

Effects of κ -casein glycosylation on heat stability of milk

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A study was conducted to determine the relationship between the degree of glycosylation of κ -casein (CN) and heat stability of milk at 140°C between pH 6.2 and 6.9. Morning milk samples from individual Holstein cows (genotypes κ -CN AA, β -CN A₁A₂, α _{s1}-CN BB, β -lactoglobulin (LG) AB) in mid-lactation were collected and analyzed for protein and somatic cell count. Samples of low somatic cell count (<150 000 cells/ml) were skimmed and dialyzed overnight at 4°C against bulk milk to equilibrate soluble components. In the first series of experiments, the heat clotting time vs pH (HCT–pH) curves were determined for 37 individual milk samples having various degrees of glycosylation of κ -CN, estimated through the *N*-acetylneuraminic acid (NANA) content in μ g/mg of κ -CN (NANA/ κ -CN). The mean NANA/ κ -CN, HCT_{max}, and HCT_{min} were 50.3 ± 22.3 μ g/mg, 17.4 ± 1.2 min and 3.3 ± 0.7 min, respectively. The results of statistical analysis showed that the variations in the degree of glycosylation of κ -CN in normal milk did not significantly affect ($P > 0.1$) the heat stability parameters. The effect of NANA depletion on the HCT–pH profile was tested in the second series of experiments. HCT–pH profiles of 14 individual milk samples, untreated and neuraminidase-treated to extensively remove NANA associated with κ -CN, were compared. The average NANA/ κ -CN before treatment, the HCT_{max}, and HCT_{min} were 78.3 ± 24.9 μ g/mg, 21.4 ± 1.5 min and 3.6 ± 0.8 min, respectively. As no effect of the desialylation was observed for HCT_{min} and pH_i, and as the effect on HCT_{max}, although significant ($P \leq 0.05$), was very low (0.7 min on average), these results indicate that the charges and the extent of hydrophilicity of the heat-induced κ -CN/ β -LG complexes are not the crucial factors for the production of κ -CN depleted micelles upon heating and that the glycosylation of κ -CN does not affect its heat-induced interaction with β -LG.

Keywords: κ -Casein, glycosylation, β -lactoglobulin, heat stability, milk.

INTRODUCTION

κ -Casein (CN) is a glycoprotein and, in milk, there is a microheterogeneity in the population of κ -CN based on the number (Kanamori *et al.*, 1980; Zevaco & Ribadeau-Dumas, 1984) and the type of oligosaccharides (Fournet *et al.*, 1979; Van Halbeek *et al.*, 1980) existing on the protein. This produces glycosylation variants of κ -CN. Within a milk sample, all the glycosylation variants are present to differing extents and a mean degree of glycosylation of κ -CN can be estimated, which can

vary greatly from milk to milk. As shown by Robitaille *et al.* (1991), the mean degree of glycosylation of κ -CN, estimated through the *N*-acetyl neuraminic acid content per mg of κ -CN (NANA/ κ -CN), is highly variable ranging from 23 to 166 μ g/mg depending on genetic and environmental factors. Although no specific roles have been assigned to the sugar moieties on κ -CN, some effects on the milk and on the functional properties of purified κ -CN have been observed. Glycosylation is known to affect milk yield (Robitaille *et al.*, 1991) and the chymosin-induced milk coagulation parameters (Robitaille *et al.*, 1993). Moreover, it affects several functions of κ -CN in an artificial micelle system such as its stabilizing activity toward the other CN (Takeuchi *et al.*, 1985; Khalifa *et al.*, 1986a), the micelle size (Doi *et*

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al., 1979a; Khalifa *et al.*, 1986b) and its susceptibility to chymosin proteolysis (Doi *et al.*, 1979b; Vreeman *et al.*, 1986; Haque & Khalifa, 1992).

The protective barrier at the micelle surface, the hairy layer (Holt & Dalgleish, 1986), that prevents micelle aggregation by a steric stabilization mechanism, is mainly made of the C-terminal portion of κ -CN, the region of the molecule that carries the oligosaccharides. Interest thus arises if the presence of sugars, such as NANA, an acidic sugar, can change the thermostability of milk by affecting the level of hydration and zeta potential of the hairy layer. The purpose of this research was to determine whether the extent of glycosylation of κ -casein of natural milk or the enzymatic treatment to decrease the sugar content of κ -CN had any consequences on the thermal stability of milk heated at 140°C.

