

Note

Preparation of Fresh Cheese from Caprine Milk as a Model for the Reduction of Allergenicity

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Summary Fresh cheese was prepared from caprine milk by isoelectric precipitation as a model experiment for reducing the allergenicity of milk. After acidic precipitation of casein, the β -lactoglobulin content in curd was determined by ELISA using monoclonal antibody (MAb- β 209). The β -lactoglobulin content was very high in the fresh cheese obtained from heat-treated (85°C) bovine or caprine milk, while that obtained from untreated milk contained none of this protein. Taking it into account that caprine milk has only a small amount of α_{s1} -casein, one of the major bovine milk allergens, the caprine fresh cheese sterilized after processing by precipitation may be useful as a protein source of low allergenicity.

Key Words caprine milk, fresh cheeses, β -lactoglobulin, allergenicity

β -Lactoglobulin (β -LG) is considered to be the major bovine milk allergen and its antigenicity has been extensively studied (1–3). Numerous procedures including various enzymatic or physical treatments have been designed to modify allergenic properties of β -LG (4). It has been reported that the IgE binding capacities of β -LG are decreased by heating at 80–100°C for 15 min, but that there remains residual allergenicity even after severe heating (5, 6). It is well established that the heating process causes structural changes in some milk proteins (7–10). The sterilization process during the preparation of milk on an industrial scale involves heat treatment. Since β -LG unfold and interact with casein to form aggregates with thermal treatment (11–13), overheating of the milk during the sterilization may be responsible for an irreversible aggregation reaction. Unlike the detailed studies on the complex formation between caseins and β -LG in bovine milk (14, 15), there have been few investigations concerning the formation of such a complex among proteins from caprine milk.

It has been reported that caprine milk in some cases was less allergenic than bovine milk (16, 17). This is probably due to the lower content of α_{s1} -casein, which is one of the major bovine milk allergens, in the former. Our previous study demonstrated that the amount of α_{s1} -casein in the milk from the Japan-Saanan goat was very low (18).

The objectives of this study were to investigate the heat-induced aggregation of β -LG in caprine milk and to prepare fresh cheese from the milk without preheat-

ing as a model for milk products of reduced allergenicity.

Materials and Methods

1. *Samples.* Fresh caprine raw milk was obtained from Japan-Saanan goats at National Livestock Breeding Center Nagano Station. Freshly bulked raw bovine milk was supplied by local farms. Four types of commercially available heat treated milk (66°C, 30 min, 80°C, 15 min, 130°C, 2 s, 140°C, 2 s) were purchased from the local market.

2. *Heat treatment of raw milk.* The milk samples were heat treated in tightly capped glass test tubes in a water bath at 85°C. After the samples were heated, they were cooled in iced water. At various time periods from 5 to 30 min, tubes were removed and immediately cooled in ice water to 4°C.

3. *Preparation of fresh cheeses.* Fresh cheeses were prepared from each milk sample by isoelectric precipitation with acetate buffer (pH 4.6) in glass test tubes. The precipitate was washed with distilled water and resolubilized in 0.2 M phosphate buffer (pH 7.0) for the determination of β -LG content. The protein content of samples was determined by Quick Start Bradford Dye Reagent (Bio Rad, Hercules, CA, USA).

4. *Preparation of monoclonal antibody.* Monoclonal antibody (MAb) to β -lactoglobulin (β -LG) was prepared as described in our previous study (19). The culture supernatants of the hybridoma population were screened for specific antibody production by ELISA with bovine β -LG used as an antigen. Positive hybrids were cloned by the limiting dilution method.

