Phosphorylation and glycosylation isoforms of bovine κ -casein variant E in homozygous Swedish Red cow milk detected by liquid chromatography-electrospray ionization mass spectrometry

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ABSTRACT

Variations in the phosphorylation and glycosylation patterns of the common κ -casein (CN) variants A and B have been explored, whereas studies on variant E heterogeneity are scarce. This study reports for the first time the detailed phosphorylation and glycosylation pattern of the κ -CN variant E in comparison with variants A and B. Individual cow milk samples representing κ -CN genotype EE ($n = 12$) were obtained from Swedish Red cows, and the natural posttranslational modifications of its κ -CN were identified and quantified by liquid chromatography-electrospray mass spectrometry. In total, 12 unique isoform masses of κ -CN variant E were identified. In comparison, AA and BB milk consisted of 14 and 17 unique isoform masses, respectively. The most abundant κ -CN E isoform detected in the EE milk was the monophosphorylated, unglycosylated [1P 0G, ~70%; where P indicates phosphorylation from single to triple phosphorylation (1–3P), and G indicates glycosylation from single to triple glycosylation (1–3G)] form, followed by diphosphorylated, unglycosylated (2P 0G, ~12%) form, resembling known patterns from variants A and B. However, a clear distinction was the presence of the rare triphosphorylated, nonglycosylated (3P 0G, ~0.05%) κ -CN isoform in the EE milk. All isoforms detected in variant E were phosphorylated, giving a phosphorylation degree of 100%. This is comparable with the phosphorylation degree of variants A and B, being also almost 100%, though with very small amounts of nonphosphorylated, glycosylated isoforms detected. The glycosylation degree of variant E was found to be around 17%, a bit higher than observed for variant B (around 14%), and higher than variant

A (around 7%). Among glycosylation, the glycan e was the most common type identified for all 3 variants, followed by c/d (straight and branched chain trisaccharides, respectively), and b. In contrast to κ -CN variants A and B, no glycan of type a was found in variant E. Taken together, this study shows that the posttranslational modification pattern of variant E resembles that of known variants to a large extent, but with subtle differences.

Key words: posttranslational modifications, isoforms, glycan type, genetic variants

INTRODUCTION

κ -Casein is essential for structure, size, and stability of casein micelles in milk (Bijl et al., 2014). Properties of κ -CN can be influenced by many factors, including genetic polymorphisms, which can lead to AA changes in the expressed proteins. Bovine κ -CN has been found to be present in at least 14 allelic variants, including A, A¹, B, B², C, D, E, F¹, F², G¹, G², H, I, and J (Caroli et al., 2009). Among these genetic variants, A (reference variant) and B are the most common, whereas variant E has been identified especially in some Nordic cattle breeds, though in low frequencies, <5% (Lien et al., 1999). Different genetic variants of κ -CN could lead to different processing properties and functionalities of milk (e.g., micelle size and coagulation properties). Variant B of κ -CN has been associated with smaller casein micelles and a higher relative concentration of glycosylated κ -CN, compared with κ -CN variant A (Bijl et al., 2014; Sheng et al., 2021). Furthermore, improved coagulation properties have been associated with κ -CN variant B (Poulsen et al., 2013). κ -Casein variant E has previously been associated with poor or even noncoagulation properties in Swedish Red (Wedholm et al., 2006; Hallén et al., 2007) and Finnish Ayrshire (Ikonen et al., 1999) cattle. Poulsen et al. (2017a) also found a higher frequency of the κ -CN AE haplotype in poorly coagu-

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lating milk compared with good coagulating milk from Danish Holstein, which probably correlated with casein micelle size. Smaller casein micelles have been shown to promote the coagulation process of milk (Glantz et al., 2010). These differences could, at least in part, relate to posttranslational modification (PTM) patterns of κ -CN by influencing casein micelle properties (Bijl et al., 2019), and eventually also its cleavage by chymosin (Jensen et al., 2015). However, studies on the topic of the PTM pattern of κ -CN variant E are limited.

