Effects of genetic variants and sialylation on *in vitro* digestibility of purified κ -casein

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ABSTRACT

Milk with different κ -casein (CN) phenotypes has previously been found to influence its gastric digestion rate. Therefore, the aim of the present study is to disentangle contributions of genetic variation and its related sialylation on the *in vitro* digestion process of κ -CN. Accordingly, κ -CN was purified from milk representing homozygous cows with κ -CN phenotypes AA, BB, or EE and used as substrate molecules in model studies using the INFOGEST 2.0 *in vitro* static digestion model. Furthermore, the effect of removal of the terminal sialic acids present on the O-linked oligosaccharides of the purified κ -CN A, B, and E protein variants were studied by desialylation enzymatic assays. The κ -CN proteins were purified by reducing anion exchange chromatography with purities of variants A, B, and E of 93.0, 97.1, and 90.0%, respectively. Protein degradations of native and desialylated κ -CN isolates in gastric and intestinal phases were investigated by sodium dodecyl sulfate-PAGE, degree of hydrolysis (DH), and liquid chromatography electrospray ionization mass spectrometry. It was shown that after purification, the κ -CN molecules reassembled into multimer states, which then constituted the basis for the digestion studies. As assessed by DH, purified variants A and E were found to exhibit faster *in vitro* digestion rates in both gastric and intestinal phases compared with variant B. Desialylation increased both gastric and intestinal digestion rates for all variants, as measured by DH. In the gastric phase, desialylation promoted digestion of variant B at a rate comparable with native variants A and E, whereas in the intestinal phase, desialylation of variant B promoted better digestion than native A or E. Taken together, the results confirm that low glycosylation degree of purified κ -CN promotes faster

in vitro digestion rates, and that desialylation of the O-linked oligosaccharides further promotes digestion. This finding could be applied to produce dairy products with enhanced digestibility.

Key words: glycosylation, sialic acid, digestion rate, degree of hydrolysis

INTRODUCTION

κ -Casein genetic variants A and B are the most common variants among different breeds (Caroli et al., 2009), including Nordic breeds (Poulsen et al., 2016). Studies have also identified κ -CN with the more rare E variant in Holstein Friesian cows in different Nordic and other European cattle breeds (Lien et al., 1999; Gustavsson et al., 2014; Ketto et al., 2017). Different genetic variants may represent variances in posttranslational modifications. The glycosylation degree (GD), the ratio of glycosylated κ -CN to total κ -CN, and the glycan combinations all show high degrees of heterogeneity between genetic variants A, B, and E (Nilsson et al., 2020; Sheng et al., 2022). Overall, κ -CN variant B is known to have a higher GD than variant A, and recent reports indicate that variant E has a GD around or slightly higher than that of B (Jensen et al., 2015; Poulsen et al., 2016; Sheng et al., 2022). Apart from glycosylation, bovine κ -CN is also known to be modified through disulfide bonds, with its 2 cysteine residues per κ -CN molecule (Cys¹¹ and Cys⁸⁸) being involved in intra- and intermolecular disulfide bonds (Mercier, Brignon, and Ribadeau-Dumas, 1973; Rasmussen et al., 1992). κ -Casein is accordingly present in raw bovine milk mainly as oligomers with 2 or more monomers linked by disulfide bonds, and only 10% of total κ -CN has been reported to be present as monomer (Huppertz, 2013).

The glycosylated part of κ -CN is located on the outside of the CN micelle and stabilizes the milk colloid system by steric hindrance and electrostatic repulsion (de Kruif, 1999; Tuinier and de Kruif, 2002). The gly-

Received September 14, 2021.

Accepted December 16, 2021.

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cans identified on bovine κ -CN consist of a combination of galactose (Gal), N-acetylgalactosamine (GalNAc) and N-acetylneuraminic (sialic) acid (NeuNAc). In bovine milk, κ -CN comprises 5 different types of glycans (a, b, c, d, and e), of which 3 (c, d, and e) contain NeuNAc—that is, are sialidated—and these account for more than 90% of the total glycans attached to κ -CN (Saito and Itoh, 1992). Because NeuNAc is negatively charged at normal milk pH conditions (~ 6.6 ; Vimr et al., 2004), it can provide a major contribution to intermicellar repulsion in milk (de Kruif, 1999; Tuinier and de Kruif, 2002). Sunds et al. (2019) reported that treatment with sialidase could efficiently remove almost all NeuNAc from the glycomacropeptide of bovine κ -CN. It has further been shown that desialylation of κ -CN affects its interactions with β -LG and other whey proteins (Robitaille and Ayers, 1995; Gaspard et al., 2020), as well its acid-induced gelation properties (Cases et al., 2003). It might be due to the disappearance of electrostatic repulsion and weak steric hindrance after removing NeuNAc.

