Interactions between Different Genetic Variants of β-Lactoglobulin and κ-Casein A during Heating of Skim Milk

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Milk samples (5 mL) from cows, homozygous for κ-casein A and either homozygous A ($n = 14$) or B ($n = 12$) or heterozygous AB ($n = 14$) for β-lactoglobulin (β-lg), were heated at 90 °C for 0−10 min, followed by ultracentrifugation at 100000*g*. The native whey proteins in the supernatant were thereafter analyzed by fast performance liquid chromatography (FPLC), and the decrease of whey proteins was recorded. The reaction orders for the heat-induced loss of β-lg were found to be 0.53, 0.65, and 0.91 for β-lg A, β-lg A+B, and β-lg B, respectively, in milk containing the A variant of κ-casein. The rate of heat-induced loss, expressed as the half-life ($t_{1/2}$) of the reaction, was found to be 340 and 270 s for β-lg A and B, respectively, in milk from homozygous cows. In milk from heterozygous cows (β-lg A+B), this value was 330 s. After correction for the casein number, the $t_{1/2}$ value for β-lg B was still notably lower than for β-lg A. The results, therefore, showed that the concentration of β-lg B decreased more rapidly than β-lg A in skim milk containing κ-casein A, probably due to differences in hydrophobicity between the genetic variants of β-lg.

Keywords: *β-Lactoglobulin; κ-casein; casein micelles; heat treatment; genetic polymorphism*

INTRODUCTION

Milk composition has an effect on the processing properties of milk and has therefore been the subject of numerous investigations. During the last decade, several investigations have focused on the genetic polymorphism of milk proteins due to the relationship found between the phenotypes of cows and the composition and yield of milk (McLean et al., 1984; Ng-Kwai-Hang et al., 1984, 1986, 1987; Kroeker et al., 1985a,b; Aleandri et al., 1990; van den Berg et al., 1992). All major milk proteins exist as different genetic variants and are transmitted by Mendelian inheritance without dominance (Aschaffenburg and Drewry, 1955; Brum et al., 1968). The genetic variants differ from each other by amino acid substitution(s) or deletion(s), which result in different net charges on the molecules and thereby also in differences in physico-chemical properties. Extensive studies have been undertaken on the influence of genetic variants of milk proteins on the cheesemaking properties of milk. Several reports have demonstrated a favorable effect of κ-casein (κ-cn) B on cheese making (Schaar, 1984; Schaar et al., 1985; Marziali and Ng-Kwai-Hang, 1986; Ng-Kwai-Hang et al., 1989; Jakob 1993).

Little information is available on the effect of genetic variants of milk proteins on the quality of fermented milk products. During the manufacture of fermented milk, the milk is heated to improve the rheological properties of the final product, i.e., increased water-holding capacity and improved "mouthfeel". The mechanism responsible for this effect has been explained by an agglomeration of whey proteins with casein micelles, as demonstrated using electron microscopy by Harwalkar and Kalab (1981), who showed that β-lactoglobulin (β-lg) filaments are formed and attached to the surface of casein micelles. The network layer was later shown to also contain α-lactalbumin (α-la) and milk salts, and its structure and properties are highly dependent on the heating temperature and pH (Harwalkar and Kaláb, 1981, 1986, 1988; Modler and Kaláb, 1983; Mottar et al., 1989). Initially, the network is caused by an interaction between β-lg and casein micelles (Mottar et al., 1989). The bonds involved are primarily intermolecular disulfide bonds between β-lg and κ-cn, but hydrophobic bonds also play a role (Smits and van Brouwershaven, 1980; Parnell-Clunies et al., 1988).