The structural background for the E variant is that it has a substitution of Ser¹⁷⁶ to Gly¹⁷⁶ compared with both variants A and B (Caroli et al., 2009). It is important to note that the Ser¹⁷⁶ is not in a position normally known to be part of PTM sites (Le et al., 2017). The phosphorylation sites of κ -CN primarily occur at Ser¹⁷⁰ and subsequently at Ser¹⁴⁸ (Talbot and Waugh, 1970; Mercier et al., 1973), but Thr¹⁶⁶ and Ser¹⁸⁷ can also be phosphorylated (Holland et al., 2006; Hernández-Hernández et al., 2011). Further distinctions between the variants are the presence of the polar residues Thr¹⁵⁷ and Asp¹⁶⁹ in variants A and E, which in variant B are substituted by the more hydrophobic Ile¹⁵⁷ and Ala¹⁶⁹ residues.

Further PTM of κ -CN include O-linked glycosylation at some specific Thr residues. The attached glycans consist of galactose (Gal), *N*-acetylgalactosamine (GalNAc), and *N*-acetylneuraminic (sialic) acid (NeuNAc). The most common type is tetrasaccharide (type e, ~56%), followed by trisaccharides with straight (type c, ~18%) or branched chain (type d, ~18%), disaccharide (type b, ~6%), and monosaccharide (type a, ~1%; Saito and Itoh, 1992). Six positions in variants A and B have been reported for glycosylation, consisting of Thr¹⁴², Thr¹⁵², Thr¹⁵⁴, Thr¹⁶³, Thr¹⁶⁶ (could also be phosphorylated), and Thr¹⁸⁶. In addition, the Thr¹⁵⁷ present in variant A has also been identified as a potential glycosylation site (Pisano et al., 1994; Holland et al., 2005; Holland et al., 2006). Threonine¹⁵⁷ is also present in variant E, but the glycosylation of all these sites in variant E is not known. In spite of this additional potential position for glycosylation in both A and E, κ -CN variant A has been shown to have a lower glycosylation degree, calculated as amount of glycosylated κ -CN isoforms relative to total κ -CN, compared with B (Bijl et al., 2014; Sheng et al., 2021). The glycosylation degree of variant E has not been reported earlier.

By liquid chromatography coupled to electrospray mass spectrometry (LC-ESI/MS), either by analysis of caseinomacropeptide (CMP) released from κ -CN variants A, B, or E (Jensen et al., 2015; Sunds et al., 2019) or the intact κ -CN molecule (Miranda et al., 2020; Nilsson et al., 2020), isoforms of κ -CN in milk were investigated. The isoforms identified in these stud-

ies covered 1 and 2P (phosphorylated) molecular forms, in combination with 0–3G (glycosylated) patterns, even in milk at the individual cow level. Specifically, a few of the individual cow milk samples were found to contain low abundant 0P or even 3P forms (Nilsson et al., 2020), though detailed PTM profiles for each variant were not shown. Therefore, it is now revealed to which extent the 3 different variants A, B, and E present with different isoform patterns, in different amounts, or both.

The aim of the present study is therefore to specifically investigate the detailed PTM pattern of κ -CN variant E in milk from individual κ -CN EE cows in comparison with milk from AA and BB cows.

MATERIALS AND METHODS

Intact proteomic analyses of individual cow milk samples of κ -CN phenotype EE ($n = 12$) were obtained from Swedish Red (Nilsson et al., 2020) and investigated by LC-ESI/MS (Poulsen et al., 2016). Deconvoluted MS spectra were used to obtain the information on qualitative and quantitative features. The detailed PTM patterns of the κ -CN EE milk were compared with those of AA ($n = 5$) and BB ($n = 4$) milk from individual Danish Holstein cows (Thesbjerg et al., 2021). The LC-ESI/MS results for mass abundances were analyzed using MassHunter 10 (Agilent; Thesbjerg et al., 2021). In short, MassHunter was used to deconvolute the ion chromatograms obtained from the individual milk samples. The deconvoluted masses were then assigned to a κ -CN specific in-house database, consisting of the κ -CN variants A, B, and E with 0–3P and 0–3G comprising glycosylation combinations as represented by the 5 different glycan types reported earlier (Saito and Itoh, 1992). To allow for process-induced modifications, the database included the possible loss of up to 3 water molecules, the addition of up to 3 deamidations, and the presence of up to 3 sodium adducts. In total, the database contained 2,700 entries. The relative abundance of each κ -CN isoform was calculated as a fraction of the specific ion intensity assigned to total κ -CN ion intensity in each sample. The glycosylation and phosphorylation degrees were calculated as a fraction of glycosylated or phosphorylated κ -CN ion intensities, respectively, relative to total κ -CN ion intensity, in each sample. Statistical analysis was performed by one-way ANOVA with Duncan's post hoc test using SPSS 25.0 software (IBM Corp.). Significant differences are shown for $P < 0.05$.