In a skim milk system representing different genetic variants of κ -CN, it has been shown that glycosylated forms of κ -CN had slower rates of hydrolysis by chymosin during renneting, as measured by release of caseinomacropeptide (CMP; Jensen et al., 2015). Accordingly, other proteolytic processes involving κ -CN, such as gastrointestinal digestion, might be affected by glycosylation. Using in vitro digestion, it was recently found that κ -CN AA milk, with lower GD of its κ -CN molecules, showed faster gastric digestion rate compared with AB and BB milk (Sheng et al., 2021). Therefore, glycosylation together with the genetic variants, and accordingly also CN micelle size (Bijl et al., 2014), may play a role in κ -CN and thereby milk protein digestibility (Sheng et al., 2021).

To further explore the contribution of κ -CN protein genetic variants and their associated posttranslational modifications in a nonmicellar system, a model study involving in vitro gastrointestinal digestion of the purified κ -CN protein genetic variants A, B, and E was carried out. The potential effect of the terminal sialic acids on κ -CN glycans on in vitro gastrointestinal digestibility was studied via comparison of in vitro digestibility using the INFOGEST 2.0 protocol (Brodtkorb et al., 2019) before and after desialylation. The protein degradation during in vitro gastrointestinal digestion was characterized using SDS-PAGE, liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) and degree of hydrolysis (DH). Ultimately, the results obtained could be exploited for production of more easily digested dairy products targeting consumers with weak digestive function or specific consumer segments.

MATERIALS AND METHODS

Purification of κ -CN Variants from Homozygous Milk

Individual cow milk with κ -CN phenotypes AA ($n = 3$) and BB ($n = 4$) were obtained from Danish Holstein cows from a single morning milking at the Danish Cattle Research Centre (Foulum, Aarhus University, Denmark) after a larger screening based on LC-ESI/MS Single Quadrupole (Q) protein analysis to identify homozygotes (Sheng et al., 2021). Fresh milk samples from the Danish cows were centrifuged at $2,643 \times g$ for 30 min at 4°C to remove fat and prepare skim milk, followed by snap freezing at -80°C for 24 h and then transferred to -20°C until further use. Individual EE skim milk samples ($n = 12$) were obtained by centrifugation of the milk samples ($2,000 \times g$, 4°C , 30 min) from Swedish Red cows (Nilsson et al., 2020). These were stored at -18°C until being shipped frozen to the laboratory at Aarhus University, Denmark, and then stored at -20°C until further use. After thawing, the individual skim milk samples representing each genetic variant were pooled according to same volume, followed by acid precipitation to prepare crude CN fractions. A total of 5 mL of 10% CH_3COOH was carefully mixed with 50 mL of each genetic pool of skim milk (A, B, and E) for 2 min until precipitate occurred (pH ~ 4.6), followed by adding 5 mL of 1 M CH_3COONa . The obtained precipitates were washed twice with Milli-Q water (Sheng et al., 2021) and then dissolved into 50 mL of Milli-Q water with pH adjusted to 7.0 to 7.5 with 5 M NaOH, followed by dialysis against buffer A [20 mM bis-tris propane, 3.3 M urea, 1 mM 1,4-dithioerythritol (DTE), pH 7.0 by HCl] until the dissolved CN preparations had same conductivities as buffer A. The DTE was included in buffer A to allow for monomer formation of κ -CN during purification (Petrat-Melin et al., 2016), as this was considered to be simpler than isolating the κ -CN in various multimer forms. The purification principle aiming for all κ -CN isoforms was based on fast protein liquid chromatography using ÄKTA fast protein liquid chromatography preparative ion exchange chromatography equipped with UNICORN operating system (GE Healthcare/Cytiva) basically as described earlier (Petrat-Melin et al., 2016) with the following modifications. The fast protein liquid chromatograph was equipped with an XK 26/40 column manually packed with Q Sepharose Fast Flow anion exchange resin (Cytiva) with a column volume of 150 mL. A volume of 250 mL of each of the dialyzed CN preparations A, B, and E were pumped and loaded onto the column at a flow rate of 5 mL/min. After equilibration with 1 column volume of buffer A, a linear gradient of 0 to 20% of buffer B (with ad-

ditional 2 M NaCl compared with buffer A) in 6 column volumes at a flow rate of 5 mL/min. Fractions of 14 mL were collected using a Frac-900 fraction collector (GE Healthcare). Eluted proteins were monitored by UV 214 nm, and peaks were analyzed by SDS-PAGE (Petrat-Melin et al., 2015). Fractions containing pure κ -CN based on SDS-PAGE pooled for each variant and dialyzed using 6 to 8 kDa cut-off dialysis membranes (Millipore) against 5 L of nonreducing 10 mM ammonium bicarbonate solution for 24 h at room temperature with 3 buffer shifts to desalt and allow for multimerization again at nonreducing conditions. After dialysis, the isolates were freeze-dried, weighed, and stored at -80°C until further use.