MATERIALS AND METHODS

A group of 12 cows in mid-lactation (50 to 250 days in milk) was selected within the Macdonald campus (McGill University) Holstein herd on the basis of milk proteins genotype (α -CN BB, β -CN A₁A₂, κ -CN AA, β -lactoglobulin (LG) AB), and α -lactalbumin (LA) AA. Milk protein phenotype determination was carried out by urea-PAGE (Ng-Kwai-Hang *et al.*, 1984).

On test day, bulk milk and milk from individual cows were collected during the morning milking, rapidly chilled and kept at 4°C. Somatic cell content was measured by a Fossomatic cell counter (Foss. Electric, Hillerod, Denmark) and samples having more than 150 000 somatic cells/ml were discarded. Milk samples were analyzed for fat and protein content with a Multi-spec II infrared analyzer (Multispec Ltd, Weldrake, UK), for total CN by the amido black dye-binding method (Ng-Kwai-Hang & Hayes, 1982), and the CN number [total CN (g/100 ml of milk)/total protein (mg/ml of milk) $\times$ 100] was calculated. The $\alpha_{s1+s2} - (\alpha_s)$, β - and κ -casein content of the whole casein, in % (w/w), and the β -LG and α -LA content of whey proteins fraction, in % (w/w), were determined by densitometry after urea-PAGE (Ng-Kwai-Hang & Kroeker, 1984). The mean NANA content, in μ g/mg of κ -CN (NANA/ κ -CN), that was used to evaluate the glycosylation extent of κ -CN, was measured as described by Robitaille *et al.* (1991). Briefly, NANA concentration was determined by the colorimetric periodic acid-thiobarbital assay (Aminoff, 1961) on CN fraction that was isoelectric precipitated, washed, and redissolved in water at 20 mg/ml.

On each test day, 5 to 6 individual milk samples having protein content close to 3.1% (w/w) were selected to determine the heat stability of natural milk or 3 milk samples selected for their high NANA/ κ -CN content were analyzed to study the effect of a neuraminidase

treatment on heat stability of milk. Milk samples were skimmed and subsamples were dialyzed against skimmed bulk milk, precisely adjusted at pH 6.2 and pH 6.9 with 1N HCl or 1N NaOH, respectively. The dialysis was carried out overnight at 4°C in the dark using a dialysis bag having molecular weight cut-off of 12 000–14 000 Da (Spectrum, Los Angeles, USA). The following morning, the two subsamples were warmed to room temperature for 2 h and mixed to obtain 6 to 9 different milk pH values. To compare the heat stability of normal and neuraminidase-treated milk, an aliquot of each milk was treated with *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St Louis, USA) at 0.04 U/ml for 2 h at 37°C. The treated aliquot and an untreated sample were then dialyzed and tested as previously described.

Subjective heat stability at 140°C was measured according to the method of Davis & White (1966). Briefly, 1.5 ml aliquots of skimmed milk were put in 3 ml Pyrex test tubes (10 $\times$ 0.75 cm) and the tubes sealed with a silicone rubber stopper and clamped in a rack. The rack held 6 tubes. The tubes were immersed in a thermostatically controlled oil bath maintained at 140 $\pm$ 0.1°C using a Julabo, VC immersion circulator heater (Canlab, Montreal, Canada), and were rocked over an arc of 60° in a horizontal plane with an oscillation of 8 cycles/min. For each milk sample, the heat clotting time (HCT), defined as the time that elapses before the first sign of protein coagulation, was evaluated at 6–9 different pHs, from pH 6.2 to pH 6.9. A curve in which HCT is plotted versus initial milk pH was drawn and the heat stability parameters were deduced: HCT maximum (HCT_{max}) occurring at about pH 6.6, the HCT minimum (HCT_{min}) obtained around pH 6.8, and the pH of inflection (pH_I) between HCT_{max} and HCT_{min}.

The general linear models procedure (PROC GLM) of SAS (SAS, 1988) was used to analyze heat stability parameters of natural milk and to compare the heat stability parameters of milks that were untreated and neuraminidase-treated.

The heat stability determination of natural milk was carried out on 37 milk samples (9 test days). The model statement used test day and cow as classified data and NANA/ κ -CN as well as casein number as covariables. The effect of test day, cow, NANA/ κ -CN (μ g/mg) and casein number on HCT_{max}, HCT_{min}, and pH_I were determined.

To study the effect of the desialylation, 14 milk samples were analyzed (5 test days). Intact (treatment 1) and neuraminidase-treated milk (treatment 2) were compared using an analysis of covariance model with treatment as repeated factor, adjusted for cow and NANA/ κ -CN. Interactions NANA/ κ -CN by treatment and cow by treatment were included into the model and tested at the 5% level of significance. If one of the interactions was found not significant, it was removed from the model and treatments were compared using

the reduced model. The same model was repeated by substituting cow by test day.

