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The relationships among bovine α_S-casein phosphorylation isoforms suggest different phosphorylation pathways

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ABSTRACT

Casein (CN) phosphorylation is an important posttranslational modification and is one of the key factors responsible for constructing and stabilizing casein micelles. Variation in phosphorylation degree of α_S-CN is of great interest because it is suggested to affect milk technological properties. This study aimed to investigate the variation in phosphorylation degree of α_S-CN among milk of individual cows and to explore relationships among different phosphorylation isoforms of α_S-CN. For this purpose, we analyzed morning milk samples from 529 French Montbéliarde cows using liquid chromatography coupled with electrospray ionization mass spectrometry. We detected 3 new phosphorylation isoforms: α_{S2}-CN-9P, α_{S2}-CN-14P, and α_{S2}-CN-15P in bovine milk, in addition to the known isoforms α_{S1}-CN-8P, α_{S1}-CN-9P, α_{S2}-CN-10P, α_{S2}-CN-11P, α_{S2}-CN-12P, and α_{S2}-CN-13P. The relative concentrations of each α_S-CN phosphorylation isoform varied considerably among individual cows. Furthermore, the phenotypic correlations and hierarchical clustering suggest at least 2 regulatory systems for phosphorylation of α_S-CN: one responsible for isoforms with lower levels of phosphorylation (α_{S1}-CN-8P, α_{S2}-CN-10P, and α_{S2}-CN-11P), and another responsible for isoforms with higher levels of phosphorylation (α_{S1}-CN-9P, α_{S2}-CN-12P, α_{S2}-CN-13P, and α_{S2}-CN-14P). Identifying all phosphorylation sites of α_{S2}-CN and investigating the genetic background of different α_{S2}-CN phosphorylation isoforms may provide further insight into the phosphorylation mechanism of caseins.

Key words: phosphorylation, casein, milk protein composition, liquid chromatography coupled with electrospray ionization mass spectrometry

INTRODUCTION

Detailed milk protein composition contributes largely to the nutritional value and technological properties of milk. Casein accounts for about 80% of total protein in cow milk, and its content and composition largely influence cheese manufacturing properties of milk (Hallén, 2008; Wedholm, 2008; Caroli et al., 2009). Casein arises from the expression of 4 genes encoding 4 distinct polypeptide chains: α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN, of which the relative amounts in milk are approximately at the ratio 4:1:4:1. Phosphorylation of caseins is an important posttranslational modification occurring after the synthesis of the polypeptide chains in the Golgi apparatus of the mammary epithelial cell under the action of protein kinases (Bingham et al., 1972). These kinases phosphorylate Ser or Thr by recognizing the tripeptide sequence Ser/Thr-X-Glu/SerP/Asp, where X represents any AA residue and P indicates phosphorylation (Mercier, 1981). This posttranslational modification allows caseins to interact with calcium phosphate to form large colloidal structures called casein micelles. Phosphoserine clusters of α_{S1}-CN, α_{S2}-CN, and β-CN are involved in stabilizing calcium phosphate nanoclusters and enable micellar growth by crosslinking between phosphorylated residues of caseins and calcium phosphate (De Kruif and Holt, 2003; De Kruif et al., 2012; Dalgleish and Corredig, 2012).

Although α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN are all phosphoproteins, they vary strikingly in their degree of phosphorylation. In bovine milk, α_{S1}-CN has 2 common phosphorylation isoforms: α_{S1}-CN-8P and α_{S1}-CN-9P; α_{S2}-CN is present with isoforms from 10P to 13P, β-CN is usually present with 5P and occasionally with 4P, and κ-CN is present with 1P to 2P and occasionally with 3P (Farrell et al., 2004; Holland and Boland, 2014). Exploring the variation in phosphorylation degree of α_S-CN is of great interest because caseins may play different roles in stabilizing the internal micellar structure (Hoagland et al., 2001; De Kruif and Holt, 2003), and affect cheese-making properties of milk (Frederiksen et

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al., 2011). Phosphorylation degree of α_{S2}-CN was found negatively correlated with phosphorylation degree of α_{S1}-CN (Heck et al., 2008). Additionally, α_{S1}-CN-8P and α_{S1}-CN-9P are suggested to be regulated by different sets of genes (Bijl et al., 2014).

This study aimed to investigate the variation in phosphorylation degree of α_S-CN among milk of individual cows and to explore the relationships among different phosphorylation isoforms of α_S-CN in Montbéliarde cows in the Franche-Comté region in France.

MATERIALS AND METHODS

Milk Samples

We sampled 576 Montbéliarde cows located on 430 herds across 3 departments (Doubs, Jura, and Haute-Saône) to obtain a good representation of the variation in milk protein composition from the current Montbéliarde cattle population in the Franche-Comté region in the east of France. We sampled cows across different parities (1–5) and lactation stages (7–652 d) based on paternal pedigree (to maximize genetic diversity) and on protein and calcium content in milk (to increase milk content diversity). During autumn/winter (October–December, 2014) and spring/summer (April–July, 2015), fresh morning milk samples (25 mL) were preserved with Bronopol after collection, transported on ice to the laboratory, and then frozen at −20°C until analysis by liquid chromatography/electrospray ionization-mass spectrometry (**LC/ESI-MS**).

