Genetic variants of bovine β - and κ -casein result in different immunoglobulin E-binding epitopes after in vitro gastrointestinal digestion

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ABSTRACT

Immunoglobulin E-mediated allergy to cow milk is a common allergy in industrialized countries, mainly affecting young children and infants. β -Casein (CN) and κ -CN belong to the major allergens in cow milk. Within these milk proteins, genetic polymorphisms occur, which are characterized by substitutions or deletions of AA, resulting in different variants for each protein. Until now, these variants have not been considered when discussing the allergenic potential of bovine milk. In this study, the focus was placed on the arising peptide pattern after in vitro gastrointestinal digestion of several β - and κ -CN variants to determine resistant fragments containing IgE-binding epitopes and to identify potential differences between these variants. β -Casein A¹, A², and B, as well as κ -CN A, B, and E, were separated and isolated from milk of cows homozygous for these variants and digested with an in vitro gastrointestinal digestion model. The resulting peptides were identified using mass spectrometry and compared with previously determined epitopes. Seven β -CN and 4 κ -CN peptides, common in all β - or κ -CN variants, remained of sufficient size to harbor IgE-binding epitopes. In addition, some peptides and, consequently, epitopes differ from each other due to the AA substitution occurring in the individual variants. The distinct peptides AA 108 to 129 of β -CN A¹ and A², AA 103 to 123 of β -CN B, as well as AA 59 to 72, AA 59 to 80, and AA 58 to 80 of all 3 β -CN variants correspond to the IgE-binding epitopes AA 107 to 120 and AA 55 to 70, respectively. In κ -CN, the 2 variant-specific peptides AA 136 to 149 (κ -CN A, E) and AA 134 to 150 (κ -CN B) are congruent with the IgE-binding epitope AA 137 to 148. The present study shows that genetic polymorphisms affected the arising peptide pattern of the caseins and thus modifications in the IgE-binding epitopes occurred. As a consequence, the casein variants could show differences in their aller-

genicity. Studies investigating the allergenic potential of these different peptides are currently in progress.

Key words: casein variants, allergenicity, in vitro gastrointestinal digestion, immunoglobulin E-binding epitope

INTRODUCTION

Milk from cows has been a valuable food source since domestication took place more than 8,000 yr ago, especially in lactose-tolerant human societies (Beja-Pereira et al., 2003). On the other hand, cow milk is one of the major elicitors of food allergy, particularly in children, affecting around 2 to 3% of this population (Skrupak et al., 2007). In adults, it is represented with a prevalence of 0.1% (Osterballe et al., 2005). Cow milk allergy (CMA) is mostly mediated by milk protein IgE, which provokes allergic reactions at the skin, respiratory tract, and gastrointestinal tract (Huang and Kim, 2012). The 2 caseins β - and κ -CN are important cow milk allergens (Docena et al., 1996; Gaudin et al., 2008) and account for 40 and 10% of total milk protein, respectively (Farrell et al., 2004). β -Casein comprises 209 AA residues. It is a phosphoprotein including a cluster of phosphoserine residues near the N terminus and it contains no cysteine. In contrast, κ -CN consists of 169 AA, and it is characterized by bound carbohydrates, the absence of phosphoserine clusters, as well as 2 cysteines, which are able to form disulfide bonds (Farrell et al., 2004). Both caseins share a high proportion of proline residues, which contributes to the fact that they exhibit an open flexible structure with significant amounts of secondary structure but with no rigid tertiary structure (Swaigood, 1993; Farrell et al., 2004). Therefore, they are very susceptible to hydrolysis by all proteases (Astwood et al., 1996; Swaigood, 2003). For β - and κ -CN, genetic polymorphisms are described as a consequence of either substitutions or deletions of AA within the polypeptide chain resulting in several protein variants (Erhardt, 1989, 1996; Caroli et al., 2009). Thus, a total of 12 β -CN variants (A¹, A², A³, B, C, D, E, F, G, H¹, H², and I) and 12 κ -CN variants (A, B, C, D, E, F¹, F², G¹, G², H, I, and J) were identified in the *Bos* genus,

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with important differences in occurrence and frequency of the variants among species and breeds (Farrell et al., 2004; Caroli et al., 2009).