RESULTS AND DISCUSSION

Figure 1 shows the detailed PTM pattern identified for κ -CN E, representing a total of 12 unique masses,

but potentially representing more isoforms as some of the glycan combinations with (b, e) and (c/d, c/d) have identical masses. These isoforms have been merged into groups, as shown for 1P 3G (b, e, e), 1P 3G (c/d, c/d, e), or both. As expected, the most abundant isoform was 1P 0G [$70.0 \pm 4.7\%$ (SD), relative to total κ -CN], which had a significantly higher abundance over the other isoforms. This was followed by the 2P 0G ($11.7 \pm 3.1\%$). The most common glycosylation patterns were 1P 2G (e, e) and 1P 1G (e), which had relative abundances of $6.7 \pm 1.5\%$ and $5.7 \pm 2.1\%$, respectively. This was followed by 1P 3G (e, e, e) ($2.3 \pm 0.7\%$). Other low abundant isoforms, including 1P 1G (c/d), 1P 2G (c/d, e), 1P 3G (b, e, e), 1P 3G (c/d, c/d, e), 1P 3G (c/d, e, e), 2P 2G (e, e), and 2P 3G (e, e, e), all had relative abundances $<1\%$ of total κ -CN. Taken together, the most common glycan was type e, followed by c/d and b. No type a glycan was identified for variant E. Furthermore, small amounts of the rare 3P 0G isoform were

identified among E isoforms. All isoforms identified for variant E were phosphorylated.

Combined results of pure qualitative mass identifications of PTM isoforms of skim milk representing homozygous milk of κ -CN EE ($n = 12$), AA ($n = 5$), and BB ($n = 4$) cows are shown in Figure 2. In total, 17 unique masses were identified for variant B and 14 unique masses for variant A. Therefore, variant B comprised a larger heterogeneity in PTM isoforms than both A and E (12 unique masses). In total, 10 to 11 PTM isoforms were identified for all protein variants, including 1P 0G, 1P 1G (e), 1P 2G (c/d, e), 1P 2G (e, e), 1P 3G (b, e, e), 1P 3G (c/d, c/d, e), 1P 3G (c/d, e, e), 1P 3G (e, e, e), 2P 0G, 2P 2G (e, e), and 2P 3G (e, e, e). Remarkable differences include the presence of the rare 3P 0G isoform in variant E, whereas no 3P forms were detected in variant A or B. Additionally, very small amounts of 0P forms (with either 2 or 3G) were found in variants A and B, but not in variant E (Figure 2).

Figure 1. Relative abundance of posttranslational modification isoforms of κ -CN variant E expressed as their specific ion intensity relative to total κ -CN intensity of the liquid chromatography-electrospray ionization-MS of skim milk samples ($n = 12$). P indicates phosphorylation from single to triple phosphorylation (1–3P); G indicates glycosylation from single to triple glycosylation (1–3G). Lowercase letters in parentheses represent the 5 glycan types: (a) *N*-acetylgalactosamine (GalNAc), (b) galactose (Gal) $\beta(1-3)$ GalNAc, (c) *N*-acetyl-neuraminic acid (NeuAc) $\alpha(2-3)$ Gal $\beta(1-3)$ GalNAc, (d) Gal $\beta(1-3)$ [NeuAc $\alpha(2-6)$]GalNAc, and (e) NeuAc $\alpha(2-3)$ Gal $\beta(1-3)$ [NeuAc $\alpha(2-6)$]GalNAc. Different lowercase letters (a–e) above the whiskers represent significant different values within each isoform across phenotypes calculated by one-way ANOVA, $P < 0.05$. Error bars represent SD. The 2G forms with (b, e) or (c/d, c/d) had same molecular masses and thus could not be discriminated.