Milk Protein Profiling

All chemicals used in the LC/ESI-MS analysis were of the highest purity commercially available and were used without further purification. Trifluoroacetic acid (**TFA**), urea, Bis-Tris, dithiothreitol, and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water (Milli-Q Plus System, >18.3 MΩcm) was produced in the laboratory.

Prior to LC/ESI-MS analysis, milk samples were skimmed by centrifugation at 2,500 × *g* for 20 min at 4°C. Skim milk samples (20 μL) were then clarified by adding 180 μL of 0.1 *M* Bis-Tris buffer pH 8.0, containing 8 *M* urea, 1.3% trisodium citrate, and 0.3% dithiothreitol (Visser et al., 1991). Next, 15 μL of clarified milk samples were injected into a Discovery BIOWide Pore (Supelco) C5 column (150 × 2.10 mm, 300Å). Reversed-phase HPLC was carried out with an Ultimate LC 3000 system (Thermo Fisher Scientific, Waltham, MA). During the analysis, the autosampler was kept at 10°C, and the column was maintained at 42°C. The column's mobile phase consisted of a gradient mixture of solvent A (0.025% TFA in ultrapure water, vol/vol) and solvent B (0.02% TFA in acetonitrile, vol/vol) at a flow rate of 0.2 mL/min. The elution condition was a linear gradient from 29.5 to 34% B in 16 min, from 34 to 35.5% B in 0.1 min, from 35.5 to 37.5% B in 14.9 min, from 37.5 to 42% B in 14 min, from 42 to 95% B in 0.1 min, followed by an isocratic elution at 95% B for 5 min, and a linear return to 29.5% B in 0.1 min. The column was then re-equilibrated at 29.5% B as the starting condition for 10 min.

Protein elutes were detected by UV absorbance at 214 nm. The column was directly interfaced with an ESI-TOF mass spectrometer micrOTOF II focus (Bruker Daltonics, Wissembourg, France). The positive ion mode was used and mass scans were acquired over a range of 50 to 3,000 m/z. End plate offset voltage was set at −500 V and capillary voltage to 4,500 V. Nebulizer gas (N_2) pressure was maintained at 250 kPa and drying gas (N_2) flow was set at 4.0 L/min at 200°C. The LC/ESI-MS system was controlled by Hystar software v.2.3 (Bruker Daltonics). The charge number of multicharged ions, the deconvoluted mass spectra, and the determination of average molecular mass ($\boldsymbol{M}_r$) were obtained from Data Analysis v.3.4 software (Bruker Daltonics). A blank sample was injected after every milk sample to avoid carryover effects. A reference milk sample was analyzed after every 10 milk samples to determine reproducibility. In total, 58 aliquots of this reference sample were analyzed during the whole series of analyses.

Identification and Quantification of Milk Protein Fractions

We implemented the LC/ESI-MS method developed at INRA to simultaneously measure the relative concentrations of the major milk proteins and their isoforms, notably their phosphorylation isoforms (Miranda et al., 2013). Peak profiles from UV 214 nm were analyzed by Chromeleon software (Chromeleon 7.0.0, Dionex, Thermo Fisher Scientific). Protein variants and isoforms of the 6 major milk proteins (α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, α-LA, and β-LG) were identified by matching measured molecular masses with an in-house calculated mass database on bovine milk proteins (Miranda et al., 2011).

A milk sample was considered degraded if peptides arising from the proteolysis of milk proteins, such as γ-casein and proteose-peptone component 5, accounted for more than 10% of total protein in milk. Degraded milk samples were discarded because degradation products co-eluted with some major protein fractions and interfered with the accuracy of quantification. As a result, 47 milk samples were discarded and 529 samples

qualified for downstream quantification of the relative concentration of each protein fraction. Fractions of individual proteins and of α_{S1}-CN phosphorylation isoforms were estimated based on dividing the peak area of an individual protein by the total integrated peak area in the chromatogram of an individual milk sample. The LC could not completely separate the α_{S2}-CN phosphorylation isoforms in our study, so quantification based on peak area in the chromatogram would not be possible for individual α_{S2}-CN phosphorylation isoforms. Coupling mass spectrometry allows identification of a specific isoform in a mixture based on its molecular mass and further quantification via its signal intensity (Tuli and Ressom, 2009). Therefore, we used mass signal intensity to estimate the proportion of each α_{S2}-CN phosphorylation isoform as a fraction of total α_{S2}-CN, and the following equation was used to estimate the relative concentration of each α_{S2}-CN phosphorylation isoform in total milk protein:

$$\frac{\text{isoform mass signal intensity}}{\Sigma\text{isoform mass signal intensity}} \times \text{total } \alpha_{S2}-\text{CN}(\%\,\text{wt}/\,\text{wt}).$$

The reproducibility of the method was assessed by calculating the coefficient of variation of the relative protein concentration for each protein and isoform fraction from the 58 aliquots of the reference milk sample.

