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Factors influencing degree of glycosylation and phosphorylation of caseins in individual cow milk samples

N. A. Poulsen,[1] H. B. Jensen, and L. B. Larsen
Department of Food Science, Aarhus University, 8830 Tjele, Denmark

ABSTRACT

The aim of this study was to examine variations in posttranslational modifications (PTM) of caseins (CN) in milk from individual cows and determine how these differ between breeds, across lactation, and between variants. Furthermore, we examined the variation of casein PTM in relation to rennet coagulation properties of milk. In total, detailed protein composition of milk from 892 Danish Holstein and Jersey cows was determined by liquid chromatography/electrospray ionization-mass spectrometry. The method measured relative contents of the main milk proteins as well as several variants and PTM. The results showed that the 2 breeds had distinct milk protein composition. Milk from Danish Holstein cows was mainly characterized by higher relative contents of β-CN, α-lactalbumin (α-LA), and β-lactoglobulin, and a higher fraction of glycosylated κ-CN (G κ-CN), whereas milk from Danish Jersey cows was characterized by higher relative contents of κ-CN, α_{S2}-CN, and the less phosphorylated forms of α_{S1}-CN and α_{S2}-CN. Univariate linear models including days in milk and parity as class effects showed variation in the detailed protein profile across and between lactations; in particular, changes in the degree of glycosylation of κ-CN were pronounced, but changes in α_{S1}-CN 8P to total α_{S1}-CN and α_{S2}-CN 11P to α_{S2}-CN were also observed over lactation for both breeds. The phosphorylated forms of α_{S1}-CN and α_{S2}-CN were, to some extent, correlated. Further, the κ-CN *BB* genotype was associated with higher relative contents of both unglycosylated κ-CN (UG κ-CN) and G κ-CN compared with κ-CN *AA*; κ-CN *AB* showed intermediate results in both breeds. The influence of protein composition on rennet coagulation properties was explored based on 4 classes for curd firming rate: noncoagulation, and poor, average, and good coagulation. The results revealed breed differences: Holstein milk, higher relative content of κ-CN to total protein, and higher content of G κ-CN were associated with improved milk coagulation. In contrast, relative content of α-LA was the main component associated with milk coagulation properties in Danish Jerseys and it was shown to affect milk coagulation properties negatively. In addition, variation in phosphorylation degrees of α_{S1}-CN also played a role. This study demonstrates that although the genetic influence of glycosylation seems to be the same in both breeds, nongenetic variation differs, which is further reflected in different associations with milk coagulation properties.

Key words: posttranslational modification, glycosylation degree, mass spectrometry, milk coagulation

INTRODUCTION

A large number of genetic casein variants (Farrell et al., 2004; Caroli et al., 2009), multiple glycosylation isoforms of κ-CN (Saito and Itoh, 1992; Holland et al., 2006), and various phosphorylation sites of, particularly, α_{S1}- and α_{S2}-CN but also κ-CN and β-CN (Holland, 2009) result in a very heterogeneous isoform pattern of the caseins, which is still not well understood even though the sensitivity of characterization techniques has been improved. Posttranslational modifications (**PTM**) affect casein micelle stability, and the highly glycosylated hydrophilic part of κ-CN, caseinomacropeptide (CMP), ensures electrostatic and steric repulsion between micelles in bovine milk (Dziuba and Minkiewicz, 1996). Several studies have documented that variation in κ-CN content affects casein micelle size (Frederiksen et al., 2011; Day et al., 2015), but the glycosylated part especially seems to play a major role (Bijl et al., 2014a). Thus, casein micelle size is strongly negatively correlated with the content of glycosylated of κ-CN (**G κ-CN**) but not with the content of unglycosylated (**U κ-CN**) in milk from Montbéliarde cows (Bijl et al., 2014a). Furthermore, κ-CN (*CSN3*) *BB* genotypes exhibit higher relative contents of κ-CN (Heck et al., 2009; Jensen et al., 2015), which seems to be related to higher levels of both U κ-CN and G κ-CN relative to *AA* genotypes (Bonfatti et al., 2014). Apart from the glycosylation of κ-CN, the relative distribution of the caseins, the associated calcium phosphate

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[1]Corresponding author: Nina.Poulsen@agrsci.dk

nanocluster, and phosphorylation of phosphoserine sites of the caseins also affect the technological properties of milk. Thus, a higher fraction of the least phosphorylated forms of α_{S1}-CN and α_{S2}-CN and a higher fraction of G κ-CN have been positively associated with rennet coagulation properties in milk from Danish Holstein (**DH**) cows (Frederiksen et al., 2011; Jensen et al., 2012), suggesting that variation in PTM plays a significant role determining the technological properties of milk.