5. *ELISA.* Standard bovine β -LG (Sigma) or cheese samples were diluted in 0.2 M phosphate buffer (pH

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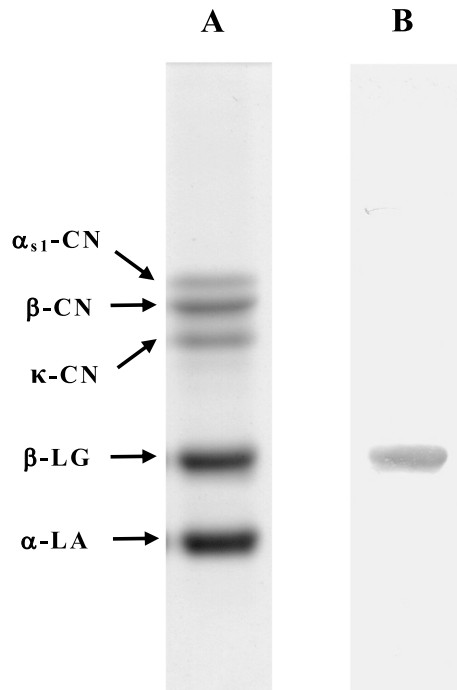


Fig. 1. SDS-PAGE analysis (A) and Western blotting (B) using anti β -lactoglobulin monoclonal antibody. SDS-PAGE was carried out on 15% polyacrylamide gel. Standard milk proteins were transferred onto the transfer membrane, and blotted β -lactoglobulin was detected by Western blotting with anti β -lactoglobulin monoclonal antibody and horseradish peroxidase-conjugated anti mouse IgG antibody. α_{s1} -CN, α_{s1} -casein; β -CN, β -casein; κ -CN, κ -casein; β -LG, β -lactoglobulin; α -LA, α -lactalbumin.

7.0), dispensed directly into a 96-well ELISA plate (9018, Costar, NY, USA) and left at 4°C overnight. The wells were blocked with 0.7% BSA for 1 h at room temperature followed by the addition of anti- β -LG MAb and incubation for 1 h. After washing four times with phosphate-buffered saline (PBS), horseradish peroxidase (HRP)-conjugated anti-mouse IgG was added and left for 1 h. Each well was washed five times with PBS, and then the substrate mixture of H_2O_2 and ABTS (Kirkegaard & Perry Laboratories, MD, USA) was dispensed into the well and left for enzyme reaction for 1 h at room temperature. The absorbance at 405 nm was measured on a model 680 microplate reader (Bio-Rad).

6. SDS-PAGE and Western blotting. SDS-PAGE was carried out on 15% polyacrylamide gel according to the method of Laemmli (20). Standard milk proteins (Sigma) were transferred onto the transfer membrane (clear blot P membrane, Atto, Tokyo, Japan), and blotted β -LG was detected by subsequent reactions with anti- β -LG mouse MAb, HRP-conjugated anti-mouse IgG antibody and the substrate mixture of 4-chloro-1-naphthol and H_2O_2 (21).

Results

1. The specificity of the monoclonal antibody

The specificity of the monoclonal antibody (MAb), which was designated MAb- β 209, was examined by

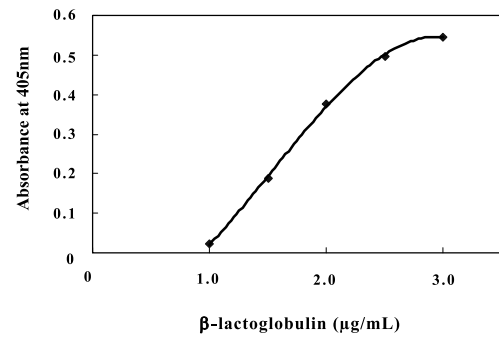


Fig. 2. Standard curve used for determination of β -lactoglobulin by ELISA. Standard β -lactoglobulin was diluted in phosphate buffer and poured directly into an ELISA plate. The diluted samples were reacted with anti β -lactoglobulin monoclonal antibody and horseradish peroxidase-conjugated anti mouse IgG. Each well was incubated with the substrate mixture and the absorbance at 405 nm was measured by a microplate reader.

Table 1. Concentrations of β -lactoglobulin in fresh cheeses from sterilized bovine milk.