Desialylation of Purified κ -CN Variants A, B, and E

Sialidase from *Clostridium perfringens* (Sigma Aldrich) was used for desialylation assays of the purified κ -CN variants A, B, and E according to Sunds et al. (2019). The κ -CN solution of each genetic variant was prepared by dissolution of purified, freeze-dried κ -CN material into Milli-Q water (Millipore) at 10 mg/mL. The protein concentrations were further measured by bicinchoninic acid assay (Thermo Scientific) as previously described (Petrat-Melin et al., 2016), revealing protein concentrations in purified materials of approximately 80%.

The pH of κ -CN solutions (pH $\sim$ 7) was not adjusted to the optimum pH for sialidase (pH $\sim$ 5), as κ -CN will aggregate at this pH, and thus the desialylation was carried out at pH $\sim$ 7. For desialylation, 2 units (U) of the sialidase with an activity of 100 U/mg of enzyme (Sigma Aldrich) was added into 14 mL of each κ -CN solution, corresponding to 1 U/70 mg of purified κ -CN, and incubated at 37°C for 24 h. This enzyme-to-substrate ratio was 8 times higher than that (1 U/570 mg of glycosylated CMP isolates) employed by Sunds et al. (2019), due to the higher pH used here.

SDS-PAGE

The protein profiles of the purified κ -CN isolates before and after desialylation and of in vitro digests were assessed by SDS-PAGE at reducing (including 5 mM DTE, final concentration in sample buffer) and nonreducing conditions (Petrat-Melin et al., 2015) using precast Criterion Tris-HCl gels (Any kDa, BioRad Laboratories). For assessment of purified κ -CN isolates before and after desialylation, 20 μg of reduced or non-reduced protein was loaded to each well (12-well gels). The digests were diluted twice in Milli-Q water, and 15 μg of reduced protein was loaded to each lane (18-well gels). The gels were stained with colloidal Coomassie

brilliant blue G-250 (Serva). Identifications of proteins were according to earlier reports (Petrat-Melin et al., 2015, 2016).

Protein Profiling by LC-ESI/MS

Detailed protein profiles of κ -CN A, B, or E preparations before or after desialylation were analyzed by a reverse-phase LC-ESI/MS Single Q system equipped with a Jupiter C4 column (250×2 mm, 5- μm particle size, 300 Å pore size; Phenomenex), essentially as described earlier (Poulsen et al., 2016; Sunds et al., 2019). Solvent A was 0.05% trifluoroacetic acid in Milli-Q water, and solvent B was 0.05% trifluoroacetic acid in acetonitrile. A total of 10 μL of 5 mg/mL purified protein preparations dissolved in Milli-Q water were analyzed in technological duplicates. Separated proteins were monitored by UV at 214 nm. Briefly, the mass peaks representing different protein isoforms were identified by matching the deconvoluted average mass against an in-house milk protein database (Jensen et al., 2015) using the ChemStation software (Rev. C.01.10, Agilent Technologies). Purity of isolated κ -CN variants A, B, and E was calculated as relative peak areas at 214 nm of total κ -CN relative to the total peak area. The GD before and after desialylation were calculated as peak area of peaks representing glycosylated κ -CN forms relative to total κ -CN peak area in the run, based on a single sample. Furthermore, data from LC-ESI/MS Single Q analyses of the original skim milk samples from individual cows before pooling for purification were further used for calculations of relative ion abundances of specific isoforms based on deconvoluted ion chromatograms obtained by MassHunter 10 (Agilent Technologies) as earlier reported (Thesbjerg et al., 2021). The obtained deconvoluted masses were matched to a κ -CN specific in-house database for identification of specific proteoforms. Relative abundance of each isoform in 3 purified κ -CN isolates were calculated by its specific ion intensity relative to total κ -CN ion intensity.

In Vitro Gastrointestinal Digestions of Purified κ -CN Variants Before and After Desialylation

Because proteases are not prevalent in saliva, the IN-FOGEST 2.0 protocol (Brodkorb et al., 2019) without the oral phase was conducted to investigate the digestibility of native and desialylated purified κ -CN variants A, B, and E. Freeze-dried κ -CN isolates dissolved into Milli-Q water (10 mg/mL) were subjected to the in vitro gastrointestinal digestion procedure with details as described earlier (Sheng et al., 2021). Final concentrations of pepsin (activity 337.6 U/mg of enzyme) and

pancreatin (activity 4.9 U/mg of enzyme) in the gastric and intestinal phases were 100 and 5 U/mL of κ -CN solution, respectively. The gastric digestion was stopped after 0, 1, 2, 5, 10, 15, 30, 60, and 120 min (designated **G0**, **G1**, **G2**, **G5**, **G10**, **G15**, **G30**, **G60**, and **G120**), and intestinal digestion, after the 2 h of gastric digestion, was stopped after 0, 1, 2, 5, 10, 15, 30, 60, and 120 min (designated **I0**, **I1**, **I2**, **I5**, **I10**, **I15**, **I30**, **I60**, and **I120**) as described earlier (Sheng et al., 2021). The whole digestion process was performed in complete biological duplicates for each sample of purified κ -CN variants A, B, and E, before and after desialylation.