The agglomeration of whey proteins with casein micelles during heat treatment is influenced by different genetic variants of the milk proteins. Imafidon et al. (1991a,b) who studied the denaturation temperatures of different genetic variants of β-lg, individaually and in solution with different genetic variants of κ-cn, came to a conclusion that genetic polymorphism significantly influences the denaturation reaction of these proteins, also depending on the buffer characteristics. McKenzie et al. (1971) showed that κ-cn reacted faster with β-lg B than with β-lg A in cacodylate buffer solutions at pH 6.6 and 74 °C. These findings have been confirmed by Hillier and Lyster (1979), Dannenberg and Kessler (1988*a*) and Parnell-Clunies et al. (1988) during heat treatment of milk.

In the studies previously reported in the literature regarding the interactions during heating of different genetic variants of β-lg with casein, the genetic variants of κ-cn have been unknown. It is likely that the genetic κ-cn variants also will have an important influence on the reaction between β-lg and κ-cn. In the present investigation we have therefore studied the heat-induced loss of different genetic variants of β-lg in skim milk from cows homozygous for κ-cn A in order to obtain a better understanding of the heat-induced aggregation of β-lg and its subsequent interactions with casein micelles.

MATERIALS AND METHODS

Milk Samples. Skimmed milk from individual cows at the Experimental Dairy Farm of the Swedish University of Agricultural Sciences was used for the experiments. The cows

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Table 1. Combinations of Genetic Variants of Milk Proteins Used in the Experiments

β-lactoglobulin	AA (14)	AB (14)	BB (12)
κ-casein	AA (14)	AA (14)	AA (12)
α_{s1}-casein	BB (12) or BC (2)	BB (11) or BC (3)	BB (11) or BC (1)
α_{s2}-casein	AA (14)	AA (14)	AA (12)
β-casein	A1A1 (3), A1A2 (9), A2A2 (1), or A1A5 (1)	A1A1 (3), A1A2 (8), A2A2 (1), A2A5 (1), or A2B (1)	A1A1 (7) or A1A2 (5)
α-lactalbumin	AA (14)	AA (14)	AA (12)

were of the Swedish Red and White (SRB, $n = 18$), Swedish Friesian (SLB, $n = 20$), and Swedish Jersey (SJB, $n = 2$) breeds. The phenotypes of the cows were determined by isoelectric focusing within the pH range 4−6.5 using Phastgel electrophoresis equipment (Pharmacia AB, Uppsala, Sweden), according to Bovenhuis and Verstege (1989). The phenotypes of the different milk proteins of the cows are given in Table 1. Only cows with κ-casein AA phenotype were used and they were divided into three groups depending on the β-lg phenotype, AA ($n = 14$), AB ($n = 14$), and BB ($n = 12$). The phenotypes for α-la and α_{s2}-casein were AA for all cows, while the phenotypes for α_{s1}- and β-caseins were combinations of the genetic variants B, C and A1, A2, B, respectively. Two milk samples from each cow were analyzed. The samples represented different lactation numbers and different stages of lactation.

The total protein, the casein and the whey protein concentrations were determined for all skim milk samples using a Milkoscan 93 NIR spectrophotometer (A/S N. Foss Electric, Hillerød, Denmark). The whey protein concentration was determined after that casein had been precipitated by rennet and the casein concentration was calculated as the difference between total protein and whey protein concentrations. The casein number (cn no.) was calculated as the ratio between the casein and total protein concentrations.

Sample Treatment. Skim milk samples (5 mL) were heated rapidly to 90 °C in a shaken thermoblock and held at this temperature for 0−10 min, at 1 min intervals. Immediately after the heat treatment, the samples were cooled to 22 °C in cold water to stop the heat-induced reactions. Casein micelles with aggregated whey proteins were separated from unaggregated whey proteins by ultracentrifugation at 100000*g* for 1 h at 5 °C. Samples of supernatant were taken by pipette, thereby avoiding the casein pellet or residual fat on the pellet surface, and frozen at −70 °C until further analysis.