Within β -CN, the variants A¹, A², and B are widely distributed in all breeds, with β -CN A² showing the highest frequency. β -Casein A³, C, and I are rather common in *Bos taurus*. The other β -CN variants are rare and described in particular breeds only (Caroli et al., 2009).

κ -Casein A is the most frequent variant in the bulk of breeds, followed by κ -CN B and H. κ -Casein C and E are less common in *Bos taurus*, κ -CN G² is specific for *Bos grunniens*, and the rest of the κ -CN variants occur infrequently in certain breeds only (Farrell et al., 2004; Caroli et al., 2009).

Genetic polymorphisms of milk proteins are used in phylogenetic studies (Ibeagha-Awemu et al., 2004; Jann et al., 2004) and animal breeding, as they have a significant effect on milk production traits and cheesemaking properties (Martin et al., 2002; Heck et al., 2009). In addition, milk protein variants play a notable role in human nutrition because they improve the functional quality of milk and dairy products regarding bioactive peptides, which are considered as ingredients of functional foods. Thus, peptides derived from genetic variants of several caseins exhibit differences in their antihypertensive, opioid, dipeptidyl peptidase-4 inhibitory, and osteoblast mineralization activities (Jinsmaa and Yoshikawa, 1999; Weimann et al., 2009; Tulipano et al., 2010, 2011). Genetic polymorphisms may also influence the allergenicity of milk proteins. However, information about the allergenic potential of bovine milk protein variants is scarce. Modifications in the immunoreactivity of different caprine caseins could already be demonstrated in goat milk samples, where the genotypes α_{s1} -CN 0₁0₁ and 0₁F exhibited lower immunoreactions compared with the goat milk samples characterized by α_{s1} -CN BB, BE, EE, and FF (Ballabio et al., 2011).

It is generally accepted that most food allergens sensitize individuals through the gastrointestinal tract (Mills et al., 2004; Moreno, 2007). Therefore, several processes such as digestion, absorption, and mucosal immune responses take place before milk proteins induce an allergic reaction. The majority of milk allergic patients showed a strong humoral and cellular response to β - and κ -CN, with high titers of IgE specific for these proteins (Shek et al., 2005). The allergenicity of these proteins is due to some small protein fragments containing IgE-binding epitopes, which are spread along the whole protein sequence, able to cross-link 2 IgE molecules, and thereby elicit an allergic reaction (Huang and Kim, 2012). Multiple IgE-binding epitopes in β - and κ -CN, ranging from 8 to 20 AA, have been

identified by SPOT-membrane-based or microarray immunoassays with sera from CMA patients (Chatchatee et al., 2001; Cerecedo et al., 2008; Lin et al., 2009). Even though the caseins are labile proteins, recent studies reported that peptides resulting from in vitro gastrointestinal digestion of β -CN and β -LG still comprise IgE-binding epitopes and, therefore, may retain allergenic potential (Dupont et al., 2010a,b; Picariello et al., 2010). However, no previous study considered the influence of the genetic polymorphisms on the digestion of casein variants in association with their IgE-binding epitopes.

Therefore, the aim of the present study was to detect resistant regions containing IgE-binding epitopes and possible differences after a simulated gastrointestinal digestion of several bovine β - and κ -CN variants by using mass spectrometry.

MATERIALS AND METHODS

Chemicals, Enzymes, and Other Reagents

Chemicals and reagents were purchased from standard suppliers: Servalyt pH 5.0 to 7.0, Coomassie Brilliant Blue R 250, diethylaminoethyl (DEAE) 52 Cellulose Servacel, and Protein Test Mixture 6 for SDS-PAGE were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany); Pharmalyte pH 2.5 to 5.0 and pH 4.2 to 4.9 carrier ampholytes were purchased from GE Healthcare Europe GmbH (Freiburg, Germany); sodium taurocholate ($\geq 95\%$ by thin-layer chromatography), sodium glycodeoxycholate ($\geq 97\%$ by thin-layer chromatography), pepsin from porcine gastric mucosa (activity: 3,200–4,500 U/mg of protein using hemoglobin as substrate), trypsin from porcine pancreas (activity: 13,800 U/mg of protein using benzoylarginine ethyl ester as substrate), α -chymotrypsin from bovine pancreas (activity: 40 U/mg of protein using benzoyltyrosine ethyl ether as substrate), and Bowman-Birk trypsin-chymotrypsin inhibitor from soybean were acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); and 2,5-dihydroxy benzoic acid and methylene-diphosphonic acid for the matrix solution were obtained from Fluka (Neu-Ulm, Germany). All chemicals were of analytical reagent grade.