Figure 2. Schematic representation of specific posttranslational modification isoforms identified of κ -CN variants E ($n = 12$), A ($n = 5$), and B ($n = 4$) by liquid chromatography-electrospray ionization-MS of skim milk. P indicates phosphorylation from single to triple phosphorylation (1–3P); G indicates glycosylation from single to triple glycosylation (1–3G). Lowercase letters in gray boxes represent the 5 glycan types, a: *N*-acetylgalactosamine (GalNAc), b: galactose (Gal) β (1–3)GalNAc, c: *N*-acetyl-neuraminic acid (NeuAc) α (2–3)Gal β (1–3)GalNAc, d: Gal β (1–3)[NeuAc α (2–6)]GalNAc, and e: NeuAc α (2–3)Gal β (1–3)[NeuAc α (2–6)]GalNAc.

Apart from the major 1P 0G isoform, κ -CN normally also has a relatively high abundance of the 2P 0G isoform (Jensen et al., 2015; Sunds et al., 2019; Nilsson et al., 2020), as also observed here for the E variant (Figure 1). The primary sites for phosphorylation are, as mentioned, Ser¹⁷⁰ and partly Ser¹⁴⁸. Additionally, a possible third phosphorylation site of κ -CN variants A and B was reported for Thr¹⁶⁶ (Holland et al., 2006). This site matches well the general motif Ser/Thr-Xxx-Glu/Asp/pSer for phosphorylation, though with Ser residues preferred over Thr (Mercier et al., 1973; Kjeldsen et al., 2003; Hernández-Hernández et al., 2011). In addition, Ser¹⁸⁷ constitutes a potential third phosphorylation site (Hernández-Hernández et al., 2011). Therefore, the third phosphorylation identified here in κ -CN variant E could be at Thr¹⁶⁶ or at Ser¹⁸⁷. It should be noted that Thr¹⁶⁶ can also be O-glycosylated, and therefore may compete with phosphorylation. Type e glycan was the most common glycan form attached in variant E, consistent with earlier observations for variants A and B (Saito and Itoh, 1992; Nilsson et al., 2020).

To compare the most prevalent PTM isoforms present in comparison for all 3 genetic variants, we determined the relative abundances for each major isoform. Figure 3 displays the overall PTM patterns of the 3 genetic variants. Variant E largely followed the pattern of variants A and B in terms of common and rare isoforms, but also some interesting differences could be observed in the relative abundances. Variants E and

B had significantly lower relative abundance of the 1P 0G isoform (around 70%) compared with variant A (around 80%). Furthermore, variant E had significantly higher relative abundance of the 1P 1G isoforms com-

Figure 3. Relative abundances of posttranslational modification groups of κ -CN in milk samples representing genetic variants A ($n = 5$), B ($n = 4$), and E ($n = 12$). The ratio was calculated as specific ion intensity of each isoform relative to total κ -CN ion intensity. P indicates phosphorylation from single to triple phosphorylation (1–3P), and G indicates glycosylation from single to triple glycosylation (1–3G). Different lowercase letters (a–c) above the whiskers represent significant different values within each isoform across phenotypes calculated by one-way ANOVA, $P < 0.05$. Error bars represent SD.

pared with both variants A and B. For 1P 2G isoforms, variant E had a significantly higher relative abundance than variant A, but significantly lower than variant B. Variants B and E had similar relative abundance of the 1P 3G isoforms, which was significantly higher than variant A.

Using LC-ESI/MS, Nilsson et al. (2020) identified various PTM isoforms for κ -CN variants A, B, and E in Swedish Red dairy cattle. However, Nilsson et al. (2020) did not specify the exact isoforms per variant or their relative abundance. In comparison, the present study identified each specific isoform and its relative abundance for the κ -CN variants A, B, and E. Additionally, Sunds et al. (2019) identified the 1P 0G, 1P 2G (e, e), and 2P 0G isoforms in a LC-ESI/MS Single Q study of commercial caseinomacropptide isolates (Lacprodan cGMP-20, Arla Foods Ingredients), cleaved from each of these 3 genetic variants, which were all found in the present study. According to Thesbjerg et al. (2021), the most common glycosylated isoforms of κ -CN A and B in Danish Holstein-Friesian cows were the 1P 2G (e, e) followed by 1P 1G (e) and 1P 3G (e, e, e).