Sterilization conditions	β -Lactoglobulin content (μ g/mg protein)
66°C, 30 min	122.0 $\pm$ 7.3
80°C, 15 min	136.8 $\pm$ 2.2
130°C, 2 s	145.1 $\pm$ 1.7
140°C, 2 s	137.0 $\pm$ 1.2

Cheese samples were diluted in phosphate buffer and poured directly into an ELISA plate. The diluted samples were reacted with anti β -lactoglobulin monoclonal antibody and horseradish peroxidase-conjugated anti mouse IgG. Each well was incubated with the substrate mixture and the absorbance at 405 nm was measured by a microplate reader.

Values are means $\pm$ SD ($n=3$).

Western blotting. Figure 1 shows the results of SDS-PAGE and Western blotting analyses for standard milk proteins. The MAb reacted specifically with β -LG. The specificity of the MAb was also determined by ELISA. Each standard milk protein solution was adsorbed onto the ELISA plate (0.3 μ g/well) and reacted with the MAb. The values of absorbance at 405 nm with α_{s1} -casein, β -casein, κ -casein, β -lactoglobulin and α -lactalbumin were 0.074, 0.094, 0.058, 1.024 and 0.060, respectively. In order to identify the difference in the reactivity with MAb between heat-treated and untreated milk, both milks were analyzed by the ELISA. The values for heat-treated and untreated bovine milk were 0.394 and 0.377, respectively.

2. Quantification of β -LG by ELISA using MAb

An ELISA was used to quantify β -LG. The bovine β -LG solution (3 μ g/mL) was serially diluted and each dilution was dispensed into a well of a plate. A typical standard curve is shown in Fig. 2. The linear portion of the curves was used to estimate the level of β -LG in the samples. The β -LG content in caprine cheese was calcu-

lated from a standard curve using bovine β -LG.

3. Determination of β -LG content in cheeses from sterilized bovine milk

The β -LG content in samples was successfully determined by the ELISA method. The values were expressed in $\mu\text{g}/\text{mg}$ protein of material. Fresh cheeses from sterilized bovine milk tested contained high concentrations of β -LG (Table 1). The β -LG content in cheeses was further confirmed by SDS-PAGE. As shown in Fig. 3, the

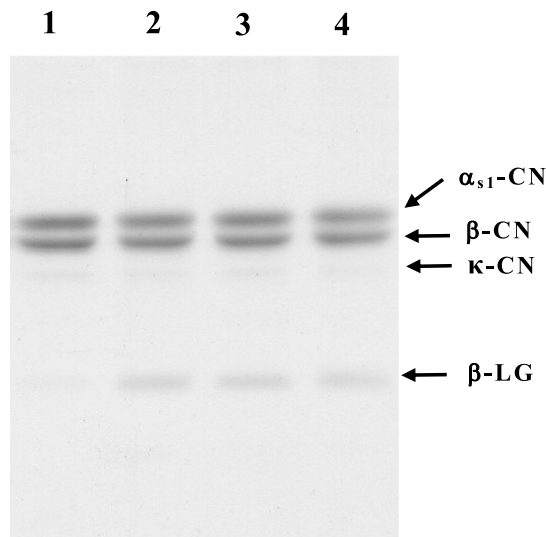


Fig. 3. SDS-PAGE analysis of fresh cheeses from sterilized bovine milk. Fresh cheeses were prepared from four types of commercially available heat-treated milk by isoelectric precipitation with acetate buffer (pH 4.6). The precipitate was washed with distilled water and resolubilized in 0.2 M phosphate buffer (pH 7.0). SDS-PAGE was carried out on 15% polyacrylamide gel. 1, 66°C, 30 min; 2, 80°C, 15 min; 3, 130°C, 2 s; 4, 140°C, 2 s.

major band corresponding to β -LG was observed on the gel.