Degree of Hydrolysis

An *o*-phthalaldehyde assay (Butré et al., 2014) was used to follow the increase in level of free amino terminals during digestions. Each sample of native or desialylated purified κ -CN variants A, B, and E after both gastric and intestinal in vitro digestion at the different time points was measured for the biological duplicates. Based on a glutamine standard curve, measured levels of free amino terminals were calculated and expressed as glutamine equivalents (mM). The calculation of DH was outlined previously (Sheng et al., 2021). The maximal concentration of free amines was obtained by hydrolyzing the protein solutions before digestion in 6 M HCl at 110°C for 24 h (Halabi et al., 2020; Sousa et al., 2020).

Data Analysis

The data were analyzed by one-way ANOVA and Duncan's post hoc test by software SPSS 25.0 IBM Corp., as described previously (Sheng et al., 2021). Significant differences were defined as $P < 0.05$.

RESULTS

Protein Profiling of Native and Desialylated Purified κ -CN Variants

Bovine κ -CN variants A, B, and E were purified from pooled skim milk from homozygous cows by reducing anion exchange chromatography, which, after purification, was thoroughly dialyzed at nonreducing conditions. The yield of purified κ -CN was measured, based on weighing of the dialyzed freeze-dried protein material. The yield was found to differ between the 3 genetic variants, with obtained yields of 484, 946, and 288 mg/L of skim milk, of variants A, B, and E, respectively.

The purity and oligomer states of the purified κ -CN A, B, and E variant proteins after dialysis and freeze

drying were initially assessed by SDS-PAGE at reducing and nonreducing conditions (Figure 1). At nonreducing conditions, it was evident that a major part of the purified κ -CN had reformed into multimers, demonstrated as ladder structures in the upper part of the gel. After reduction with DTE, these multimer structures disappeared and condensed into a protein band at the position of κ -CN monomer, with a molecular mass around 20 kDa. Based on the SDS-PAGE analysis, it was seen that the purified κ -CN fractions were quite pure but also contained minor bands representing small amounts of the whey proteins, including BSA, β -LG, and α -LA. Furthermore, even smaller amounts of protein appeared in expected positions of α_{S1} - and α_{S2} -CN and β -CN regions, showing only minor contamination. Similar patterns were found for the native and desialylated κ -CN variants (Figure 1).

As it is known that SDS-PAGE is not quantitative in its nature, the purity and furthermore the isoform compositions of the isolates of κ -CN variants A, B, and E before and after desialylation but before the in vitro digestibility studies were subjected to LC-ESI/MS Single Q proteomic analysis (Figure 2). Using UV peak areas at 214 nm, variant B was calculated to have the highest purity (97.1%), followed by variants A (93.0%) and E (90.0%). The LC-ESI/MS analysis thus confirmed high purity of the purified variants, as well as comparable UV chromatograms before and after desialylation, with the major peak, constituting monophosphorylated, non-glycosylated κ -CN (κ -CN A and E 1P, and κ -CN B 1P) for all genetic variants moved a little toward the left, showing shorter retention time after desialylation (Figure 1). Based on UV peak area, the calculated GD for variant B 14.3% is higher than those of variants A and E, appearing with comparable GD of 13.2% and 13.4%, respectively. As expected, the desialylation did not change the overall calculated GD for each genetic variant, as based on UV 214 nm, by the sialic acid removal.

Furthermore, isoform identification and relative compositions were investigated based on their deconvoluted masses matched against an in-house database for identifications and using relative ion intensities for relative calculations of composition based on MS signals. From this, the ion intensity-based GD values were calculated, in addition to the UV-based GD previously described. Results for GD based on MS ion intensities were again variant B with highest GD (15.0%), followed by variant E (11.5%) and variant A (7.6%). The proteomic footprint of each genetic variant based on the deconvoluted masses and the prevalence of each form, including identified glycoforms before and after desialylation based on the relative ion intensities, are shown in Table 1. For all 3 genetic variants, A, B, and E, the vast

Figure 1. Sodium dodecyl sulfate-PAGE of purified κ -CN variants A, B, and E under nonreducing and reducing conditions, and before and after desialylation (-de). For each lane, 20 μ g of protein was loaded. Molecular mass markers are indicated in kDa to the left.

majority of the molecules were phosphorylated (Table 1), as observed both before and after desialylation. The most common glycan type present in all 3 variants before desialylation was the e form, with κ -CN B having the highest relative abundance of type e. This analysis confirmed the change in glycosylation type from the sialylated e or c and d forms into b, with no sialylations after the enzymatic treatment, showing that sialylation successfully removed all NeuNAc from κ -CN. One contaminant potential protein peak in each purification, with masses of 22,802, 20,523, and 22,843 in variants A, B, and E, respectively, were also found, but none of these matched any of the known major milk proteins, and therefore these masses were not known.

