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GENETIC POLYMORPHISM OF KAPPA CASEIN AND CASEIN MICELLE SIZE IN THE BULGARIAN RHODOPEAN CATTLE BREED

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Abstract: The present study aimed to compare the size of casein micelle in cow milk sample in function of kappa casein (*CSN3*) genetic polymorphism. Sixteen cows from Bulgarian Rhodopean cattle breed were genotyped by PCR-RFLP analysis. Milk samples from the three found *CSN3* genotypes (AB, AA and BB) were employed for the determination of casein micelles size by Dynamic Light Scattering (DLS). The results showed differences in the size and polydispersity of the casein micelles between the milks of cows with different genotypes. Hydrodynamic radii of micelles at a scattering angle of 90 °C varied from 80 to 120 nm and polydispersity varied from 0.15 to 0.37. In conclusion casein micelle size of *CSN3* AA cows (~ 120 nm) exceed with about 60% cows with AB (~ 80 nm) and BB genotype (~ 70 nm). These results could be useful for improving technological properties of the milk.

Keywords: casein micelle, Dynamic Light Scattering, kappa casein polymorphism

Introduction

The bovine casein locus contains four milk protein genes: αs1-casein (*CSN1S1*), β-casein (*CSN2*), αs2-casein (*CSN1S2*), and κ-casein (*CSN3*) (*Threadgill et al., 1990*). The genes are organized in a cluster of approximately 250 kB (*Rjinkels et al., 1997*). Among all known *CSN3* variants A and B are with highest frequency in *Bos taurus* (*Caroli et al., 2009*). In milk, the caseins exist as polydisperse, large, roughly spherical colloidal particles, 50–600 nm in diameter (mean ~ 150 nm), called ‘‘casein micelles’’ (*Fox et al., 2008*). The size, form and

structure of the casein micelle are of great importance for cheese-making properties of the milk (*Di Stasio et al., 2000*).

Since milk protein genes' polymorphism in genus *Bos* have been discovered and characterized its development is associated mainly to milk practice to clarify association between genetic variants and milk quantitative and qualitative traits (*Tsiaras et al., 2005*). This gives opportunity to usage some allelic variants as markers for milk composition and manufacturer properties of milk. Also researches of milk proteins polymorphism are focused to clarify origin, domestication and biogeography of modern cattle breeds (*Jann et al., 2004*)

Genetic variants of milk proteins can be detected by various identification methods. These techniques include, e.g., acrylamide electrophoresis in denaturing (SDS PAGE) or native conditions, isoelectric focusing (IEF), HPLC chromatography, Cryo-scanning electron microscopy etc. (*Hallen et al., 2009; Ren et al., 2013*). An inexpensive and fast method to determine the size distribution profiles of small particles in suspension or solution is Dynamic Light Scattering (DLS) (*Gebhardt et al., 2006; de Kruif et al., 2012*). DLS is an optical detection method that can directly measure some important structural parameters of biomacromolecules, such as hydrodynamic radius (R_h) and diffusion coefficient in solution. It exhibits many advantages in the analysis process, including sensing very small amounts of samples without destruction; real-time monitoring of the specimens under different conditions (temperature, pH etc.) and in addition is relatively simple and convenient for operating. Thus, DLS has been widely applied to the structural researches of proteins, polysaccharides and other bio-macromolecules (*Chu et al., 1995; O'Connell et al., 2001*).

In the present work, the correlation between *CSN3* different genotypes and the hydrodynamic radius (Rh) of casein micelles from individual milk samples are investigated.

Materials and Methods

Animals and sample collection

The experiments were performed on nasal swab and milk samples from pure breed cows of Bulgarian Rhodopean cattle (BRC). That breed originated from autochthonous Shorthorn cattle, upgraded mainly with Jersey cow.

From a herd of 80 Bulgarian Rhodopean cows a total of 16 unrelated cows were selected; number of lactations (2-5), age of the cows (3−8 years). All animals were genotyped for the *CSN3* gene by PCR-RFLP analysis as described previously (*Hristov et al., 2013*). *CSN3* genotyping showed three genotypes: AA (four cows), AB genotype (eight cows) and BB genotype (four cows). Milk production of each animal was recorded monthly for 305-d lactation period and protein and fat content were determined with MilkoScan 133-B (Foss Electric, Denmark). For DLS

analyses milk samples from each cow in mid lactation (100-130 days) were taken after morning milking and sodium azide (0.02%, m/m) was added to all tubes to prevent microbial growth.