Whey Protein Analysis. After thawing, the native whey proteins in the supernatant were analyzed by fast protein liquid chromatography (FPLC; Pharmacia, Uppsala, Sweden) using a Mono Q HR/R 5/5 column at a flow rate of 1.0 mL/min (modified after Humphrey and Newsome, 1984). Before the samples (500 μL) were injected onto the FPLC column, they were diluted 11-fold in a 0.02 M piperazine-HCl buffer (pH 6.2) and filtered through a 0.45 μm filter. Elution was by a linear NaCl gradient, 0.0−0.4 M, in a 0.02 M piperazine-HCl buffer (pH 6.2) over 25 min. The eluates were quantified by measuring the absorbance at 280 nm using a UV-1 monitor (Pharmacia, Uppsala, Sweden) connected to a PC. For each ultracentrifuged sample, duplicate FPLC analysis was performed.

Extinction coefficients at 280 nm were determined to be 0.215 and 0.220 cm^{-1}, respectively, for β-lg A and B, which were prepared from whey (FPLC separation, dialysis, and lyophilization) from cows homozygous for the respective β-lg variant. The extinction coefficients found were used to calculate the β-lg concentrations in the supernatants from the FPLC chromatograms using the JCL6000 Chromatography Data System (Jones Chromatography, Mid-Glamorgan, U.K.) on the basis of area integration.

Calculations. The concentrations of native β-lg at every time point were grouped according to the genetic combination of β-lg/κ-cn in the sample. Mean values of every group were used for the kinetic evaluations. The reaction order for the loss of native β-lg during heating was determined for the different genetic variants by plotting the above means in the coordinates log c_t versus log v_t (c_t is the β-lg concentration at time t, and v_t is the reaction rate at time t). The slope of the line obtained from the plot, using the least squares method for best fitting, was equal to the reaction order. To facilitate comparison of the rate of the heat-induced loss of different genetic variants of β-lg, the reaction half-life ($t_{1/2}$) values of all three data sets (β-lg A, β-lg B, and β-lg A+B) were calculated.

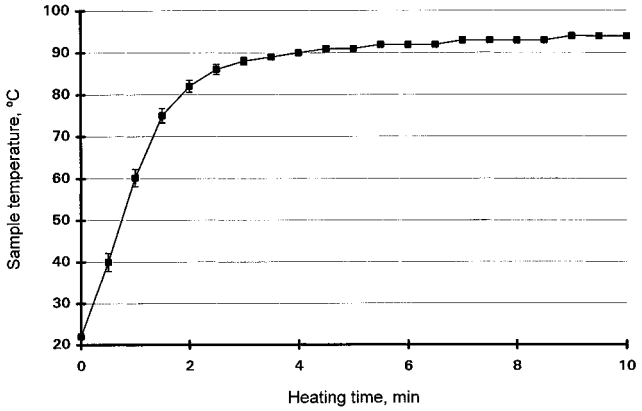

Figure 1. Temperature of samples (5 mL) during heating in a shaken thermoblock (112 °C).

RESULTS

There was relatively little variation in pH of the skim milk samples, all being within the range of 6.67−6.70 prior to heating. The rate of increase in temperature for a 5 mL sample, heated in a thermoblock placed on a shaker, is shown in Figure 1.

Figure 2 shows an FPLC chromatogram of α-la, β-lg B, and β-lg A in the ultracentrifuged supernatant from skim milk samples from a heterozygous cow heat-treated for different times; satisfactory resolution of α-la and the β-lg variants A and B was obtained. Due to its higher net negative charge, β-lg A was eluted later than β-lg B from the anion exchange column. There was no significant decrease of the concentration of α-la in the supernatant from ultracentrifuged skim milk samples heat-treated at 90 °C for 10 min. As shown in Figure 2, the heights of the void volume peak, α-la peak, and an unidentified peak were equal in the unheated sample and the sample heated for 7 min while much of the β-lg was lost from the supernatant.