A limitation of these previous studies (Sunds et al., 2019; Nilsson et al., 2020; Thesbjerg et al., 2021) and the present study is that the intact protein analysis performed using different types of LC-MS methodologies did not provide or report data on the specific modification sites of κ -CN, and this could be interesting to investigate for variant E using a MS/MS-based analysis. The 2 main phosphorylation sites (Ser¹⁴⁸ and Ser¹⁷⁰), as well as the 6 shared glycosylation sites (Thr¹⁴², Thr¹⁵², Thr¹⁵⁴, Thr¹⁶³, Thr¹⁶⁶, and Thr¹⁸⁶) and the additional Thr¹⁵⁷ for variants A and E. These sites had been confirmed by bottom-up proteomics using tandem MS from enzymatic hydrolysis of κ -CN separated by 2-dimensional gel electrophoresis (Pisano et al., 1994; Holland et al., 2005, 2006). However, the quantitative PTM patterns of the different genetic variants were not reported.

Taken together, among κ -CN with different levels of phosphorylation from 0 to 3P, the 1P κ -CN molecules displayed more diversity of glycosylated isoforms compared with the other phosphorylation isoforms. All κ -CN variant E isoforms were found to be phosphorylated, giving a phosphorylation degree of 100%, and the phosphorylation degrees of variants A and B were almost 100%. Another important characteristic, the glycosylation degree can be calculated either by the relative peak area based on UV 214 nm (Poulsen et al., 2016), or by the ion intensity values as in the present study. From the present study, κ -CN variant E was calculated to have a glycosylation degree of $17.5 \pm 6.1\%$, and for κ -CN variant B of $14.2 \pm 3.6\%$, both

being significantly higher ($P < 0.05$) than that of variant A ($6.9 \pm 2.5\%$). The difference in glycosylation degree between A and B is consistent with previous studies based on UV peak areas; for example, Poulsen et al. (2016) found 23 and 28% for variants A and B in Danish Holstein cows, respectively, and 17 and 21% for variants A and B in Danish Jersey cows, respectively. The different value between each study might be due to different samples and more importantly methodologies. To the best of our knowledge, neither the phosphorylation nor glycosylation degree of κ -CN variant E have been reported earlier.

κ -Casein genetic variants A, B, and E showed different PTM with varying levels, which could influence casein micelle size and cause variation in the total concentration of κ -CN in milk (Bijl et al., 2014; Poulsen et al., 2016; Sheng et al., 2021). In combination, these factors could affect the technological properties of milk, for example, rennet-induced coagulation (Jensen et al., 2012) and formation of acid-induced milk gels (Gustavsson et al., 2014; Glantz et al., 2015; Nilsson et al., 2020). In this study, the casein micelle size of EE homozygous milk still remains to be elucidated, whereas Poulsen et al. (2017b) found that the AE haplotype displayed smaller casein micelle size than haplotypes A and B. However, the exact effect of phosphorylation and glycosylation pattern of κ -CN on these technological properties still needs to be investigated, especially in the context of good, poor, and noncoagulating milk. Investigations have pointed out the importance of glycosylation degree for coagulation properties of milk samples, for which coagulation ability increased with a higher fraction of glycosylated κ -CN (Bonfatti et al., 2014; Poulsen et al., 2016), but apparently not of importance in noncoagulating milk (Nilsson et al., 2020). Complying with the observation here of a glycosylation degree of variant E comparable with variant B, the potential association of variant E with good coagulation needs further investigation.

Recently, it was shown in an in vitro study that κ -CN AA milk had faster gastric digestion rate than BB and AB milk (Sheng et al., 2021). Therefore, it is also worth investigating the implications of the variabilities in the PTM modifications of κ -CN genetic variants that could affect their digestibility and peptide release to ascertain if some isoforms or variants are more digestible than others.

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