RESULTS

In the first series of experiments, 37 natural milk samples were analyzed for their heat stability. Overall means and standard deviation for milk protein composition for these samples are presented in Table 1. The mean NANA/ κ -CN was 50.3 ± 22.5 $\mu\text{g}/\text{mg}$ with a range from 22 to 105. Typical HCT/pH profiles of type

Table 1. Overall mean and standard deviation of protein composition of individual milks ($n = 37$)

Components	Mean	Standard deviation
Protein (%)	3.1	0.1
Casein number (%)	80.4	3.8
α_s -casein (%)	47.4	3.3
β -casein (%)	44.1	3.8
κ -casein (%)	8.5	1.8
β -lactoglobulin (%)	63.4	5.6
α -lactalbumin (%)	17.9	2.2
NANA/ κ -casein ($\mu\text{g}/\text{mg}$) ^a	50.3	22.5

^aNANA/ κ -casein: *N*-acetylneuraminic acid content in μg per mg of κ -casein.

Table 2. Mean squares and *F* values for the effect of test day, *N*-acetylneuraminic acid content of κ -casein, and casein number on the heat stability parameters (HCT_{max}^a, HCT_{min}^b, pH_I^c)

Source	Degrees of freedom	Mean square	<i>F</i>
HCT_{max}			
Test day	8	3.6700	4.60**
Cow	6	1.0200	1.28
Casein number	1	0.3072	0.38
NANA/ κ -casein ^d	1	2.7663	3.47
HCT_{min}			
Test day	8	0.8624	1.98
Cow	6	0.5652	1.30
Casein number	1	0.1688	0.39
NANA/ κ -casein	1	0.4143	0.95
pH_I			
Test day	8	0.0012	3.70**
Cow	6	0.0014	4.28**
Casein number	1	3.41×10^{-5}	0.10
NANA/ κ -casein	1	5.88×10^{-5}	0.18

** $P \leq 0.01$.

^aHeat clotting time at the maximum of stability reached at pH ~6.7.

^bHeat clotting time at the minimum of stability reached at pH ~6.8.

^cpH of the inflexion point of the HCT-pH curve between HCT_{max} and HCT_{min}.

^dNANA/ κ -casein: *N*-acetylneuraminic acid content in μg per mg of κ -casein.

A (Rose, 1961*a,b*) were obtained for all milk analyzed. The mean for HCT_{max} and HCT_{min} was 17.4 ± 1.2 min (range: 14.4–19.4 min), and 3.3 ± 0.7 min (range 2.0–6.3 min), respectively, with a pH_I centered at 6.74 ± 0.03 (range: 6.6–6.78). The analysis of variance to test the significance of the effect of NANA/ κ -CN on HCT_{max}, HCT_{min}, and pH_I is presented in Table 2. Test day had a significant effect on HCT_{max} ($P \leq 0.01$), both cow and test day had an effect on pH_I ($P \leq 0.01$) and none of the covariables tested were found to affect HCT_{min}.

The effects of κ -CN glycosylation were studied by comparing the heat stability parameters of 14 untreated and enzymatically desialyzed milks. The overall mean value for NANA/ κ -CN was 78.3 ± 24.9 $\mu\text{g}/\text{mg}$ and the extent of NANA removal was 76.9%. Typical HCT/pH profiles of type A (Rose, 1961*a,b*) were also obtained and the mean values for HCT_{max}, HCT_{min} and pH_I were 21.4 ± 1.5 min, 3.6 ± 0.8 and 6.74 ± 0.04 , respectively, for untreated milk and 20.7 ± 1.1 min, 3.7 ± 1.0 and 6.74 ± 0.03 , respectively, for treated milk. The statistical analysis is presented in Table 3. As the interactions NANA/ κ -CN by treatment and cow by treatment were not significant ($P > 0.1$), they were removed from the model and treatments were compared using the reduced model. Treatments were found to significantly affect ($P \leq 0.05$) HCT_{max} while cow and test day had a significant effect ($P \leq 0.01$) on HCT_{min} and pH_I, respectively.