The decrease in the concentration of β-lg A and B in skim milk, from cows homozygous (AA or BB) or heterozygous (AB) for β-lg, after heating at 90 °C is shown in Figure 3. During the first 2 min of heating, no significant decrease in β-lg concentration was found, which was probably related to the temperature−time curve shown in Figure 1. Between 3 and 7 min, a significant decrease in the concentration of native β-lg was observed: about 95% for β-lg B and 70% for β-lg A, the decrease starting earlier for β-lg B. The rate of decrease of β-lg in milk from the heterozygous cows (i.e., β-lg A+B) was closer to β-lg A rather than B.

The reaction orders for the heat-induced loss of β-lg was found to be 0.53, 0.65, and 0.91 for β-lg A, β-lg A+B, and β-lg B, respectively, in milk containing the A variant of κ-cn (Figure 4). The distinct difference in

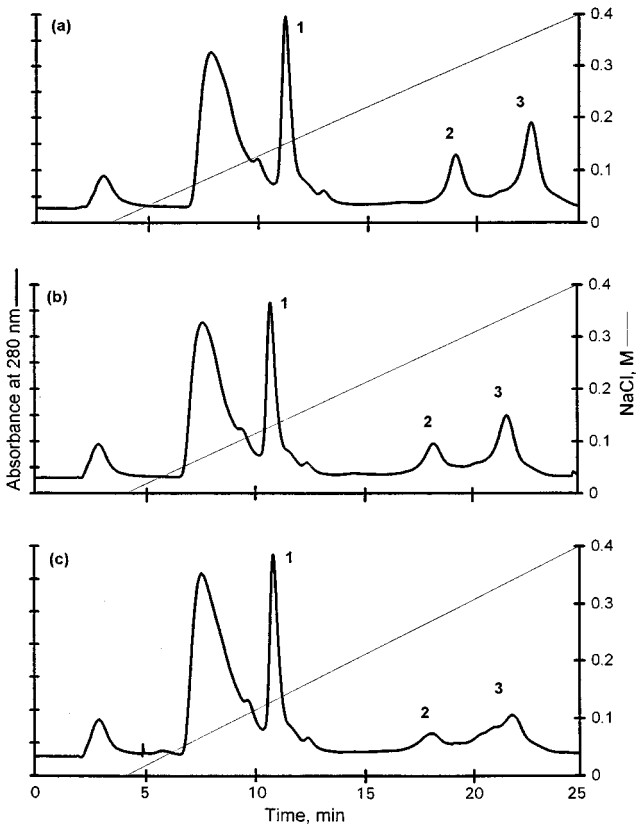

Figure 2. FPLC (Mono Q) chromatograms of ultracentrifuged supernatants from skim milk samples from a heterozygous cow heated at 90 °C for 0 (a), 5 (b), or 7 min (c). (—) Protein; (—) NaCl. Peak 1, α-lactalbumin; peak 2, β-lactoglobulin B; peak 3, β-lactoglobulin A. For details of the chromatographic conditions, see Materials and Methods.

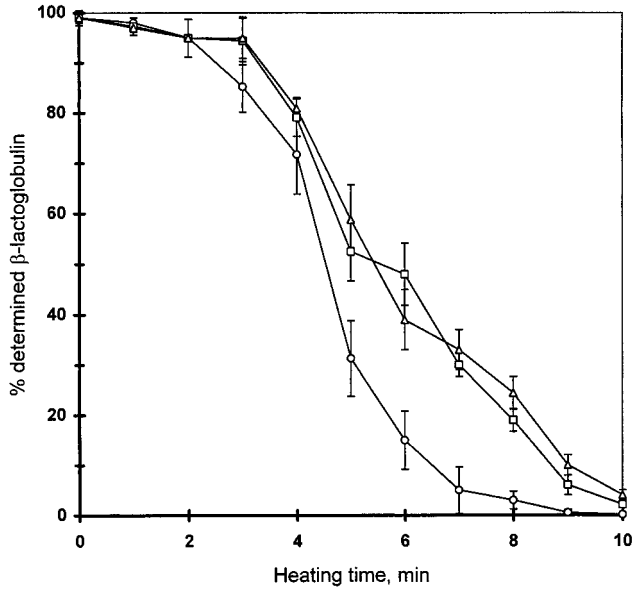

Figure 3. Decrease in the concentration of β-lactoglobulin in the ultracentrifugal supernatant from skim milk after heating at 90 °C for 0−10 min. (□) β-lg A; (○) β-lg B; (△) β-lg A+B.