Phenotyping of Milk Samples by Isoelectric Focusing

Individual milk samples were collected from different bovine breeds derived from several associations and private dairy farms in Germany. Genetic variants of β - and κ -CN were determined by isoelectric focusing (IEF) on 0.3-mm thin polyacrylamide gels using car-

rier ampholytes according to Erhardt (1989). The gels ($t = 5.01$; $C = 3.75$; 7.09 M urea) contained 0.644 mL of the following mixture of carrier ampholytes: 0.30% (wt/vol) Pharmalyte pH 2.5 to 5.0, 1.10% (wt/vol) Pharmalyte pH 4.2 to 4.9, and 1.19% (wt/vol) Servalyt pH 5.0 to 7.0. After fixation in 20% (wt/vol) TCA and staining with a solution of 0.1% (w/v) Coomassie Brilliant Blue R 250, 45% (vol/vol) ethanol, and 10% acetic acid (vol/vol), β - and κ -CN variants were identified manually by using cow milk samples with known variants as reference samples.

Preparation of Caseins from Milk

Milk samples from cows with defined casein phenotypes (β -CN A¹, A², and B, and κ -CN A, B, and E) were skimmed by centrifugation at $1,620 \times g$ for 10 min at 4°C . Preparation of whole casein was performed by acid precipitation. Briefly, skim milk samples were acidified with 50% (vol/vol) acetic acid to pH 4.6 and centrifuged at $1,620 \times g$ for 10 min. The casein precipitate was dissolved in distilled water with 1.0 M NaOH. Steps of precipitation and washing were repeated 3 times. Whole casein was finally lyophilized and stored at -20°C until analysis.

Purification of Caseins by Anion-Exchange Chromatography

β -Casein and κ -CN variants were separated from the whole casein fraction by anion-exchange chromatography according to a modified method of Andrews and Alichanidis (1983). The DEAE 52-cellulose resin was packed into a column ($46.5 \times 2.5\text{ cm}$) to reach a column volume of 50 mL . Equilibration (EQ) took place with 0.01 M Tris/imidazole (pH 7.0), 3.3 M urea, 0.01 M 2- β -mercaptoethanol, and 3.1 mM sodium azide (EQ buffer). An amount of 1.0 to 1.5 mg of lyophilized whole casein was dissolved in 30 to 40 mL of EQ buffer and applied onto the DEAE cellulose column. Buffers with different salt concentrations were prepared by mixing 1.0 M NaCl and EQ buffer. Caseins were eluted stepwise with 0.075 , 0.13 , and 0.17 M NaCl buffer at a flow rate of 0.6 mL/min using a peristaltic pump (Ismatec Ip-4; Ismatec SA, Zürich, Switzerland). The absorbance of the column effluents were monitored at 280 nm (Uvicord SII detector; LKB, Bromma, Sweden) and fractions of 7 mL were collected. The main peak fractions of the resulting chromatogram, corresponding to the different purified caseins, were determined by IEF in the way described above, and then separately pooled and dialyzed 3 times against 5 L of distilled water for 24 h . After lyophilization, the purity and identity of pooled casein fractions were assessed by SDS-PAGE

and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Samples were frozen (-20°C) for storage until used.