Dynamic light scattering measurements

DLS measurements on milk solutions were carried out on a Brookhaven Instruments 90Plus (Brookhaven Instruments Corporation, NY, USA) apparatus at 22.0 °C and scattering angle of 90 °C (wavelength 657 nm and 35 mW). Time dependent fluctuations in the scattered intensity were measured using an avalanche photo detector (APD) and a digital correlator. To check for sedimentation or aggregation data collections were performed in triplicate as 2 minutes co-added runs (total time of 6 min). NIST traceable polystyrene solutions 3020 A, 22 nm ± 1.8 and 3090 A, 92 ± 2 nm (Thermo Scientific) and a blank, 0.02 μm filtered ultrapure water, were used as standards. The used buffer solution (50 mM TBS, pH 7.2) was filtered through 0.44 μm filter and also examined by DLS to account for eventual "dust" particles. Prior to DLS data collection and in order to remove major aggregates (fat fraction and unspecific precipitates) individual milk samples were centrifuged at 2 000 x g for 3 minutes at 4 °C. Then the "skim milk" fraction was diluted 100 times with 50 mM TBS (pH 7.2) and filtered through 0.44 μm syringe filters (*de Kruif and Huppertz, 2012*). DLS employs the Stokes−Einstein relationship between the diffusion coefficient (D) and the hydrodynamic radius (R_h):

$$D = \frac{kT}{6\pi\eta R_h}$$

where η is the viscosity. For obtaining size distributions the autocorrelation functions were deconvoluted using the non-negatively constrained least squares fit (multiple pass NNLS) algorithm. In addition, the intensity of scattered light is proportional to the particle size to the sixth power resulting in a higher scattered intensity for larger particles. Thus the intensity weight distributions measured by DLS were converted to number weighted distributions using the analysis software provided by Brookhaven (Brookhaven Instruments Corporation, NY, USA).

Statistical analysis

Descriptive statistics was used concerning the milk productivity and qualitative milk traits data. The calculated mean values (shown as mean value ± SEM) for milk productivity and qualitative traits were compared within different genotypes and evaluated by Student's t-test. These statistical assays were performed with GraphPad Prism version 5.04 (GraphPad software).

Results and Discussion

Effect of κ-CN genotypes on milk quantitative and qualitative traits

PCR-RFLP analysis showed three genotypes AB (eight cows), AA (four cows) and BB (four cows) for the 16 selected cows. To determine milk production, butter milk, fat and protein contents during the lactation period (305 d) a total of ten milk samples were collected on a 30 days basis from each animal. The average composition of milk (butter milk, fat and protein) for the different κ-CN genotypes is shown in Table 1. The results clearly demonstrate correlation between genotypes and milk production (AB > AA > BB). Milk production of heterozygous AB animals significantly exceeds that of homozygous BB cows, with 12% or about 500 L ($P < 0.01$) and with 5% (about 200 L, $P < 0.01$) that of animals homozygous by A allele of the gene. Regarding fat and protein contents there are only slight differences amongst the three genotypes (Table 1).

Table 1. Influence of the *CSN3* genetic polymorphism on the milk production and the milk quality traits in cows of the Bulgarian Rhodopean cattle

Genotype	Milk production L	Butter milk kg	Protein %	Milk fat %
AB	4099 ± 78.6^{a}	185.5 ± 0.5	3.54 ± 0.04	4.58 ± 0.09
AA	3896 ± 14.5^{b}	178 ± 2.5	3.76 ± 0.04	4.78 ± 0.07
BB	3598.5 ± 44.5^{c}	172.1 ± 0.9	3.56 ± 0.03	4.60 ± 0.2

Values express as means ± standard deviation; values within the same row not sharing a common letter differ significantly, $P < 0.01$.

Our results support the data by (*Bovenhuis et al., 1992*) suggesting a 15 % decrease of the milk production of the BB homozygous cows compared to the AB heterozygous cows. Some studies claim that the BB genotype is associated with higher (*Van Eenennaam et al., 1991*) or lower (*Bovenhuis et al., 1992*) milk yield whereas other studies indicated no effect (*Comin et al., 2008*). One should be very careful when crosschecking the results of different studies as in most cases they are not comparable due to differences in population size, breed of cows, frequency of occurrence of specific genetic variants under consideration, methods of expressing traits (whether test day or lactation averages) and the effect of other genetic variants.

DLS measurement of milk samples

Previous studies has linked the size distribution of casein micelle to the lactating stage (*de Kruif and Huppertz, 2012*), have investigatd the influence of the feeding regimes and investigated the micelle size of native and heated milk samples (*Devold et al., 2000*). This study focuses on the correlation between κ-CN genotypes and casein micelle size in individual milk samples. The resulting data from DLS measurements (hydrodynamic radius, polydispersity and multimodal distribution) is presented in Table 2.