Divisive Hierarchical Clustering Analysis

A dendrogram was constructed using divisive hierarchical clustering analysis to graphically visualize phenotypic correlations among α_S-CN phosphorylation isoforms. The dissimilarity between the isoforms was calculated as $\sqrt{1-\text{r}}$, where r is the correlation coefficient between isoforms, to account for the direction and intensity of the relationships. The distance matrix was calculated using Euclidean distance. The analysis was performed using the R package Cluster (Maechler et al., 2015; R Core Team, 2015).

RESULTS AND DISCUSSION

Milk Protein Profiling by LC/ESI-MS

Milk protein composition of 529 individual cows was determined by the LC/ESI-MS method. In this method, the 6 major milk proteins were separated by RP-HPLC, and their protein variants and posttranslational isoforms were identified by measured molecular masses ($\boldsymbol{M}_\mathbf{r}$) using ESI-MS. The LC/ESI-MS reproducibility was based on the coefficient of variation of 58 aliquots of a reference milk sample analyzed during the whole series of sample analyses, which were below 5% for the 6 major milk proteins and below 8% for the α_{S1}-CN and α_{S2}-CN phosphorylation isoforms (Table 1). The LC/ESI-MS reproducibility for the 6 major milk proteins obtained in this study is within the range of reproducibilities reported for previous studies (Bobe et al., 1998; Bordin et al., 2001; Bonfatti et al., 2008; Heck et al., 2008).

The 6 major milk proteins eluted in the following order: κ-CN, α_{S2}-CN, α_{S1}-CN, β-CN, α-LA, and β-LG (Figure 1A). κ-Casein eluted as 3 separate peaks: the first one contained glycosylated κ-CN from different protein variants, whereas the second one with a minor shoulder contained nonglycosylated κ-CN A-1P+2P (M_r = 19,037 Da for 1P; M_r = 19,117 Da for 2P) or κ-CN E-1P+2P (M_r = 19,007 Da for 1P; M_r = 19,087 Da for 2P), and the last peak with a minor shoulder contained nonglycosylated κ-CN B-1P+2P (M_r = 19,005 Da for 1P; M_r = 19,085 Da for 2P). Although κ-CN A-1P+2P and E-1P+2P had similar retention times, they could be discriminated based on their molecular masses. The α_{S2}-CN eluted into 2 major peaks as the retention time increased with its phosphorylation degree: one with α_{S2}-CN-11P (M_r = 25,228 Da) as the major component, together with α_{S2}-CN-10P (M_r = 25,148 Da) and occasionally with α_{S2}-CN-9P (M_r = 25,069 Da; Figure 1.B1), and the other with α_{S2}-CN-12P (M_r = 25,308 Da) as the major component and α_{S2}-CN-13P (M_r = 25,388 Da), α_{S2}-CN-14P (M_r = 25,468 Da), and sometimes α_{S2}-CN-15P (M_r = 25,548 Da; Figure 1.C2). To our knowledge, this is the first study to show the presence of α_{S2}-CN-9P, α_{S2}-CN-14P, and α_{S2}-CN-15P (Figure 1.B1 and 1.C2). The α_{S1}-CN separated into α_{S1}-CN-8P (M_r = 23,615 Da for the B variant; M_r = 23,543 Da for the C variant) and α_{S1}-CN-9P (M_r = 23,695 Da for the B variant; M_r = 23,623 Da for the C variant). The B and C variants had the same retention times, but they could be differentiated by their molecular masses. Four protein variants of β-CN eluted in the following order: β-CN B-5P (M_r = 24,092 Da), β-CN A1–5P (M_r = 24,023 Da), β-CN A2–5P (M_r = 23,983 Da), and β-CN I-5P (M_r = 23,965 Da). Two whey proteins without posttranslational modifications eluted after the caseins in the following order: α-LA (M_r = 14,186 Da), β-LG D (M_r = 18,280 Da), β-LG B (M_r = 18,281 Da), and β-LG A (M_r = 18,367 Da).

Variation in Major Milk Proteins

Detailed milk protein composition of Montbéliarde cows is of great interest because cheese-making properties are considered important in this breed. In Franche-Comté, milk from the Montbéliarde breed is used to produce regional cheeses with Protected Designation of Origin certification. However, not much information is

available about the Montbéliarde breed. Table 1 summarizes the descriptive statistics for milk production traits and milk protein composition, and frequencies of milk protein variants. α-Lactalbumin was the least abundant protein, and α_{S1}-CN the most abundant protein among milk samples from 529 Montbéliarde cows. The coefficient of variation values ranged between 7 and 20%, implying substantial variation in milk protein composition among milk of individual cows. The average relative protein concentrations for the 6 major milk proteins are in the range of results from previous studies in other breeds (Bobe et al., 1998; Heck et al., 2008).