SDS-PAGE

Sodium dodecyl sulfate PAGE was performed according to Laemmli (1970) with 1.5-mm thin polyacrylamide gels ($16 \times 14\text{ cm}$) consisting of a 2.5% stacking gel and a 12% separating gel using the Dual Gel Electrophoresis System, Emperor Penguin (P9DS OWL; Thermo Fisher Scientific, Rochester, NY). Fifteen microliters of the sample preparations containing 11.5% (vol/vol) casein fraction, 0.069 M Tris-HCl (pH 6.8), 2.2% SDS, 11.1% glycerol, 5.5% 2- β -mercaptoethanol, and traces of bromophenol blue were loaded onto each lane of the gel. The electrophoretic run was done in running buffer [25 mM Tris, 192 mM glycine, and 0.1% (wt/vol) SDS, pH 8.3] at 400 V . After fixation with 50% TCA, gels were stained with 0.1% (wt/vol) Coomassie Brilliant Blue R 250. A molecular weight marker (Protein Test Mixture 6) was run with each gel.

In Vitro Gastrointestinal Digestion of Casein Variants

In vitro gastrointestinal digestion of β - and κ -CN variants (β -CN A¹, A², and B, and κ -CN A, B, and E) was performed in duplicate as described by Moreno et al. (2005). Each digestion was repeated twice and control incubations in the absence of proteases were done.

In Vitro Gastric Digestion (Phase 1)

β -Casein and κ -CN variants (10 – 15 mg/mL) were dissolved in simulated gastric fluid (SGF; 0.15 M NaCl, pH 2.5), adjusted to pH 2.5 with 1.0 M HCl, and incubated at 37°C for 15 min . Then, a solution of 0.32% (wt/vol) porcine pepsin in SGF was added at an approximately physiological ratio of enzyme:casein ($1:20$, wt/wt), giving 165 U of pepsin/mg of protein. After incubation of the samples at 37°C for 60 min , the pH was raised to 7.5 to irreversibly inactivate pepsin by using 1.0 M NaOH and aliquots ($40\text{ }\mu\text{L}$) in triplicate were taken for MALDI-TOF-MS analysis.

In Vitro Duodenal Digestion (Phase 2)

Phase 2 digestion took place with the gastric digests of phase 1 as starting material. Before duodenal proteolysis, the pH of the digests was adjusted to 6.5 with 0.1 M NaOH. To simulate a duodenal environment, samples were mixed with a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate and sodium glycodeoxycholic acid, 1.0 M CaCl_2 , and

Figure 1. Separation of bovine milk samples with different β - and κ -CN genotypes by isoelectric focusing. The positions of the major bands of the β -CN variants are indicated by an x and the main fractions of the κ -CN variants are marked by a dot. Color version available in the online PDF.

0.25 M Bis-Tris (pH 6.5). Finally, solutions of 0.1% (wt/vol) trypsin and 0.4% (wt/vol) α -chymotrypsin in water were added at approximately physiological ratios of casein:trypsin: α -chymotrypsin = 1:400:100 (wt/wt/wt), respectively (1 mg:34.5 U:0.40 U). Duodenal digestion was performed for 60 min at 37°C and stopped by addition of a 2-fold excess of soybean Bowman-Birk trypsin-chymotrypsin inhibitor, calculated to inhibit trypsin and chymotrypsin in the digestion mix. At the end, aliquots (40 μ L) in triplicate were removed for MALDI-TOF-MS analysis.

MALDI-TOF-MS

Samples collected at the end of gastric (phase 1) and duodenal (phase 2) digestion, as well as isolated casein fractions from anion-exchange chromatography, were analyzed by MALDI-TOF-MS to identify the peptides remaining after digestion. Prior to analysis, casein digests were desalted using ZipTip C₁₈ columns (Millipore GmbH, Eschborn, Germany). After preparing the matrix solution by dissolving 2,5-dihydroxy benzoic acid and methylene-diphosphonic acid at concentrations of 5 mg/mL in water, respectively, the samples were crystallized by the dried droplet method. The MALDI-TOF-MS analyses were carried out using an Ultraflex TOF/TOF I mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a UV nitrogen laser (wavelength = 337 nm, 3-ns pulse width) to desorb/ionize the matrix/analyte material. Spectra were acquired in positive ion reflectron mode over the m/z range 400 to 4,000. Approximately 250 spectra were accumulated.

External calibration was performed with a peptide standard mixture (Bruker Daltonics).