4. Effect of heating milk on β -LG content in fresh cheeses

In order to identify the difference in aggregation of β -LG between heat-treated and untreated milk, fresh cheeses from both milks were analyzed both by ELISA and SDS-PAGE. No β -LG was detected by ELISA in the fresh cheese obtained from untreated bovine or caprine milk (Table 2). In contrast, the value of β -LG in the fresh cheese from heated milk (85°C) proved to be high. It was also revealed by SDS-PAGE analysis that the fresh cheese from untreated milk of bovine or caprine origin contained no β -LG (Fig. 4). The band intensities of β -LG detected by SDS-PAGE were relatively strong after heating at 85°C. Furthermore, the fresh cheese from untreated caprine milk was demonstrated to contain a

Table 2. Concentrations of β -lactoglobulin in fresh cheeses from heat-treated bovine and caprine milk.

Heating time (min)	Bovine ($\mu\text{g}/\text{mg}$ protein)	Caprine ($\mu\text{g}/\text{mg}$ protein)
0	ND	ND
5	216.0 $\pm$ 11.1	147.6 $\pm$ 9.7*
10	245.2 $\pm$ 15.0	191.5 $\pm$ 4.0*
20	246.9 $\pm$ 8.2	189.1 $\pm$ 8.3*
30	214.5 $\pm$ 4.8	184.7 $\pm$ 5.4*

The milk samples were heat treated in test tubes in a water bath at 85°C. At various time periods from 5 to 30 min, tubes were removed and immediately cooled in ice water to 4°C. Cheese samples were diluted in phosphate buffer and analyzed on ELISA.

Values are means $\pm$ SD ($n=3$).

ND: not detected.

* as bovine β -lactoglobulin.

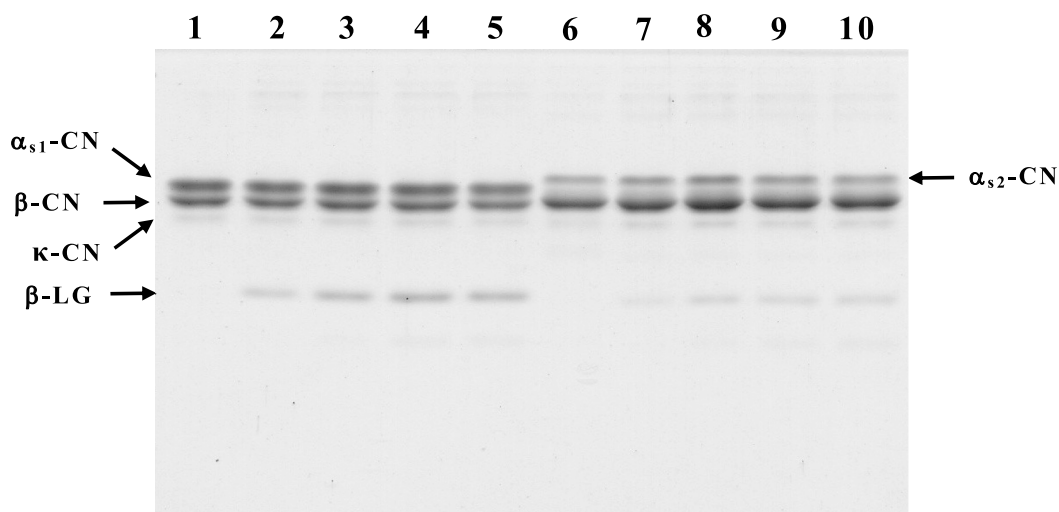


Fig. 4. SDS-PAGE analysis of fresh cheeses from heat-treated bovine and caprine milk. The milk samples were heat treated in test tubes in a water bath at 85°C. At various time periods from 5 to 30 min, tubes were removed and immediately cooled in ice water to 4°C. Fresh cheeses were prepared from heat-treated milk by isoelectric precipitation. Cheese samples were diluted in phosphate buffer and analyzed on SDS-PAGE. Milk: 1–5, bovine; 6–10, caprine. Heating time: 1, 0 min; 2, 5 min; 3, 10 min; 4, 20 min; 5, 30 min; 6, 0 min; 7, 5 min; 8, 10 min; 9, 20 min; 10, 30 min.

much lower amount of α_{s1} -casein.