calculated reaction order found for β-lg B compared to A and A+B was supported by high regression coefficients for β-lg A and β-lg B.

The total protein concentrations in the milk from the β-lg AA, β-lg AB, and β-lg BB phenotypes cows were, on average, 3.38%, 3.27%, and 3.29%, respectively, i.e., the total protein concentration in β-lg AA milk was higher than that in β-lg AB and β-lg BB milks. However, the casein concentrations in the samples were approximately the same (between 2.75% and 2.79%), thus resulting in a higher casein number (cn no.) for β-lg BB milk. In Table 2, the calculated $t_{1/2}$ values, as well as the $t_{1/2}$ values corrected for the cn no., are listed for the three phenotypes. For the sake of clarity, the $t_{1/2}$ values are also indexed, taking the value for the β-lg AA phenotype equal to 1.00. As seen from the indexed $t_{1/2}$ values, both corrected for the cn no. and not, the rate of heat-induced loss of native β-lg B was found to be clearly higher than that for β-lg A.

Table 2. Rate of Heat-Induced Loss Expressed as the Half-Life ($t_{1/2}$) of Native β-lg in Supernatants of Skim Milk Heated at 90 °C

β-lg/κ-cn	AA/AA	AB/AA	BB/AA
$t_{1/2}$, s	340 ± 12.0	330 ± 12.8	270 ± 29.1
$t_{1/2}$, indexed	**1.00** ± .035	**0.97** ± 03.5	**0.79** ± .085
cn no.	0.82 ± .02	0.84 ± .02	0.85 ± .05
$t_{1/2}$/cn no.	415 ± 16.6	393 ± 17.4	318 ± 24.7
$t_{1/2}$/cn no., indexed	**1.00** ± 04.0	**0.95** ± .042	**0.77** ± .060

DISCUSSION

The thermal denaturation of whey proteins, i.e., β-lg and α-la, and their subsequent interaction with caseins have been studied thoroughly over many years. However, the different experimental conditions used, make comparison of results difficult. Details regarding this topic are found in the comprehensive reviews of Jelen and Rattray (1995), Mulvihill and Grufferty (1995), and Singh (1995). The results presented in this paper show the rate of heat-induced loss of the genetic variants of β-lg (A and B) in skim milk containing exclusively κ-cn A during heating at 90 °C. However, these results involve all the complex reactions that occur between the proteins, carbohydrates and salts during in-vat heat treatment of skim milk.

Only α-la A was present in the samples investigated and no significant decrease in its concentration was found in the milk serum after heat treatment at 90 °C for 10 min (Figure 2). This conflicts with the findings of Parnell-Clunies et al. (1988), who found that about 75% of the α-la in milk was denatured after 10 min at 85 °C. However, Parnell-Clunies et al. (1988) acidified the samples to pH 4.6 after the heat treatment, while our samples were cooled to 22 °C until ultracentrifuged at 5 °C. Since Rüegg et al. (1977) showed that the denaturation of α-la is reversible, holding time of our samples at lower temperatures thus gave the denatured α-la a possibility to renature. Renaturation of α-la has also been observed by Baer et al. (1976) and de Wit (1990).

The larger peak of β-lg A compared with β-lg B (Figure 2) indicates a higher concentration of β-lg A in the milk from cows heterozygous for β-lg. This is in agreement with the results presented by Ford et al. (1993), who found, on average, 1.2 times more β-lg A than β-lg B in milks from heterozygous cows.

