumin in skim milk was not substantially affected by heat treatment. High molecular weight immunoreactive κ-casein was present in nonreduced samples from each temperature treatment, including incubation at room temperature, but not in samples electrophoresed under reducing conditions. This immunoblot method should be valuable for further study of intermolecular interactions of milk proteins.

INTRODUCTION

Milk contains a complex mixture of proteins. Interactions between milk proteins are important to the biochemical and functional properties of milk. For example, intermolecular interactions among caseins are critical for micelle stability (Farrell, 1988), and interactions between κ-casein and β-lactoglobulin seem to be a feature of the protein chemistry in heated skim milk or in β-lactoglobulin–casein mixtures (Farrell, 1988; Noh et al., 1989a,b; Jang and Swaisgood, 1990). Heating skim milk or whey proteins has dramatic effects on the biochemical and physiochemical properties of milk proteins (Farrell, 1988). Effects of heating on denaturation and aggregation of β-lactoglobulin and of other whey proteins have been described (McKenzie, 1971; Noh et al., 1989a,b; Laligant et al., 1991; Parris et al., 1991). We report the use of an immunoblot method to characterize the effects of heating skim milk on the migration of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and κ-casein separated under reducing and nonreducing conditions in polyacrylamide gels.

MATERIALS AND METHODS

Preparation of Skim Milk. Milk samples were collected from four cows at the University of Illinois dairy herd. Skim milk was prepared by centrifugation at 10000*g* for 20 min at 22 °C to remove fat and debris. Skim samples were pooled from each cow and 200-μL aliquots heated at 22, 55, 65, 75, or 85 °C for various times. After heating, skim samples were diluted with distilled water (1:20 or 1:40), and 5 volumes of diluted skim were combined with 1 volume of dye solution (70% glycerol with 0.12% bromophenol blue) plus 1 volume of concentrated gel sample solution [nonreducing solution contained 14.5% sodium dodecyl sulfate (SDS) and 0.28 M Tris, pH 6.8]. Concentrated gel sample solution for reducing conditions also contained 25% β-mercaptoethanol. Immediately prior to loading on acrylamide gels, the samples were heated at 90 °C for 10 min. In some reactions, purified β-LG was mixed with total caseins (at approximately 1 mg of β-LG to 4 mg of caseins) and heated as for the skim samples. Purified β-lactoglobulin was the generous gift of Dr. B. L. Larson (University of Illinois, Urbana). Casein was prepared by acid precipitation from skim milk, followed by resuspension of casein in water at pH 7.2.

Gel Electrophoresis and Immunoblotting. Samples were electrophoresed on 17% SDS–polyacrylamide gels as described by Laemmli (1970). Proteins were electrophoretically transferred to nitrocellulose and detected by immunoblotting with specific antisera for milk proteins as described previously (Ventling and Hurley, 1988). Rabbit anti-bovine β-lactoglobulin and rabbit anti-α-lactalbumin also were supplied by Dr. B. L. Larson. Rabbit anti-bovine κ-casein was a generous gift from Dr. A. Guidry (USDA, ARS, Beltsville, MD).

RESULTS AND DISCUSSION

Preliminary experiments indicated that heating skim milk at 85 °C, but not at 22 or 65 °C, for 15 min resulted in partial precipitation of proteins. Sonication of these heated samples did not result in signifiant resolubilization of the proteins (results not shown). Electrophoresis of these samples was compared when the heat-treated samples, combined with nonreducing gel sample solution, were either unheated or heated at 90 °C immediately prior to loading on the gel. Preloading heat treatment increased the high molecular weight immunoreactive β-LG bands visible in blots of the 85 °C samples, while samples heated at 22 or 65 °C generally were unchanged (results not shown). The preloading heat treatment in the presence of nonreducing gel sample solution did not seem to alter the effects of the earlier heat treatment of undiluted skim milk; rather, it apparently enhanced the resolubilization of precipitated proteins in skim milk samples receiving the 85 °C heat treatment. As a result, all subsequent samples were routinely heated to 90 °C in gel sample solution immediately prior to loading on acrylamide gels.