Table 2. DLS measurement for individual milk samples

	Sample No / genotype	hydrodynamic radius (combined)	poly-dispersity	Multimodal distribution (size and relative intensity %)	
				Peak 1	Peak 2
		nm		nm*	nm
1	2682 AA	193.8 (1.5)	0.246	122	474 (<1)
2	2309 AA	182.5 (1.4)	0.193	117	324 (1.5)
3	2672 AA	234(6.8)	0.327	127	586(<1)
4	2688 AA	254(17)	0.339	111	643(<0.1)
5	2339 AB	174.7(2.4)	0.258	59	244 (<1)
6	2819 AB	201.6(6.8)	0.302	93	477(~10)
7	2152 AB	320.7(29.6)	0.342	67	-
8	2695 AB	188.7(2.0)	0.231	112	368 (1.5)
9	2663 AB	169.6 (0.4)	0.160	38	201 (<0.1)
10	2595 AB	284.1(20)	0.355	93	859 (7.6)
11	2296 AB	143.8 (5)	0.233	66	237 (<0.1)
12	2717 AB	167.6 (3.5)	0.215	99	292(<0.1)
13	2687 BB	162.9(0.9)	0.151	64	202(<1)
14	2726 BB	410.3(25.9)	0.377	70	1320 (<1)
15	2691 BB	161.0(3.5)	0.295	77	350 (<1)
16	2680 BB	191.2(2.9)	0.202	83	269 (<0.1)

* the relative intensity for peak 1 is 100%

Hydrodynamic radii (R_h) of micelles at a scattering angle of 90 °C varied from 40 to 120 nm. These values are in accordance with micelle size for Norwegian Red cattle (*Devold et al., 2000*) and Holstein-Friesian cows (*de Kruif and Huppertz, 2012*). DLS measurments were performed on 16 milk samples with distinct *CSN3* genotypes (AA/AB/BB) and the variation of the micelle size in function of genotype is shown on Figure 1. One can see that the micelle size for AA and BB genotypes is not fluctuating a lot. In contrast the micelle size of cows with AB genotype varies a lot. An over simplification of the data interpretation, links the highest observed values of casein micelle size (over 110 nm) to AA genotype. This finding is in agreement with data reported by *Bijl et al. (2014)* for skimmed milk of Holstein-Friesian cows. According to the data from Table 1 one can eventually suggest a correlation only to the highest protein and fat content in

milk for *CSN3* AA genotype (3.76 ± 0.04 %; 4.78 ± 0.07 %, respectively). However, there is no clear correlation between casein micelle size and observed milk quantitative and qualitative traits.

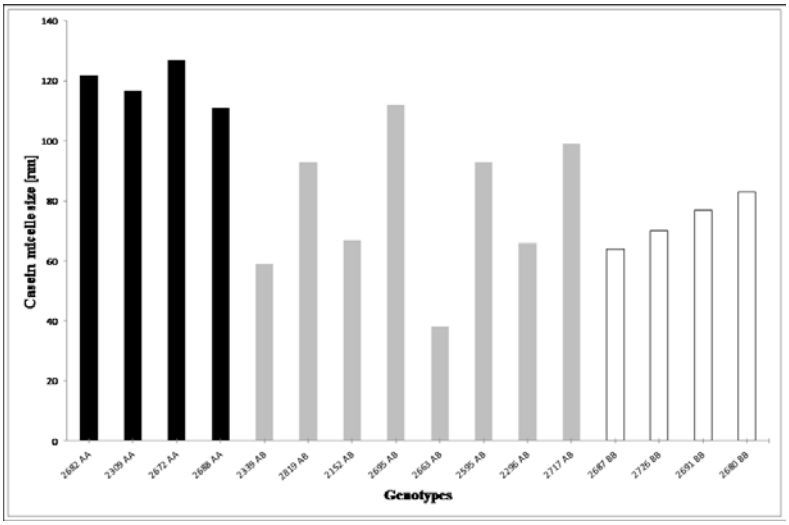

Figure 1. Genotypes vs. particle size (radius) distributions (normalized) of micelles obtained with dynamic light scattering

A major complication of light scattering studies is due to the presence of dust particles in the sample, therefore careful filtering procedures have to be applied. In the case of casein micelles an efficient filtering is not always possible, since dust particles and micelles are of similar size. It is thus essential to work with relatively concentrated solutions (resulting in higher polydispersity index (PDI)). Habitually polydispersity values below 0.1 are suited for DLS experiments while more elevated values of polydispersity are linked to either more concentrated samples or a multi modal distribution. As can be seen from Table 2, polydispersity of casein micelles varied from 0.15 to 0.37 related to a bimodal distribution. As one can see from the multimodal distribution “number vs. diameter” (Figure 2) the number average reveals that the dominant species has smaller size, consistent with the lower average intensity (Peak 1 in Table 1) while the contribution of aggregates with bigger size is minimal.