Heat-induced protein complexes in milk include all major whey proteins and κ-cn (Creamer et al., 1978), but the minimum requirement for the formation of complexes is the presence of β-lg and κ-cn (Smits and van Brouwershaven, 1980). Since Dalgleish (1990) has reported a direct relationship between the formation of β-lg/κ-cn aggregates and the loss in milk serum protein concentration, the decrease of β-lg in the milk serum samples in our experiments could thus be related as aggregated with κ-cn in the following discussion.

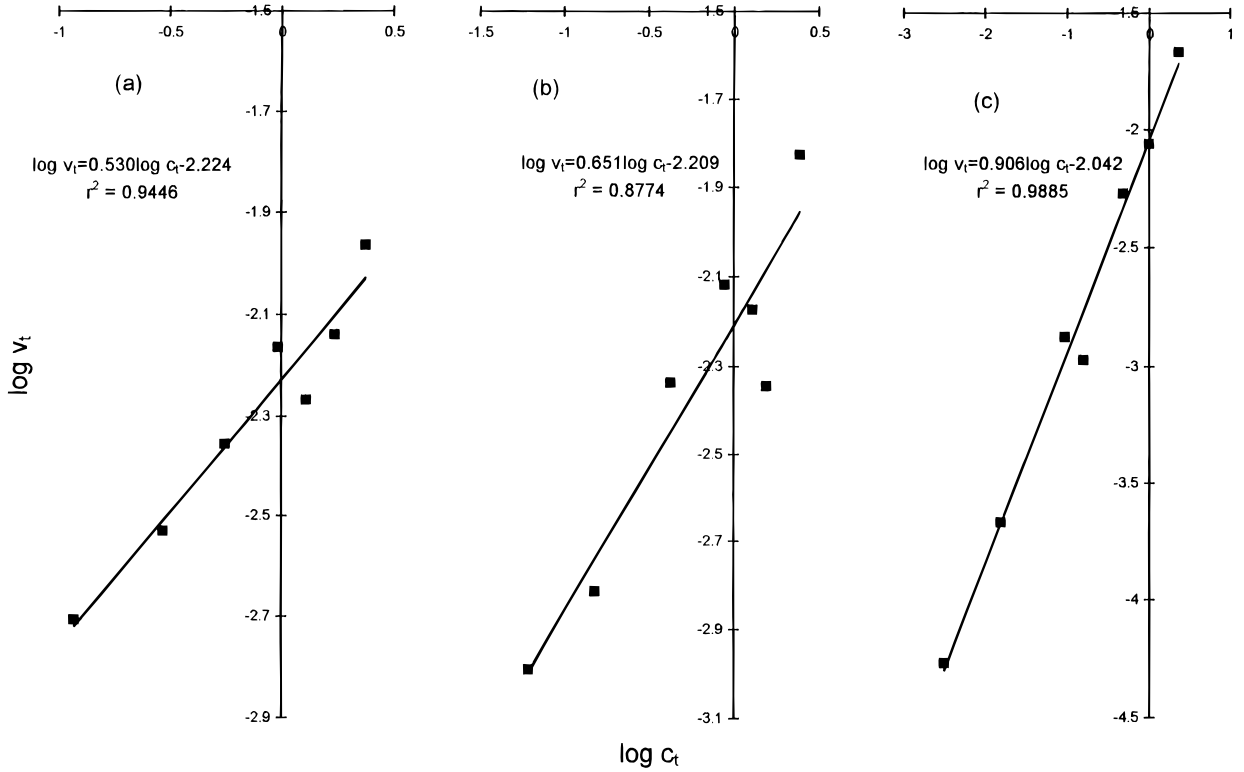

Figure 4. Decrease with time in the concentration of β-lactoglobulin A, B, and A+B in the supernatant of ultracentrifuged skim milk after heat treatment at 90 °C, expressed in the coordinates log v_t versus log(c_t/c_o). (a) β-lg A; (b) β-lg A+B; (c) β-lg B.