β-Lactoglobulin has a free cysteine sulfhydryl group arising from either residue 119 or 121 (Farrell, 1988). Reactivity of this free sulfhydryl group increases upon denaturation of the protein, resulting in formation of disulfide complexes with other protein molecules. Denaturation of β-LG, induced by temperature, pH, denaturants, or inorganic ions, facilitates aggregation with other β-LG molecules or with other proteins (Farrell, 1988). In the present study, the amount of high molecular weight immunoreactive β-LG increased with increasing temperature from 22 to 85 °C (Figure 1) when proteins were

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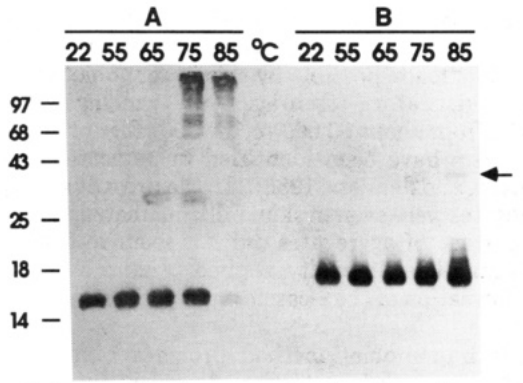

Figure 1. Immunoblot demonstrating the effects of temperature on immunoreactive forms of β-lactoglobulin in skim milk. Skim milk was heated at 22, 55, 65, 75, or 85 °C for 15 min. Heat-treated samples were electrophoresed under nonreducing (A) or reducing conditions (B). Note that monomeric β-lactoglobulin migrates at a lower apparent molecular weight under nonreducing conditions compared with under reducing conditions. The arrow indicates the putative β-lactoglobulin dimer in reduced samples. Size standards (left side) are in kilodaltons.

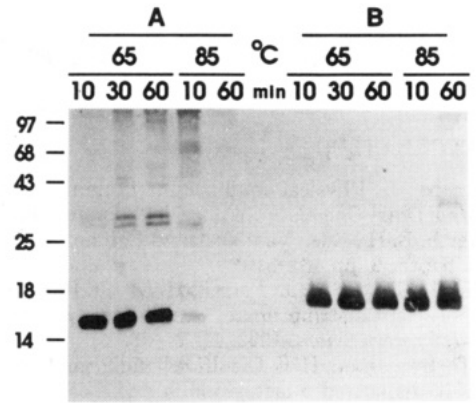

Figure 2. Immunoblot demonstrating time–temperature effects on immunoreactive forms of β-lactoglobulin in skim milk. Skim milk was heated at 65 °C for 10, 30, or 60 min or at 85 °C for 10 or 60 min. Heat-treated samples were electrophoresed under nonreducing (A) or reducing conditions (B). Size standards (left side) are in kilodaltons.

separated under nonreducing conditions. The increased high molecular weight immunoreactive β-LG coincided with a decline in immunoreactive monomeric β-LG at the 85 °C heat treatment. When separated under reducing conditions, essentially all immunoreactive β-LG migrated as the monomeric form (about 18 kDa), including the samples heated at 85 °C. This suggests that disulfide bonds were responsible, at least in part, for the intermolecular interactions of β-LG induced by heating and observed on the immunoblots. An exception was in the samples heated at 85 °C (Figure 1), where another band was observed at approximately 36 kDa, perhaps representing a dimerized form of β-LG which remained stable under the reducing conditions. Heat-induced denaturation of β-LG is thought to be irreversible at temperatures over 70 °C (Farrell, 1988). Results from the immunoblots (Figure 1) are consistent with a significant increase in aggregation of β-LG occurring between 65 and 75 °C.