SDS-PAGE Analysis of In Vitro Gastric Digests of Native and Desialylated Purified κ -CN Variants

To monitor the degradation of κ -CN during the in vitro gastric digestion, both native and desialylated variants A, B, and E were analyzed by SDS-PAGE during the time course of the digestions (Figure 3). It was seen that after 1 min of gastric in vitro digestion (G1) the κ -CN band had already started to disappear, and after 5 min of gastric digestion (G5) almost all intact κ -CN had been cleaved (Figure 3A). However, after 1 min of gastric digestion, almost all κ -CN in desialylated samples was hydrolyzed, and new bands appeared between the masses of the 10- and 25-kDa marker proteins, as shown in the G1 samples (Figure

3A). Except for β -LG, almost all proteins and large peptides were hydrolyzed into small peptides after 10 min of in vitro gastric digestion, although some bands with masses between 10 and 15 kDa remained in native κ -CN variants A and B (Figure 3A, G10). After 30 min of gastric digestion (G30), only the protein bands representing β -LG were observed, and all other protein bands disappeared (Figure 3A). No obvious change in β -LG was found during the whole in vitro gastric digestion, which therefore appeared quite resistant for the gastric in vitro digestion. The SDS-PAGE analysis showed that the gastric digestion rate of the purified κ -CN for the 3 genetic variants increased after desialylation.

Degree of Hydrolysis of Native and Desialylated Purified κ -CN Variants During in Vitro Gastrointestinal Digestion

Calculation of DH during in vitro gastrointestinal digestion of native and desialylated κ -CN A, B, and E was based on the level of free amino terminals determined by *O*-phthaldialdehyde assay at different time points (Figure 4). During the in vitro gastric digestion (G0–G120, Figure 4A), the DH of all samples were seen to continuously increase until 90 min, where a plateau was reached, with no further hydrolysis occurring toward G120. The intact κ -CN variants A and E and all desialylated samples (A-de, B-de, and E-de) had similar DH at all time points during gastric di-

gestion. However, native κ -CN variant B had a slower gastric digestion rate, as native κ -CN variant B had significantly lower ($P < 0.05$) DH at G90, compared with other samples. At the end of gastric digestion, all samples had comparable DH ($\sim 10\%$).

Furthermore, significant differences relative to genetic variant and desialylation were observed over intestinal digestion (I0–I120, Figure 4B). The DH of all samples continuously increased until 30 min, where a plateau was reached and no further hydrolysis was observed. For desialylated samples, the DH of 3 genetic variants were significantly higher ($P < 0.05$) than those of native samples at all intestinal digestion time points from I2 onward (Figure 4). Variants A and E followed same trends, both before and after sialidase treatment, with their DH being significantly higher ($P < 0.05$) than that of variant B. At the end of intestinal digestion, the order of DH from highest to lowest were the desialylated variants A and E ($\sim 44\%$), desialylated variant B ($\sim 41.5\%$), native variants A and E ($\sim 40\%$), and native variant B ($\sim 38.5\%$).

DISCUSSION

The aim of this study was to investigate the influence of the primary structures of different protein genetic

variants of κ -CN on its in vitro digestibility. For this purpose, we used an ion exchange to purify κ -CN A, B, and E protein genetic variants from homozygous milk and use these isolates in in vitro gastric and intestinal digestion studies using the INFOGEST 2.0 protocol. By using κ -CN isolates it is possible to disentangle primary structure related differences from the potential contribution of different CN micelle sizes as present when studying κ -CN in milk systems. Further, the effect of stripping off the negatively charged sialic acids from the O-linked glycans attached was furthermore studied by in vitro gastrointestinal studies of intact and desialylated purified κ -CN.

Using urea-based reducing anion exchange chromatography it was possible to obtain isolates of κ -CN A, B, and E genetic variants using homozygous milk as starting material. Using a concentration of κ -CN of 3,300 mg/L of milk (Remblay et al., 2003), the yields of κ -CN ranged from 9% for variant E to over 15% for A and 29% for variant B. The reason for lower yield from the E milk is not known. Even though the E milk samples were transferred in frozen state from Sweden to our laboratory in Denmark, there may be effects from a longer freezing storage period and shipping history compared with the Danish sampled A and B milk samples. The protein contents of the κ -CN isolates were

Figure 2. Liquid chromatography electrospray ionization MS chromatogram of reference milk sample, and of native and desialylated purified κ -CN variants, indicated as A, B, and E, and as A-de, B-de, and E-de, respectively. The protein isoforms are indicated by arrows. P indicates phosphorylation from single to triple phosphorylation.