The genetic influence on protein composition and distribution of the major milk proteins seems pronounced and thereby less affected by different feeding and management practices. The effect of the genetic amino acid polymorphisms of the caseins (or composite genotypes or haplotypes thereof) on protein composition and coagulation properties is well documented (Heck et al., 2009; Jensen et al., 2012), but other candidate genes, including genes associated with PTM, have been identified (Tyrisevä et al., 2008), suggesting that the genetic influence on coagulation properties is more complex. This is in line with the high heritabilities found for different phosphorylation forms of α_{S1}-CN, which suggest that variation in PTM are under strong genetic influence and that even highly similar isoforms such as α_{S1}-CN 8P and 9P (where P indicates the number of phosphorylated groups attached) can be regulated by different genes (Bijl et al., 2014b). Changes in PTM over a lactation are less understood, however. Bonfatti et al. (2014) showed that the content of UG κ-CN remained unchanged over lactation, whereas the relative concentration of G κ-CN increased over lactation, also reflected in increasing κ-CN contents and a higher degree of glycosylation. These findings suggest that the underlying mechanisms controlling regulation of PTM change over lactation.

Compared with DH cows, milk from Danish Jersey (**DJ**) cows has superior rennet coagulation properties, which previously have been associated with specific composite α_{S1}-, κ-, β-CN genotypes (Poulsen et al., 2013). Thus, DJ cows have higher frequencies of genetic protein variants associated with higher protein content, higher κ-CN content, and good rennet coagulation properties (Jensen et al., 2012). The objectives of the present study were to examine variation in casein PTM and distribution in relation to breed, protein variants, and milk coagulation properties in milk from healthy mid-lactation cows from 2 major Danish dairy breeds.

MATERIALS AND METHODS

Morning milk samples were collected from 22 Danish Jersey and 20 Danish Holstein herds as described in Poulsen et al. (2013).

Relative Quantification of Milk Proteins and Isoforms

The liquid chromatography/electrospray ionization-mass spectrometry (**LC/ESI-MS**) procedure used in the present study was outlined in Jensen et al. (2012). This protocol was developed from previous studies (Bobe et al., 1998; Bonfatti et al., 2008; Bonizzi et al., 2009) and modified according to Frederiksen et al. (2011). Variation of the major milk proteins were determined using a reversed phase LC-based method, where protein variants and isoform PTM of selected proteins were identified with the use of ESI/MS. Proteins were separated by reversed-phase HPLC using an HPLC 1100 system (Agilent Technologies, Santa Clara, CA) with a Jupiter C4 column (250 mm × 2 mm, 5 μm particle size, 300 Å pores; Phenomenex, Torrance, CA) operated at 40°C and a G1315A diode-array detector with UV detection at 214 nm coupled to a mass selective detector for identification and relative quantification of the milk proteins. Average molecular masses of the milk proteins were obtained using the deconvolution algorithm of the ChemStation software (rev.B.04.01 SP [650], Agilent Technologies). The procedures for sample preparation, settings for the HPLC, ESI source, and for the mass selective detector were as described by Frederiksen et al. (2011). All milk samples were analyzed in duplicate. Method reproducibility was determined by calculation of the coefficient of variation (**CV**) of the relative amount of individual milk proteins in a reference milk sample, run as part of each separate HPLC series. The relative protein content of the major milk proteins was calculated as the integrated peak area of a certain compound compared with total integrated peak area within each LC chromatogram.

Rheological Analyses for Determination of Milk Coagulation Properties

Rennet-induced coagulation of skim milk samples was determined by a ReoRox4 rheometer (MediRox AB, Nyköping, Sweden), as outlined in Poulsen et al. (2013). Briefly, milk samples were adjusted to pH 6.5 with 10% (vol/vol) lactic acid and incubated for 30 min at 33°C before rheological analysis. Thereafter, each milk sample was set into free oscillation, and amplitude damping and frequency changes were measured continuously for 1 h after addition of chymosin to a final concentration of 0.04 IMCU (international milk clotting units) per mL. Each milk sample was measured as technical duplicates. Milk coagulation properties for individual samples were described as rennet coagulation time (**RCT**) and curd firming rate (**CFR**) with the ReoRox software (version 1.5.0.1055). Rennet coagulation time was defined as time from chymosin