Heating skim milk at 65 °C for 10, 30, or 60 min resulted in a time-dependent increase in the presence of high molecular weight immunoreactive β-LG when separated under nonreducing conditions (Figure 2), but no change was apparent when proteins were separated under reducing conditions. Heating skim milk at 85 °C for 10 min resulted in a dramatic decrease of the monomeric β-LG band. When heated at 85 °C for 60 min, a nearly complete loss of

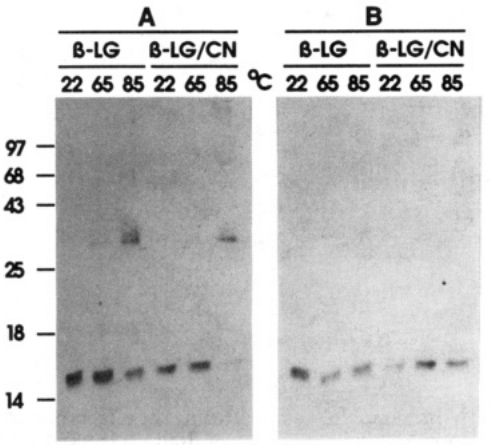

Figure 3. Immunoblot demonstrating the effects of temperature on immunoreactive β-lactoglobulin in the presence of reducing agent. Mixtures of isolated β-lactoglobulin (β-LG) or β-lactoglobulin plus total casein (β-LG/CN) were heated for 15 min at 22, 65, or 85 °C either in the absence (A) or in the presence of 2 mM DTT (B). All samples were electrophoresed under nonreducing conditions. Size standards (left side) are in kilodaltons.

immunoreactive β-LG was observed on the blot when proteins were run under nonreducing conditions (Figure 2), suggesting that polymeric aggregates too large to enter the gel were formed. However, these large aggregates seem to have been completely resolubilized when the samples were electrophoresed in the presence of reducing agent (Figure 2).

To verify that disulfide bond formation was involved in formation of the high molecular weight immunoreactive β-LG in skim milk, purified β-LG or a mixture of β-LG and total casein was heated at 22, 65, or 85 °C for 15 min in the presence or absence of 2 mM DTT (Figure 3). High molecular weight immunoreactive β-LG bands (lightly stained in Figure 3) were apparent when the β-LG or β-LG–casein mixtures were heated to 85 °C in the absence of DTT. However, all β-LG migrated as the monomer form when the heating reactions were conducted in the presence of DTT (Figure 3). The similarity of high molecular weight immunoreactive β-LG patterns between reaction mixtures of only β-LG and of β-LG plus casein suggests that the high molecular weight immunoreactive β-LG bands in heated skim milk samples are formed independently of the presence of casein. Interactions between κ-casein and β-LG involve intermolecular disulfide bonds and can be induced by heat treatment, especially at temperatures of 85 °C or greater (Jang and Swaisgood, 1990; Noh et al., 1989a,b). β-Lactoglobulin also forms complexes with α_{s2}-casein under heat-induced denaturing conditions (Noh et al., 1989a). We were not able to identify the formation of κ-casein–β-LG complexes under the time–temperature conditions used in the present study. That is, there were no bands in the 35 000–45 000 range that migrated at approximately the same apparent molecular weight on both the β-LG and κ-casein immunoblots. These results suggest that the interaction of β-LG with casein, especially κ-casein, may not be as strong as the β-LG dimer formation under the heat intensities used in these experiments. This immunoblot method should be adaptable to detect such multiple-protein complexes which seem to form primarily at the higher temperatures.