Discussion

The allergenicity of bovine milk is related to heterogeneity of protein constituents, and β -LG is generally considered as the principal allergen because of its absence from human milk. The findings in this study may offer additional evidence for reduction of the allergenicity of milk.

In the present study, we developed a simple ELISA system using MAb for the quantification of β -LG in fresh cheese. The MAb reacted specifically with β -LG and there was no cross reaction with other milk proteins. The difference in reactivity with MAb between heat-treated and untreated bovine milk was not observed by the ELISA. The β -LG content in the sample was successfully determined by the ELISA. The detection limit of this method has proved to be 1 ppm. Some researchers have argued that foods containing more than 10 ppm of undeclared allergen might pose a significant threat to food-allergic individuals (22). This ELISA system would be a useful tool for evaluating finished products in the development of allergen-free food.

We summarized the values of β -LG content in fresh cheese from bovine milk which was heated in various ways (Table 1). Whey proteins containing β -LG can be separated by isoelectric precipitation, but fresh cheese from commercially available sterilized bovine milk contained a large amount of β -LG. It is probable that β -LG are precipitated from the whey because of their thermostability. It is well known that thermal treatment of milk induces changes in the composition of the proteins in whey and their aggregation as well (7, 8, 10, 23). These aggregates are acid precipitable (24–26). In the present study, the values of β -LG content of fresh cheeses were high when bovine milk was sterilized at higher temperatures. However, fresh cheeses from the milk sterilized at 66°C for 30 min also contained a fair amount of β -LG. It has been reported that β -LG, α -lactalbumin, bovine serum albumin, and the immunoglobulin unfold at temperatures above 65°C and interact with κ -casein to form heat-induced protein aggregates (27, 28).

In order to identify the difference in aggregation of β -LG between heat-treated and untreated milk, fresh cheeses from bovine milk were analyzed. No β -LG was detected by ELISA in the fresh cheese obtained from untreated bovine milk. In contrast, the β -LG content in the fresh cheese was high when bovine milk was heat treated. These results indicate that β -LG in unsterilized bovine milk can be separated by isoelectric precipitation. However, α_{s1} -casein still remains in the cheese and is also one of the major allergens of bovine milk, accounting for about one-third of the total protein. Previously, we demonstrated that the amount of α_{s1} -casein in the milk from the Japanese-Saanen goat, a major species in Japan, was very low. In the present study, no β -LG was detected in the fresh cheese from untreated caprine milk, implying that the thermolabile nature of whey proteins in caprine milk is similar to that in bovine milk. Henry et al. reported that a covalent com-

plex between β -LG and κ -casein in caprine milk is formed during heating (29).

As shown by the SDS-PAGE profiles (Fig. 4), fresh cheese from untreated bovine milk had major bands corresponding to α_{s1} -casein, β -casein and κ -casein, while that from untreated caprine milk had major bands corresponding to α_{s2} -casein, β -casein and κ -casein. The absence of the α_{s1} -casein band in caprine fresh cheese as well as the α_{s2} -casein band in bovine is probably due to its low content in each sample. These results indicate that fresh cheese from unsterilized caprine milk may be a protein source of low allergenicity. Many studies have been performed on the thermal stability of the β -LG allergen structures, and significant reduction in β -LG allergenicity was observed following the thermal procedures (30, 31). In our experimental condition, however, the content of β -LG in fresh cheeses was increased by heat treatment. It is possible that heat treatment of milk is responsible for the high allergenicity of β -LG in fresh cheeses.

In conclusion, we prepared fresh cheeses from caprine milk and β -LG content in cheeses was determined by ELISA using MAb. The fresh cheese from unsterilized caprine milk was found to contain little or no β -LG. The cheese might be useful for milk allergic patients, although low allergenicity should be confirmed by clinical tests such as RAST.

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