Data Analysis

The MALDI-TOF-MS data analysis took place with Compass 1.2 software (Bruker Daltonics). Based on the known sequences of the different caseins, the observed ions were assigned to the corresponding AA sequences by using the protein database Swiss-Prot and TrEMBL, as well as the tools FindPept and Peptide Mass (<http://www.expasy.org>). For identification of the peptides, the following search parameters were chosen: (a) peptide masses were indicated as monoisotopic and $[M + H]^+$; (b) cysteines were treated with nothing and methionines oxidized; (c) the mass tolerance was set to 50 ppm with a maximum of 75 ppm; and (d) enzymes pepsin, trypsin, and chymotrypsin were selected.

RESULTS

Separation and Purification of β - and κ -CN Variants

Figure 1 shows the separation pattern of different β - and κ -CN genotypes of bovine milk samples determined by IEF. The known genetic variants β -CN A¹, A², and B, as well as κ -CN A, B, and E could be demonstrated in homo- and heterozygous forms.

After steps of precipitation and anion-exchange chromatography, the purity of β - and κ -CN variants was assessed by IEF (data not shown), SDS-PAGE, and MALDI-TOF-MS. From these analyses, it was evident that some β - and κ -CN variants contained slight contaminations with other caseins. Nevertheless, in both casein fractions pure samples were obtained. As summarized in Figure 2, the β -CN variants (lanes 1 to 5) occurred as one band at a relative molecular mass of around 28,000 Da, with exception of the samples β -CN A¹ and A² (lanes 1 and 4), where a small second band was visible on the SDS-PAGE gel. This indicated contamination with κ -CN. Compared with the other β -CN variants, β -CN B (lane 5) had slightly faster electrophoretic mobility. The κ -CN variants (lanes 6 to 8) were located below β -CN with one main band at a relative molecular mass of around 26,000 Da. A further very light band was identifiable above κ -CN E (lane 6), supposing the presence of some contamination with β - or α_{s2} -CN.

To confirm these findings, the casein variants were additionally analyzed by MALDI-TOF-MS. For this purpose, the samples were digested with trypsin and their purity was determined on the basis of the intensity coverage (%) of the matched tryptic peptides. Three of 5 β -CN samples showed some contamination

Figure 2. Sodium dodecyl sulfate PAGE analysis of different β - and κ -CN fractions obtained from anion-exchange chromatography. M = molecular weight marker. Lanes 1 and 2 = β -CN A¹; lanes 3 and 4 = β -CN A²; lane 5 = β -CN B; lane 6 = κ -CN E; lane 7 = κ -CN A; lane 8 = κ -CN B. Color version available in the online PDF.

with κ - or α_{s2} -CN. As already assumed by SDS-PAGE, impurities with κ -CN were measurable in β -CN A¹ and β -CN A² (lanes 1 and 4, Figure 2). The intensity coverage of the matched κ -CN peptides in the samples were 15.1% for β -CN A¹ and 6.3% for β -CN A². In contrast, β -CN A¹ (lane 2, Figure 2) revealed small impurities of α_{s2} -CN with a signal intensity of 5.9%, which was not visible with SDS-PAGE. The absence of contamination with other caseins could only be demonstrated in the samples β -CN A² and β -CN B (lanes 3 and 5, Figure 2).

For κ -CN A and B (lanes 7 and 8, Figure 2), no impurities with other caseins were found. The existence of a contamination with α_{s2} -CN casein could be detected in κ -CN E (lane 6, Figure 2), which was already apparent in the SDS-PAGE gel. A signal intensity of 32% was measured for matched α_{s2} -CN peptides.

In Vitro Gastrointestinal Digestion of β - and κ -CN Variants

The MALDI-TOF-MS analysis allowed the characterization of the comprising peptide pattern after gastric and duodenal digestion of the β - and κ -CN variants. In addition to the peptides detected together, variant specific peptides occurred in β -CN A¹, A², and

B, as well as κ -CN A, B, and E. However, a total of 44 peptides (β -CN: 35 peptides; κ -CN: 9 peptides) could not be unambiguously determined in both phases of the digestion, as for some masses, more than 1 peptide was assigned during the FindPept database search. Thus, these peptides were not included in further evaluations.