Heating skim milk at temperatures ranging from 22 to 85 °C resulted in only a minor increase in high molecular weight immunoreactive α-LA under nonreducing conditions and no change under reducing conditions (Figure 4). The stability of α-LA is considered to be greater than that

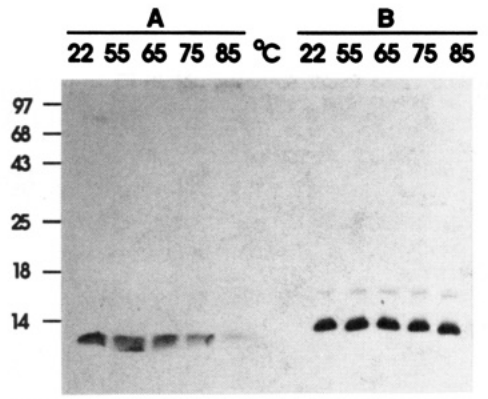

Figure 4. Immunoblot demonstrating the effects of temperature on immunoreactive forms of α-lactalbumin in skim milk. Skim milk was heated at 22, 55, 65, 75, or 85 °C for 15 min. Heat-treated samples were electrophoresed under nonreducing (A) or reducing conditions (B). Note that α-lactalbumin migrates at a lower apparent molecular weight under nonreducing conditions compared with under reducing conditions. Size standards (left side) are in kilodaltons.

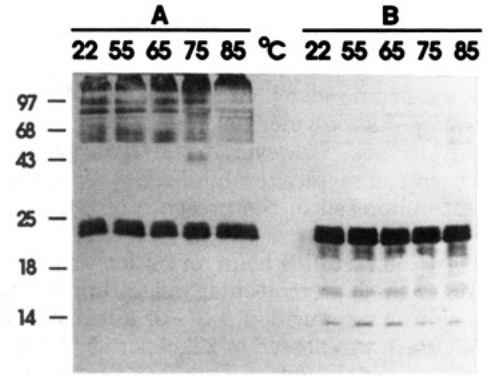

Figure 5. Immunoblot demonstrating the effects of temperature on immunoreactive forms of κ-casein in skim milk. Skim milk was heated at 22, 55, 65, 75, or 85 °C for 15 min. Heat-treated samples were electrophoresed under nonreducing (A) or reducing conditions (B). Size standards (left side) are in kilodaltons.

of β-LG, partly because of the lack of free sulfhydryl groups (Farrell, 1988), and our results using the immunoblotting method were consistent with this relatively greater stability of α-LA to heat treatment.

In contrast, high molecular weight forms of immunoreactive κ-casein were apparent in samples heated at each temperature and separated under nonreducing conditions (Figure 5). No high molecular weight forms of κ-casein were apparent when samples were electrophoresed under reducing conditions (Figure 5). Groves et al. (1992) described intermolecular interactions of isolated κ-casein which resulted in formation of polymeric aggregates. Those aggregates did not form in the presence of reducing agent. The high molecular weight immunoreactive κ-casein seen on our blots of heat-treated skim milk samples electrophoresed under nonreducing conditions may correspond to the polymeric aggregates observed by others (Walstra and Jenness, 1988; Groves et al., 1992); however, we cannot rule out the presence of interactions with other skim milk proteins in these heating experiments. In any case, the presence of high molecular weight immunoreactive κ-casein was abolished by electrophoresing the heated proteins in the presence of β-mercaptoethanol. An interesting observation, made possible by this immunoblot technique, is the evidence for κ-casein aggregates ranging in molecular weight from about 60 000 to 150 000 (Figure 5). Similar multimers have been identified in untreated raw milk (Walstra and Jenness, 1988). In the present study, these aggregates were even in skim milk incubated at 22 °C and the pattern of aggregates did not seem to change with increasing heat intensity applied (Figure 5), suggesting that formation of the κ-casein aggregates may not be heat sensitive.

This immunoblot method provides a sensitive and specific approach for detection of covalently linked protein complexes formed in response to heat treatment. The method can be extended to characterizing protein–protein interactions induced by other changes in the normal molecular environment of specific milk proteins. The method should be of value to investigators interested in milk protein chemistry and may be useful to the food industry in the quality control of milk products.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; α-LA, α-lactalbumin; DTT, dithiothreitol.

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