Two protein variants were found for α_{S1}-CN (B and C) and α_{S2}-CN (A and D), 3 for κ-CN (A, B, and E) and β-LG (A, B, and D), and 4 for β-CN (A1, A2, B, and I). Protein variants κ-CN E and β-CN I have not been reported in the French Montbéliarde population before. These variants may have been missed in earlier studies (Grosclaude, 1988) because the analytical technique used (gel electrophoresis) could not detect these 2 protein variants (Miranda et al., 1993; Jann et al., 2002). The frequency of κ-CN E is lower, whereas the frequency of κ-CN B is higher in the studied Montbéliarde population compared with other cattle breeds, such as Dutch Holstein Friesian, Danish Holstein, Italian Holstein Friesian, and Swedish Red (Heck et al., 2009; Gustavsson et al., 2014; Chessa et al., 2014). κ-Casein B has been associated with better coagulation properties of milk, whereas for κ-CN E the opposite has been shown (Hallén, 2008). Milk from the Montbéliarde breed is considered to possess better cheese-making properties than milk from Holstein and Nordic cattle breeds because of this characteristic (OS Montbéliarde, 2014). Regarding β-CN, the frequency of the B variant was moderate (0.355) and has increased at the expense of the A1 variant since 1976 (Grosclaude, 1988). The β-CN B variant has been positively associated with rennet coagulation and cheese-making properties (Caroli et al., 2009), whereas the β-CN A1 variant has been negatively associated with milk yield and protein yield (Ikonen et al., 1999; Visker et al., 2011). Because selection based on specific milk protein variants has not

Table 1. Mean, SD, CV, liquid chromatography coupled with electrospray ionization mass spectrometry (LC/ESI-MS) reproducibility, and frequencies of protein variants for the 6 major milk proteins and the different phosphorylation isoforms of α_{S1}-CN and α_{S2}-CN measured on test-day morning milk samples from 529 Montbéliarde cows

Trait	Mean	SD	CV	LC-ESI/MS reproducibility[1]	Frequency of protein variant[2]
Milk protein composition (% wt/wt of total protein in milk)					
α_{S1}-CN					
Total	32.92	2.22	7	1	B (0.935); C (0.065)
8P	25.27	2.18	8	1	
9P	7.65	0.96	13	5	
α_{S2}-CN					
Total	8.41	0.72	11	4	A (0.999); D (0.001)
9P[3]	0.24	0.05	21	NA	
10P	0.72	0.30	42	6	
11P	3.03	0.55	18	8	
12P	2.68	0.34	13	7	
13P	1.56	0.31	20	8	
14P	0.40	0.14	36	8	
15P[4]	0.20	0.10	49	NA	
β-CN	28.14	2.69	10	2	B (0.355); A1 (0.087); A2 (0.405); I (0.155)
κ-CN	9.03	0.86	9	2	A (0.537); B (0.459); E (0.004)
α-LA	3.54	0.70	20	5	B (1)
β-LG	12.16	1.87	15	4	A (0.444); B (0.554); D (0.002)
Σcasein[5]	78.50	2.60	3		
Σwhey[6]	15.71	1.83	12		
Milk production					
Milk yield (kg/d)	23.9	7.3	31		
Protein (%)	3.32	0.37	11		
Fat (%)	3.84	0.59	16		
Lactose (%)	4.93	0.31	6		

[1]Calculated based on the CV (%) of 58 repeated analyses of a reference milk sample.

[2]Frequencies of protein variants were calculated from 576 Montbéliarde cows.

[3]α_{S2}-CN-9P is 0.0 in 508 out of 529 samples; mean, SD, and CV are calculated based on 9 samples.

[4]α_{S2}-CN-15P is 0.0 in 525 out of 529 samples; mean, SD, and CV are calculated based on 4 samples.

[5]Σcasein = α_{S1}-CN + α_{S2}-CN + β-CN + κ-CN.

[6]Σwhey = α-LA + β-LG.