Table 1. The phosphorylation and glycosylation forms attached (b, c, d, e) to native and desialylated purified κ -CN variants A, B, and E, as analyzed by liquid chromatography electrospray ionization MS Single Quadrupole (Q); relative abundances based on ion intensities

Sample ID ¹	Modification of κ -CN ²	Retention time (min)	Deconvoluted mass (Da)	Theoretical mass (Da)	Relative abundance ³ (%)	Glycosylation degree ⁴ (%)
A	1P	5.9	19,037.4	19,035.5	90.3	3.8
	2P	5.8	19,117.1	19,115.5	5.9	
	1P 1G (e)	4.8	19,985.2	19,983.6	3.8	
B	1P	8.7	19,905.4	19,003.6	85.4	7.5
	2P	8.8	19,084.8	19,083.5	7.1	
	1P 1G (e)	7.7	19,952.7	19,951.6	6.7	
	1P 1G (c/d)	7.8	19,661.9	19,660.0	0.8	
	None	7.8	18,926.7	18,925.5	5.3	
E	1P	6.1	19,007.1	19,005.5	78.4	5.5
	2P	6.2	19,086.2	19,085.5	10.8	
	1P 1G (e)	4.9	19,554.3	19,953.5	4.3	
	1P 2G (e, e)	4.6	20,901.2	20,901.4	0.8	
	1P 2G (c/d, e)	4.5	20,610.2	20,609.8	0.4	
	1P	5.7	19,034.5	19,035.5	84.4	
	2P	5.7	19,114.9	19,115.5	9.0	
A-de	1P 1G (b)	4.6	19,377.5	19,400.8	6.6	6.6
	1P	8.3	19,003.0	19,003.6	86.4	
	2P	8.3	19,082.5	19,083.5	6.2	
B-de	1P 1G (b)	7.1	19,367.4	19,368.9	6.4	7.4
	1P 2G (b, b)	4.1	19,732.5	19,734.0	1.0	
	None	7.0	18,923.4	18,925.5	3.7	
E-de	1P	6.0	19,004.3	19,005.5	78.8	3.9
	2P	6.1	18,084.1	19,085.5	12.6	
	1P 1G (b)	4.4	19,369.0	19,370.8	3.9	

¹A, B, E, A-de, B-de, and E-de represent purified κ -CN variants A, B, and E, and desialylated A, B, and E, respectively.

²P indicates phosphorylation from single to triple phosphorylation (1P–2P), and G indicates glycosylation from single to triple glycosylation (1G–2G). Lowercase letters in the parentheses represent 5 different glycan types, as follows: b = galactose (Gal) β (1–3) *N*-acetylgalactosamine (GalNAc), c = *N*-acetyl-neuraminic acid (NeuNAc) α (2–3)Gal β (1–3)GalNAc, d = Gal β (1–3)[NeuNAc α (2–6)]GalNAc, and e = NeuNAc α (2–3)Gal β (1–3)[NeuNAc α (2–6)]GalNAc.

³Relative abundance was calculated by dividing specific ion intensity of each κ -CN isoform relative to total κ -CN ion intensity, respectively.

⁴Glycosylation degree was calculated by dividing ion intensity of total glycosylated κ -CN relative to total κ -CN ion intensity, respectively.

high, and $\geq 90\%$ wt/wt for all variants, as assessed by protein assay and weighing of the desalted, freeze-dried isolates. As mentioned, the κ -CN was purified under reducing conditions during the ion exchange, as earlier employed, and then considered to reform into the oligomer forms after desalting and concomitant removal of the reducing agent (Groves et al., 1998; Farrell et al., 2003; Petrat-Melin et al., 2016). This was confirmed by our SDS-PAGE analyses. Via SDS-PAGE, and further by the LC-ESI/MS Single Q analyses based on UV at 214 nm, it was confirmed that the purity of the isolates was quite good. Some minor contaminating bands were observed in the SDS-PAGE by Coomassie brilliant blue staining, but in the LC-ESI/MS analysis, only very minor contamination by 214 nm was observed, at the position between β - and γ -CN peaks (Figure 2). The proteins in these peaks were not identified but are thought to represent degradation products of β -CN, which is in accordance with the presence of some β -CN contamination, as also evident in the gels. The contaminating peak was highest in the variant E preparation. This is in line with a possibly slightly higher level of degradation in the κ -CN E material. It is noted that