McKenzie et al. (1971) suggested that the interaction between β-lg and κ-cn does not follow true first- or second-order kinetics. Dalgleish (1990) proposed that the reaction follows pseudo-first-order kinetics, while in investigations by Lyster (1970) and Hillier and Lyster (1979), the reaction order was found to be 2. Dannenberg and Kessler (1988b) found that the order of denaturation of β-lg in skim milk was 1.4 for β-lg A and 1.5 for β-lg B. One major reason for the different results found of the aggregation kinetics might be different experimental conditions in addition to mixed genetic variants of both β-lg and κ-cn in the earlier experiments. Our results showed that the kinetics of aggregation of β-lg with κ-cn followed a reaction order of ∼0.5 for the A and A+B variants of β-lg, while the reaction order was ∼1.0 for β-lg B. These data clearly give reason to believe that the reaction is so complex that it cannot be described with a particular reaction order and one can only propose that the calculated reaction orders are pseudo-orders.

The differences in reaction order and reaction rate for decrease of native β-lg may depend on availability of thiol (-SH) groups in respective genetic variant of β-lg, since one of the amino acid substitutions is located close to the free thiol group (McKenzie et al., 1972). Phillips et al. (1967), however, have shown that the reaction rate with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is similar for both β-lg A and β-lg B, i.e., the available thiol groups after denaturation are the same for both genetic variants. Furthermore, Kirchmeier et al. (1985) demonstrated that the maximum number of available thiol groups was formed within 1 min of heating milk at 90 °C, while most of the aggregation of β-lg with casein micelles occurred after heating for 3 min. This implies that the shorter $t_{1/2}$ of the loss of native β-lg B than β-lg A in the milk serum shown in this investigation, must be due to other properties of the genetic variants of β-lg than the number of available thiol groups.

Casein micelles in milk from cows homozygous for β-lg B have been found to be smaller than those from cows homozygous for β-lg A (Devold et al., 1994). Therefore the former have a larger total surface and a relatively higher content of κ-cn (Schmidt and Payens, 1976; Schmidt, 1980) than casein micelles in milk from β-lg AA phenotype cows. In addition, Mohammad and Fox (1987) have found that more β-lg precipitated onto the surface of small casein micelles than on the surface of larger ones after heating the serum protein free casein micelles in the same solution with β-lg at 130 °C for 10 min. However, the average casein concentrations in the milk of the three phenotypic groups in this investigation were not found to be significantly different. The relative amount of κ-cn was not determined in the milk samples and any influence of small casein micelles (e.g., high κ-cn content) on the shorter $t_{1/2}$ of heat-induced loss of native β-lg B could thus not be concluded from the results in this paper, although smaller micelles could have been expected in the β-lg BB milk than in the β-lg AA milk.

The difference between β-lg A and B is the substitution of Asp_{64} and Val_{118} in variant A by Gly_{64} and Ala_{118} in variant B (Bell et al., 1981). This results in a higher net hydrophobicity for the B variant, since glycine and alanine are more hydrophobic than aspargine and valine (Kyte and Doolittle, 1990). Haque and Kinsella (1987) found increased surface hydrophobicity for β-lg and κ-cn in separate solutions and a reduced surface hydrophobicity in a mixture of β-lg and κ-cn at increased temperatures. These findings suggest that the difference in the $t_{1/2}$ of heat-induced loss of β-lg A and β-lg B during β-lg–κ-cn-complex formation, reported in this investigation, may be related to the difference in hydrophobicity of the genetic variants of β-lg. A larger total surface of casein micelles in milk from β-lg BB phenotype cows, as discussed above, would also enhance any effect

caused by hydrophobicity of the proteins and thus result in a more rapid aggregation of β-lg B with κ-cn A.

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