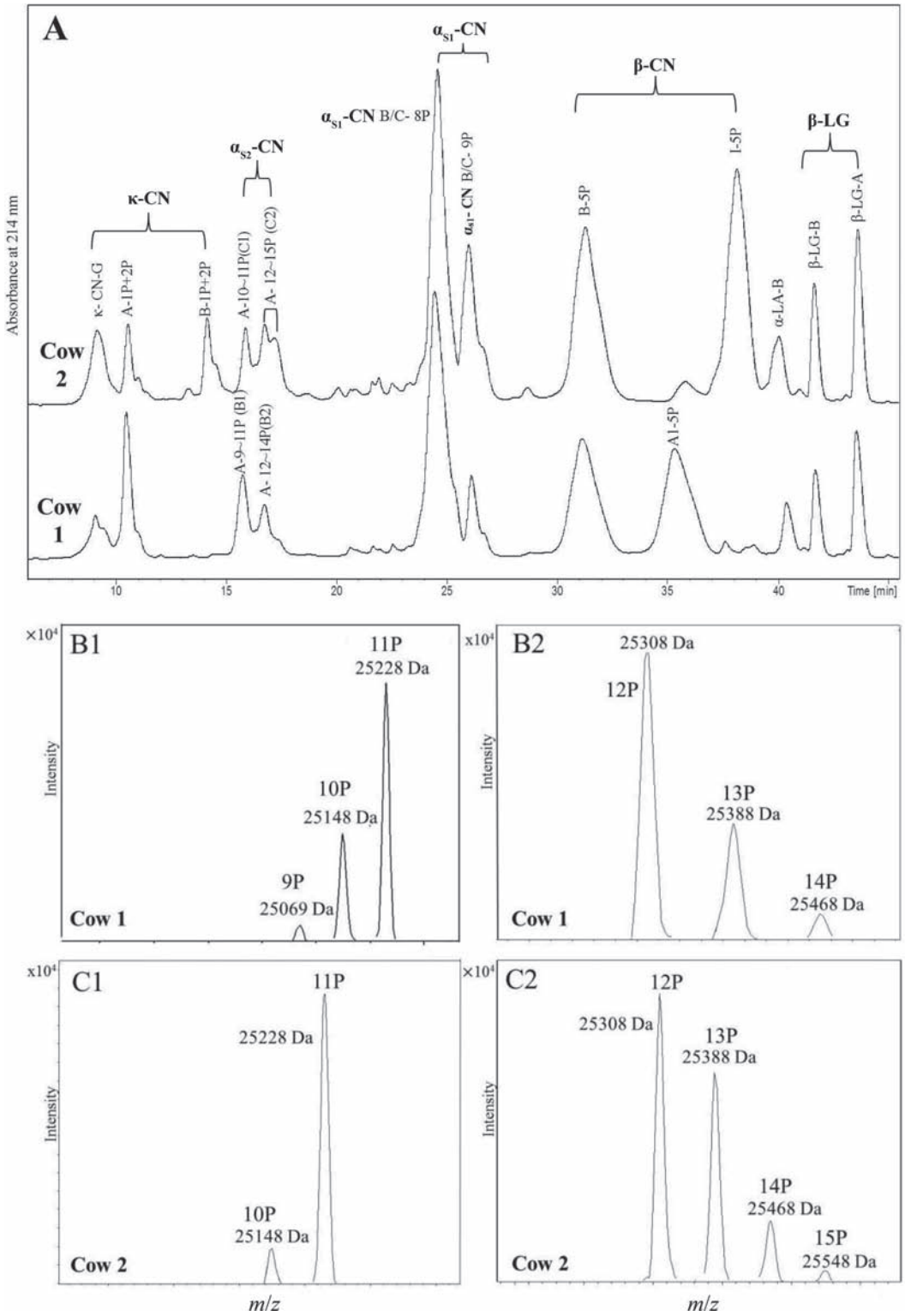

Figure 1. Milk protein profiling by liquid chromatography coupled with electrospray ionization mass spectrometry (LC/ESI-MS) of 2 individual bovine milk samples with distinct α_{S2}-casein profiles. (A) Peaks of protein variants and isoforms (G = glycan, P = phosphate group) of major milk proteins are identified by LC/ESI-MS. (B1 and B2) α_{S2}-Casein profile of cow 1 from deconvolution of multicharged-ion spectra corresponding to chromatographic peak B1 and B2. The B1 spectra show the presence of α_{S2}-CN-9P. (C1 and C2) α_{S2}-Casein profile of cow 2 from deconvolution of multicharged-ion spectra corresponding to chromatographic peak C1 and C2. The C2 spectra show the presence of α_{S2}-CN-15P.

Table 2. Phenotypic correlations[1] among the 6 major milk proteins (% wt/wt of total protein in milk) measured on test-day morning milk samples from 529 Montbéliarde cows

Item	α_{S1}-CN	α_{S2}-CN	β-CN	κ-CN	Σcasein	α-LA	β-LG
α_{S2}-CN	0.07						
β-CN	−0.33	−0.32					
κ-CN	−0.14	0.11	−0.17				
Σcasein[2]	0.41	0.00	0.63	0.07			
α-LA	0.15	0.14	−0.16	−0.10	−0.03		
β-LG	−0.37	0.01	−0.35	−0.18	−0.74	−0.24	
Σwhey[3]	−0.32	0.06	−0.42	−0.22	−0.77	0.14	0.93

[1]SE (0.004–0.04).