the contaminations of the κ -CN preparations seemed more prevalent according to the SDS-PAGE analysis compared with the LC-ESI/MS Single Q analysis, but it is known that SDS-PAGE is only semiquantitative, and therefore this analysis was used mainly to interpret the reformation of κ -CN oligomers at nonreducing conditions after purification. The distribution of isoforms in the purified materials was further assessed by LC-ESI/MS using ion intensities of the different isoforms to calculate relative abundances. The main isoforms, 1P0G, 2P0G, 1P1G (e), and 1P2G (e, e), were found to comply well with the isoform patterns recently reported for κ -CN A ($n = 5$), B ($n = 4$), and E ($n = 12$) molecules as present in individual skim milk (Thesbjerg et al., 2021; Sheng et al., 2022). This indicates that even though only a fraction of the collective pool of κ -CN molecules present in the used skim milk was purified from each milk source (AA, BB, and EE milk pools), the main isoform distribution of the purified fractions mirrors that of the native skim milk. One minor difference was that, as shown in our previous work (Sheng et al., 2022), in milk, native κ -CN contained glycan types c, d, and e, as well as low abundances of a and

Figure 3. SDS-PAGE of in vitro gastric digests of purified κ -CN variants A, B, and E before and after desialylation (-de). (A) Gastric digests at time points G0 (control), G1, and G2; (B) at time points G5, G10, and G15; and (C) at time points G30, G60, and 120 (min). For each lane, 15 μ g of protein was loaded. Variants, enzymatic treatment, and gastric digestion times (in min) are given. Molecular mass markers are indicated in kDa to the left. All samples were reduced with dithioerythritol.

Figure 4. Degree of hydrolysis (DH) for in vitro digestion of native and desialylated purified κ -CN variants A, B, and E: (A) gastric phase for G0, G1, G2, G5, G10, G15, G30, G60, and G120 (min); (B) intestinal phase for I0, I1, I2, I5, I10, I15, I30, I60, and I120 (min). Each sample was measured in biological duplicates, and SD is indicated. A, B, E, A-de, B-de, and E-de represent intact and desialylated purified κ -CN variants A, B, and E, respectively. * indicates statistically different values at the indicated time point ($P < 0.05$). In (A) * shows the significant difference between native variant B and other samples at G60. In (B) * shows the significant difference between native and desialylated samples at all intestinal digestion time points.

b. However, only κ -CN with e, c, and d were identified in this study after purification, potentially due to their concentration being below the limit of detection.

In the present work, the GD of the purified materials were found to be in good compliance for variants B and E, whether calculated based on UV 214 integrations or relative ion intensities from MS, whereas for variant A a slightly higher GD based on UV compared with ion intensity-based calculation was obtained (UV-based GD here of 13, 14, and 13%, and MS-based GD of 8, 15, and 12%, for variants A, B, and E, respectively). It could be speculated that the response factors for different isoforms relative to their UV absorption, as well as their ionization in the MS-based detection and quantification may vary, but this does not seem to be contributing here to large variances as based on the observations of the GD in the purified κ -CN molecules here, but could be the subject of future studies. What may be of greater significance relative to earlier reports regarding GD values for different protein forms of κ -CN may relate to its integration at UV level when analyzing complex samples such as skim milk. In earlier studies GD values of, for instance, 23 and 28% for variants A and B, respectively, as based on UV at 214 nm, have been reported (Poulsen et al., 2016), compared with 7, 14, and 17% as based on ion intensities (Sheng et al., 2022). This may indicate that the peak ascertained for glycosylated κ -CN might contain not only glycosylated κ -CN, but also some other proteins or protein fragments, and thus the quantification of κ -CN glycosylation calculated by LC and based on UV may be overestimated, whereas, using MS-based quantification for GD calculations, masses not complying with κ -CN are filtered off. In spite of these differences, according to the UV-based GD calculation confirmed in this study, as would be expected, the enzymatic desialylation did not change the GD for any of the purified genetic variants.

Desialylation was found to remove all the sialic acid from κ -CN for the 3 variants, which increased the gastric digestion rate and increased the intestinal DH for all 3 variants in such a way that the levels of B-de were comparable with the levels of A-de and E-de. Glycosylation has earlier been reported to influence hydrolysis of κ -CN with proteases. Jensen et al. (2015) reported that glycosylated CMP isoforms had a slower release rate from κ -CN by chymosin in a milk system compared with non-glycosylated. The same phenomenon has also been previously shown and further discussed by Van Hooydonk et al. (1984) and Ferron-Baumy et al. (1992). They proposed that the hydrolyzing rate of chymosin on κ -CN could be decreased when κ -CN had more glycans, and especially negatively charged and hydrophilic glycans comprising NeuNAc, such as

glycan types c, d, and e. Several previous studies have suggested that the cleavage access of chymosin could be hindered by glycan chains, which further inhibited the release of CMP (Saito and Itoh, 1992; Dziuba and Minkiewicz, 1996; Brulé et al., 2000). Therefore, the present study is in line with the suggestion that low GD, and especially fewer sialylated glycans, promotes gastric digestion rate, possibly due to less electrostatic repulsion and especially steric hindrance. This is in line with the observations in our previous study on pooled skim milk (Sheng et al., 2021), where κ -CN phenotype AA showed a significantly higher gastric digestion rate compared with AB and BB. It was speculated that this was due to AA milk having lower GD.