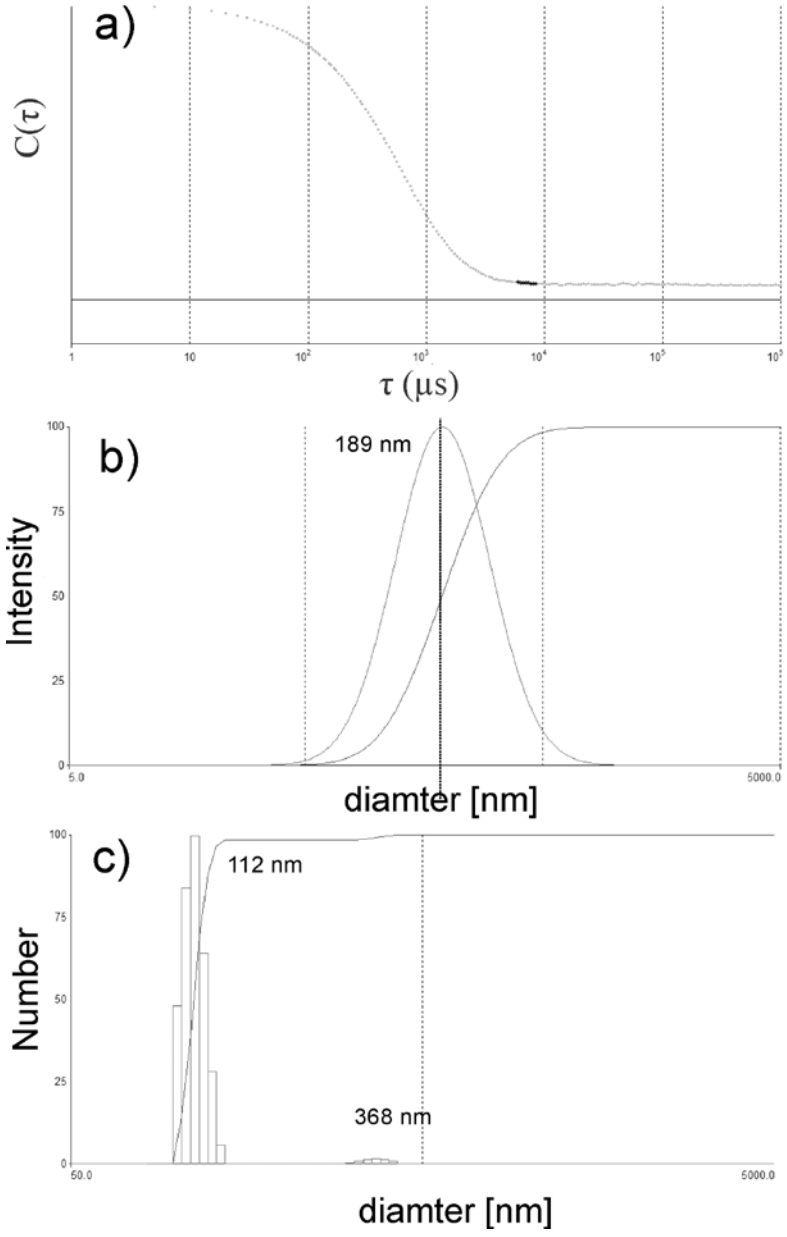

Figure. 2. Representative DLS distributions for sample 2695 a) correlation function b) intensity particle size distribution and c) multimodal number particle size distribution

Conclusion

This study reveals for the first time the correlation between κ-CN genotypes and casein micelle size in individual milk samples. *CSN3* AB genotype showing distinct variations of micelle size. DLS data suggest that there is a correspondence with *CSN3* genotype e.g. AA genotype shows bigger size of casein micelle. In contrast, protein and fat content in milk cannot be correlated to casein micelles size.

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Genetski polimorfizam kapa kazeina i veličina kazein micela u goveda bugarske rodopske rasa

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Rezime

Ova studija ima za cilj da uporedi veličinu kazein micela u uzorku kravljeg mleka u funkciji kapa kazein (CSN3) genetičkog polimorfizma. Šesnaest krava bugarske rodopske rasa goveda su genotipizirane korišćenjem PCR-RFLP analize. Uzorci mleka tri pronađena *CSN3* genotipa (AB, AA i BB) su upotrebljeni za određivanje veličine kazein micela metodom Dynamic Light Scattering (DLS). Rezultati su pokazali razlike u veličini i polidisperzitetu kazeina micela između mleka krava različitih genotipova. Hidrodinamički radijusi micela pod uglom rasejanja od 90°C varirali su od 80 do 120 nm a polidisperzitet od 0,15 do 0,37. U zaključku, veličina kazein micela *CSN3* AA krava (~ 120 nm) prelazi sa oko 60% krava sa AB (~ 80 nm) i BB genotipa (~ 70 nm). Ovi rezultati mogu biti korisni za poboljšanje tehnoloških svojstava mleka.

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