[2]Σcasein = α_{S1}-CN + α_{S2}-CN + β-CN + κ-CN.

[3]Σwhey = α-LA + β-LG.

been implemented in Montbéliarde breed, the increased frequency of the β-CN B variant could be either due to genetic drift or selection on milk production traits.

Phenotypic Correlations Among 6 Major Milk Proteins

Table 2 presents the phenotypic correlations among the 6 major milk proteins. Strong negative correlation was found between β-LG and Σcasein (−0.74). This correlation supports previous findings (Wedholm et al., 2006; Ng-Kwai-Hang, 2006; Schopen et al., 2009). As for the relationships among the 4 caseins, the phenotypic correlations were relatively low and ranged from −0.33 to +0.11. Generally, these low correlations among the caseins are in line with those reported by Gebreyesus et al. (2016) and Schopen et al. (2009) with 2 exceptions. Schopen et al. (2009) reported a moderate and negative phenotypic correlation between α_{S1}-CN and α_{S2}-CN (−0.50 vs. 0.07 in this study), and Gebreyesus et al. (2016) reported a moderate and positive phenotypic correlation between α_{S1}-CN and β-CN (0.48 vs. −0.33 in this study). The discrepancies between these studies might be explained by genetic differences between the 3 breeds (Montbéliarde, Holstein Friesian, and Danish Holstein) and use of different analytical methods [capillary zone electrophoresis (**CZE**) and LC/ESI-MS)]. As some protein variants have been shown to be associated with the relative concentrations of certain protein fractions, differences in frequencies of protein variants between breeds could affect the concentrations and, subsequently, the phenotypic correlations between protein fractions (Heck et al., 2009; Bonfatti, 2010). In terms of analytical methods, CZE may have provided ambiguous estimations of α_{S2}-CN, β-CN, and κ-CN because α_{S2}-CN-13P was not detected, measurement of β-CN was partially disturbed by compounds co-migrating with β-CN such as glycosylated and multi-phosphorylated κ-CN, and measurement of κ-CN involved only the mono-phosphorylated isoform (Heck et al., 2008). Furthermore, measurements of protein fractions using the same analytical method, such as LC, could differ because of different separation conditions.

Variation in α_{S1}-CN and α_{S2}-CN Phosphorylation Isoforms

Among milk samples from 529 Montbéliarde cows, we found 77% of α_{S1}-CN carrying 8P and 23% carrying 9P, on average. Thus, α_{S1}-CN-8P was the abundant isoform whose mean concentration accounted for 25.3% of total protein in milk (Table 1). For α_{S2}-CN phosphorylation isoforms, we estimated only the relative concentrations of α_{S2}-CN-10P to α_{S2}-CN-14P from the A variant because only one milk sample contained the D variant (which is present with isoforms from 7P to 11P). The α_{S2}-CN-9P and α_{S2}-CN-15P occurred in trace amounts, and they were not observed in all milk samples. Out of 529 milk samples, α_{S2}-CN-9P was detected in 21 samples, and α_{S2}-CN-15P was detected in 4 different samples. For the remaining samples, the concentrations of these 2 isoforms may have been too low to be detected by MS. Among milk samples from 529 Montbéliarde cows, we found 8% of α_{S2}-CN having 10P, 36.5% having 11P, 32% having 12P, 19% having 13P, and 4.5% having 14P, on average. Thus, α_{S2}-CN-11P and α_{S2}-CN-12P were the abundant isoforms, whose mean concentrations accounted for 3.1 and 2.7% of the total protein in milk, whereas α_{S2}-CN-13P had a mean concentration of 1.6%. The coefficient of variation values of α_S-CN phosphorylation isoforms ranged between 8 and 42%, suggesting large variation in relative concentrations of different α_S-CN phosphorylation isoforms. The estimated mean concentrations of α_{S1}-CN-8P and α_{S1}-CN-9P and of α_{S2}-CN-10P to α_{S2}-CN-12P, as well as their coefficient of variation values, were comparable with the results reported by Heck et al. (2008). However, Heck et al. (2008) did not detect α_{S2}-CN-9P, α_{S2}-CN-13P, α_{S2}-CN-14P, and α_{S2}-CN-15P because of the technique used (CZE) in that study.