Degree of hydrolysis and SDS-PAGE are different and to some degree, complimentary to each other. Gels (Figure 3) are suitable to assess the disappearance of intact protein and appearance of large peptides in the digests. During intestinal digestion, large peptides will be hydrolyzed into small peptides or even free amino acids, which cannot be detected by any kinds of gels. Therefore, the in vitro intestinal digests were not measured by gels in this study. Instead, the detailed digestion process was monitored by DH (Figure 4). Probably due to differences in specificities of the responsible digestive enzymes for the in vitro gastric and intestinal phases, their hydrolysis rates and DH were affected by GD and desialylation to different degrees in the 2 phases. The gel results indicated that desialylation increased the gastric digestion rate of intact κ -CN for all genetic variants, whereas according to DH, the levels of A, E, A-de, B-de, and E-de were comparable, with only the intact B variant being statistically significantly different and lower. Furthermore, no significant differences in DH were found by comparing all samples at the end of gastric digestion, which may be due to the fact that there is only one principal cleavage site of pepsin in the glycosylated part of κ -CN (Rawlings et al., 2018).

On the other hand, desialylated κ -CN showed significantly higher DH than native κ -CN throughout the in vitro intestinal digestion. Furthermore, with same treatment, variants A and E had comparable levels of intestinal DH, which was significantly higher than that of variant B. Therefore, taken together, low glycosylation and further desialylation are suggested to promote the digestibility of κ -CN. Furthermore, in the intestinal phase, desialylation of variant B promoted a better increase in digestibility than that observed for native A or E.

Although using a different in vitro gastrointestinal digestion protocol, Petrat-Melin et al. (2016) also found that the purified κ -CN with the highest glycosylation showed the lowest final intestinal DH. By adding reducing sugars to proteins through Maillard reaction, Zhao

et al. (2019) observed significantly slower hydrolysis rates and digestibility at increasing levels of glycation, which may exert effects comparable to those of natural glycosylation. Therefore, glycans, especially larger structures containing NeuNAc, could hinder the access of proteases to cleavage sites on κ -CN.

As proven previously, 3 amino acids in variant A (Thr¹³⁶, Asp¹⁴⁸, and Gly¹⁵⁵) are different from 2 amino acids in variant B (Ile¹³⁶ and Ala¹⁴⁸) and 1 in variant E (Ser¹⁵⁵; Caroli et al., 2009). By contrast, phosphorylation normally happens at Ser and Thr, and glycosylation for κ -CN only happens at Thr. However, all these amino acids are not the hydrolyzing sites of pepsin, trypsin, nor chymotrypsin, which are the main proteases in human gastrointestinal digestion system (Rawlings et al., 2018), and accordingly the amino acid differences in the primary structures of the 3 genetic variants are not expected to be the primary reasons for the different digestibilities observed.

It is possible that the presence and organization of the κ -CN oligomers in milk or purified systems potentially affect the digestibility of κ -CN. Using purified κ -CN protein variants A, B, and E, Petrat-Melin et al. (2016) found the GD to affect the in vitro gastrointestinal digestion with lower DH at higher GD. In that study the κ -CN molecules were reduced during purification, as here, and then allowed to reassemble by removal of the reducing agent, but in that study the reformation into oligomers was not assessed. The potential implications of possible heterogeneities in κ -CN disulfide bonding patterns and implications on oligomer states, or even differences between monomer and oligomer digestibilities could be the focus of future studies. Furthermore, it would be interesting to study in more detail the released peptides via peptidomics to understand the dynamics of the system relative to cleavage sites and contributions of posttranslational modifications in more detail.

CONCLUSIONS

A modified method of purifying bovine κ -CN was developed and used for purification of κ -CN from AA, BB, and EE milks. After purification, κ -CN variants A and E showed similar GD, which was lower than that of variant B. In vitro digestion showed that variants A and E had faster rates of both gastric and intestinal digestion than variant B. After enzymatic treatment with sialidase, desialylation significantly promoted the in vitro gastric digestion rate of κ -CN B to levels comparable with A and E, as well as with desialylated A and E. In the intestinal phase, desialylation of variant B promoted a better digestion than that of native A or E. This showed that the digestibility of κ -CN varies

between genetic variants, even in nonmicellar purified systems, and that digestibility can be enhanced by removal of sialic acids on the attached glycans.

ACKNOWLEDGMENTS

The authors thank Gitte H. Kristiansen (Department of Food Science, Aarhus University, Aarhus, Denmark) for operating the LC-ESI/MS. We thank the Chinese Scholarship Council (Beijing) for enabling Bulei Sheng to carry out his PhD studies at Aarhus University, Denmark. The authors have not stated any conflicts of interest.

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