Table 3. Mean squares of *F* values for the effect of neuraminidase treatment of milk on the heat stability parameters (HCT_{max}^a, HCT_{min}^b, pH_I^c)

Source	Degrees of freedom	Mean square	<i>F</i>
HCT_{max}			
Cow	3	1.5171	0.45
Test day	4	4.4687	1.93
NANA/ κ -casein ^d	1	2.2309	4.20
Treatment	1	4.0066	7.53*
HCT_{min}			
Cow	3	4.5071	8.30**
Test day	4	0.2650	0.13
NANA/ κ -CN	1	0.0544	0.38
Treatment	1	0.0238	0.17
pH_I			
Cow	3	0.0007	0.28
Test day	4	0.0072	39.01**
NANA/ κ -CN	1	4.5×10^{-5}	0.15
Treatment	1	0.0001	0.30

* $P \leq 0.05$.

** $P \leq 0.01$.

^aHeat clotting time at the maximum of stability reached at pH ~6.7.

^bHeat clotting time at the minimum of stability reached at pH ~6.8.

^cpH of the inflexion point of the HCT-pH curve between HCT_{max} and HCT_{min}.

^dNANA/ κ -casein: *N*-acetylneuraminic acid content in μg per mg of κ -casein.

DISCUSSION

In the aim of measuring the effects of κ -CN glycosylation on thermostability of milk, the HCT-pH curves of 37 individual milks having various NANA/ κ -CN were determined and the consequences of a neuraminidase treatment on the HCT-pH curves of 14 individual milks were analyzed. To minimize compositional differences between milk samples that are known to probably affect the HCT-pH curve (Fox, 1982 for review; McLean *et al.*, 1987) milk samples were carefully selected at each test day. Milk samples were obtained from healthy cows (somatic cell count <150 000/ml) in mid-lactation (50 < day < 250), that were genotypically identical for caseins, β -LG and α -LA. Moreover, protein content of milks that were analyzed was very similar, and dialysis against bulk milk was used to adjust the concentration of small dialyzable components such as urea, lactose and salts.

In the first series of experiments, the mean NANA content of κ -CN was 50.3 μ g/mg, the minimum and maximum NANA/ κ -CN values that were obtained corresponded to about 1.5 and 6.7 moles NANA/moles κ -CN, respectively. The statistical analysis of the effects of NANA/ κ -CN on heat stability parameters for natural milks suggested that the extent to which κ -CN is glycosylated does not affect the overall thermostability of the milk. Indeed, neither HCT_{max}, HCT_{min} nor pH_I varied significantly ($P > 0.1$) with NANA/ κ -CN. These results suggest firstly that if the hairy layer is modified by the glycosylation extent of κ -CN, it does not result in a change in the heat stability of milk. Secondly, these results support the idea that the initial interaction between κ -CN and β -LG is hydrophobic in nature (Doi *et al.*, 1987; Haque & Kinsella, 1988). Indeed, the κ -CN population carrying an average of 6 moles NANA/mole of κ -CN should be as efficient as a κ -CN population having only 3 moles NANA/mole of κ -CN to interact with β -LG and to form a stable κ -CN/ β -LG complex.

The heat stability of milk has been the subject of many investigations and the importance of electrostatic repulsions between the κ -CN/ β -LG complex and the micelles for the solubilization of micellar κ -CN resulting in κ -CN-depleted micelles that rapidly aggregate, was always highlighted. For instance, Singh & Fox (1987a,b) reported dramatic changes in the HCT-pH curve of reconstituted milk after chemical modification of micelles CN or β -LG to alter the net charge. The second series of experiments in the present study were conducted to study that point. We were able to extensively modify the charge and hydrophobicity of the micelle surfaces by the enzymatic depletion of NANA which decreases the length of the oligosaccharidic side chain associated to κ -CN by up to 33%, and remove up to 10 negative charges on the κ -CN molecule. The statistical analysis of the data shows an effect of neuraminidase treatment

on HCT_{max} only ($P \leq 0.05$). Considering that the difference in HCT_{max} obtained between untreated and treated samples was very small (~ 0.7 min) and that HCT_{min} and pH_I were not affected at all, our results do not support the conclusion of Singh & Fox (1987a,b). The variations in charges carried by the micelle surface are of little importance for the heat stabilization at pH < 6.8 and destabilization process at pH > 6.8 brought about by κ -CN/ β -LG complex formation. The discrepancy between our results and those of Singh & Fox (1987a,b) can be explained by the fact that the CN interactions are not affected by the enzymatic treatment. This is not the case in the study of Singh & Fox (1987a,b) where the milk protein backbone was chemically modified by succinylation or amidation of amino acids.

In conclusion, substantial changes in the micelle surface charge and hydrophilicity are necessary to modify the interactions between κ -CN and β -LG, the dissociation rate of heat-induced κ -CN/ β -LG complexes from micelles, and the heat-induced micelle aggregation.

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