Phenotypic Correlations Among α_S-CN Phosphorylation Isoforms and the 6 Major Milk Proteins

As 3 new α_{S2}-CN phosphorylation isoforms were detected, we further explored the relationships among different α_S-CN phosphorylation isoforms and with other major milk proteins (Table 3). Correlations between the relative concentrations of different α_S-CN phosphorylation isoforms and those of the other 4 major milk proteins were relatively low (from −0.34 to 0.25). Correlations related to α_{S1}-CN-8P concentration were in line with those reported by Gebreyesus et al. (2016) except for the correlation between α_{S1}-CN-8P and β-CN (0.42 vs. −0.28 in this study). The discrepancy between the 2 studies could be explained by differences between studied breeds and analytic methods used as mentioned above. A strong positive correlation was found between α_{S2}-CN-13P and α_{S2}-CN-14P (0.92). The α_{S2}-CN-10P correlated positively with α_{S2}-CN-11P (0.62), but correlated negatively with α_{S2}-CN-13P and α_{S2}-CN-14P (−0.58 and −0.55, respectively). The α_{S2}-CN-12P correlated weakly with the rest of α_{S2}-CN phosphorylation isoforms (−0.28 to 0.19). Correlation between α_{S1}-CN-8P and α_{S1}-CN-9P was weak (−0.12), which agrees with previous findings reported by Bijl et al. (2014).

The relationships between phosphorylation of α_{S1}-CN and phosphorylation of α_{S2}-CN have rarely been investigated before due to the limitations of analytic methods for quantifying α_{S2}-CN phosphorylation isoforms. The dendrogram constructed from hierarchical analysis shows 2 main groups: one group of α_{S1}-CN-8P, α_{S2}-CN-10P, and α_{S2}-CN-11P, and another group of α_{S1}-CN-9P, α_{S2}-CN-12P, α_{S2}-CN-13P, and α_{S2}-CN-14P (Figure 2). Both phenotypic correlations and hierarchical clustering suggest that α_S-CN phosphorylation might involve at least 2 different regulatory systems. One system might be involved in forming isoforms with a lower degree of phosphorylation (α_{S1}-CN-8P, α_{S2}-CN-10P, and α_{S2}-CN-11P), whereas another system favors a higher degree of phosphorylation (α_{S1}-CN-9P, α_{S2}-CN-12P, α_{S2}-CN-13P, and α_{S2}-CN-14P). This is in line with Bijl et al. (2014), who showed that 2 different chromosomal regions are associated with α_{S1}-CN-8P and α_{S1}-CN-9P concentration, respectively. Consequently, they hypothesize that a different set of genes regulates the phosphorylation of α_{S1}-CN-8P and α_{S1}-CN-9P. Our results support this hypothesis and further suggest that one regulatory system is more effective than the other depending on the individual cow. As illustrated by the relationships between α_{S1}-CN-9P and the other α_S-CN isoforms, the proportion of α_S-CN isoforms with a lower degree of phosphorylation correlated negatively with the proportion of α_S-CN isoforms with a higher degree of phosphorylation. In other words, in the milk of an individual cow, either α_S-CN isoforms with a lower degree of phosphorylation would be more abundant than those with a higher degree of phosphorylation or vice versa.

Phosphorylation could be influenced by many factors, such as genetic expression and efficiency of protein kinases, substrate availability, and accessibility of phosphorylation sites as a result of the conformational structure of proteins (Holland and Boland, 2014). It is still unclear what factors regulate the differences in α_S-CN phosphorylation among milk of individual cows. Two casein kinases with different substrate specificity could explain the hypothesis of one regulatory system responsible for isoforms with a lower degree of phosphorylation and another responsible for isoforms with a higher degree of phosphorylation. The casein kinases from the Golgi-enriched fraction of the lactating mammary gland (termed G-CK) are thought to phosphorylate caseins in vivo (Bingham and Farrell, 1974; Moore

Table 3. Phenotypic correlations[1] among α_S-CN phosphorylation isoforms and the 6 major milk proteins (% wt/wt of total protein in milk) measured on test-day morning milk samples from 529 Montbéliarde cows

Item	α_{S1}-CN-8P	α_{S1}-CN-9P	α_{S2}-CN-10P	α_{S2}-CN-11P	α_{S2}-CN-12P	α_{S2}-CN-13P	α_{S2}-CN-14P
Major milk protein							
α_{S1}-CN	0.90	0.33	0.11	−0.08	−0.01	−0.19	−0.14
α_{S2}-CN	0.22	−0.32	0.49	0.66	0.17	0.14	0.08
β-CN	−0.28	−0.16	−0.09	−0.08	−0.24	−0.10	−0.04
κ-CN	−0.17	0.04	−0.17	0.10	0.01	0.23	0.17
α-LA	0.22	−0.12	0.25	0.19	0.18	−0.30	−0.32
β-LG	−0.34	−0.11	−0.09	−0.09	0.06	0.01	−0.04
Phosphorylation isoform							
α_{S1}-CN-9P	−0.12						
α_{S2}-CN-10P	0.43	−0.68					
α_{S2}-CN-11P	0.22	−0.67	0.62				
α_{S2}-CN-12P	−0.10	0.19	−0.07	−0.28			
α_{S2}-CN-13P	−0.43	0.50	−0.58	−0.28	0.11		
α_{S2}-CN-14P	−0.38	0.49	−0.55	−0.27	−0.11	0.92	

[1]SE (0.01–0.04).

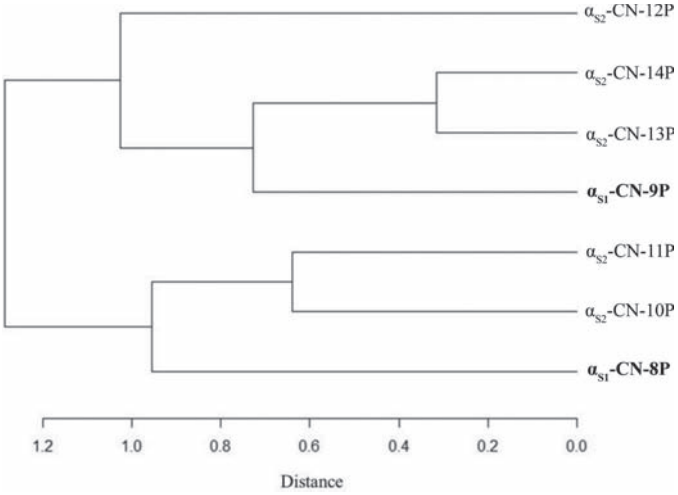

Figure 2. Dendrogram based on divisive hierarchical clustering analysis of phenotypic correlations among α_S-CN phosphorylation isoforms (% wt/wt of total protein in milk) measured on test-day morning milk samples from 529 Montbéliarde cows.

et al., 1985). The G-CK has been shown to phosphorylate consensus phosphorylation sites (Ser/Thr-X-Glu/SerP/Asp) with different efficiency. Ser-X-Glu is the most effective motif, Thr-X-Glu and Ser-X-Asp are less effective to be phosphorylated, and Thr-X-Asp has never been shown to be phosphorylated (Mercier, 1981; Meggio et al., 1988; Lasa-Benito et al., 1996). Bovine α_{S1}-CN has 10 potential phosphorylation sites, but only α_{S1}-CN-8P and α_{S1}-CN-9P are the common isoforms. The α_{S1}-CN-9P carries an extra phosphorylated serine at Ser_{41} in the Ser-X-Asp motif of the mature peptide chain (Manson et al., 1977). Furthermore, bovine α_{S2}-CN has 18 potential phosphorylation sites, but only 12 serine residues are in the consensus motifs. Until now, only 10 phosphorylation sites of α_{S2}-CN are identified, and they are in Ser-X-Glu/SerP motifs (Imanishi et al., 2007; Baum et al., 2013). Consequently, some threonine residues among the 2 Thr-X-Asp and 4 Thr-X-Glu motifs in α_{S2}-CN have to be phosphorylated for α_{S2}-CN carrying more than 12P. Additionally, threonine in Thr-X-Glu motifs would be more likely to be phosphorylated because phosphorylated threonine in Thr-X-Glu motifs has been identified in caprine, human, and equine β-CN when they are phosphorylated at the highest level (Greenberg et al., 1984; Neveu et al., 2002; Matéos et al., 2010). Recently, Fam20C has been identified as a bona fide G-CK, and *FAM20C* is also highly expressed in human breast (Lizio et al., 2015; The GTEx Consortium, 2015). However, Fam20C phosphorylates only Ser-X-Glu/SerP motifs (Ishikawa et al., 2012; Tagliabracci et al., 2012; Tibaldi et al., 2015; Tagliabracci et al., 2015), so it could be responsible only for forming α_S-CN isoforms with a lower degree of phosphorylation. To form α_S-CN isoforms with a higher degree of phosphorylation, another mammary G-CK should be responsible for phosphorylating serine or threonine in less favorable motifs, such as those in Ser/Thr-X-Asp or Thr-X-Glu motifs. This hypothesis is further supported by the phosphorylated serine in the Ser-X-Asp motif of α_{S1}-CN-9P. Identifying all phosphorylation sites of α_{S2}-CN and investigating the genetic background of different α_{S2}-CN phosphorylation isoforms may provide further insight into the phosphorylation mechanism of caseins.

CONCLUSIONS

We characterized the bovine α_{S1}-CN and α_{S2}-CN phosphorylation profile using the LC/ESI-MS method. This is the first study to show the presence of α_{S2}-CN-9P, α_{S2}-CN-14P, and α_{S2}-CN-15P, in addition to α_{S1}-CN-8P and α_{S1}-CN-9P and α_{S2}-CN-10P, α_{S2}-CN-11P, α_{S2}-CN-12P, and α_{S2}-CN-13P. Relative concentrations of α_{S1}-CN and α_{S2}-CN phosphorylation isoforms varied considerably among milk of individual cows. Interestingly, our results suggest 2 regulatory systems for the phosphorylation of α_S-CN: one responsible for isoforms with a lower degree of phosphorylation and the other responsible for isoforms with a higher degree of phosphorylation. Investigating the genetic background of α_{S2}-CN phosphorylation would help to elucidate the phosphorylation mechanism of caseins.

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