Gastric Digestion of β -CN (Phase 1)

A total of 42 common peptides could be demonstrated for β -CN A¹, A², and B, with molecular masses ranging from 551 to 2,893 Da. Thirty peptides were exactly assignable (Figure 3a). Compared with the residual protein sequence, no peptides were identified in region AA 21 to 42. As shown in Table 1, peptide AA 113 to 121 was present in all 3 variants, as glutamic acid (E) is a preferential cleavage site for pepsin. Thus, the AA substitution at position 122, causing a replacement of a serine (S) in β -CN A¹ and A² by an arginine (R) in β -CN B, has no negative effect on the cleavage of the enzyme. A further substitution distinguishes variant A¹ and B (histidine) from A² (proline) at position 67. Therefore, peptide AA 59 to 80 was released from all 3 variants, with a mass shift of about 40 Da resulting from the varying molecular weights of histidine (155.16 Da) and proline (115.13 Da). Nevertheless, peptide AA 118 to 127 was solely observed in digests of β -CN B.

Duodenal Digestion of β -CN (Phase 2)

Duodenal digestion of β -CN A¹, A², and B gave rise to a significant number of 93 identical peptides with molecular masses from 414 to 3,039 Da, covering almost the whole protein sequence. Among these 93 peptides, 70 were unambiguously identified (Figure 3b). It is interesting to note that fragments with up to 3,039 Da could not be found after gastric digestion. According to this, it is likely that peptides larger than 3,500 Da appeared in the gastric phase, which were not detectable with MALDI-TOF-MS in this study. Duodenal digestion led to further degradation of the proteins and, hence, to the formation of a larger number of smaller fragments. Similar to the gastric phase, only a few enzyme cleavages occurred in area AA 21 to 42. Furthermore, 17 peptides resulting from gastric digestion were identified at the end of this phase and avoided the progressive hydrolysis by trypsin and chymotrypsin. In total, 13 peptides comprising the variant specific AA substitutions at positions 67 and 122 could be demonstrated in the digests of the β -CN variants (Table 1). Peptides AA 59 to 68, AA 59 to 72, AA 59 to 80, and AA 58 to 80 existed in all 3 variants, with a mass shift of about 40 Da between β -CN A¹, B, and A² and with peptide AA 59 to 80 originating from gastric digestion. Peptide

a

b

Figure 3. Identification of peptides derived from gastric (a) and duodenal (b) digestion of β -CN A¹, A², and B by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; continuous lines). Peptides with AA substitutions occurring in all β -CN variants are represented as small dashed lines (-----), peptides only present in β -CN B are indicated with dotted lines (.....), and peptides detected solely in β -CN A¹ and A² are shown with large dashed lines (---). The positions of the amino acid substitutions and IgE-binding epitopes (Figure 3b) are highlighted in bold.

Table 1. Identification of peptides with AA substitutions (bold letters) after gastric and duodenal digestion of β -CN A¹, A², and B by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Phase	Experimental mass [M + H] ¹	Theoretical mass [M + H] ¹	Position	Sequence	β -CN variant
Gastric digestion (phase 1)	1,109.55	1,109.55	113–121	¹¹³ KYPVEPFTE ¹²¹ /(S)	A ¹ , A ²
	1,109.56	1,109.55	113–121	¹¹³ KYPVEPFTE ¹²¹ /(R)	B
	1,191.63	1,191.64	118–127	¹¹⁸ PFTERQSLT ¹²⁷	B
	2,390.19	2,390.28	59–80	⁵⁹ VYFPFGPI P NSLPQNIPPLTQT ⁸⁰	A ²
	2,430.18	2,430.28	59–80	⁵⁹ VYFPFGPI H NSLPQNIPPLTQT ⁸⁰	A ¹ , B
Duodenal digestion (phase 2)	1,100.57	1,100.57	59–68 ²	⁵⁹ VYFPFGPI P N ⁶⁸	A ²
	1,140.57	1,140.58	59–68	⁵⁹ VYFPFGPI H N ⁶⁸	A ¹ , B
	1,283.58	1,283.58	114–124 ²	¹¹⁴ YPVEPFTE S QSL ¹²⁴	A ¹ , A ²
	1,525.77	1,525.81	59–72 ²	⁵⁹ VYFPFGPI P NSLPQ ⁷²	A ²
	1,565.78	1,565.81	59–72	⁵⁹ VYFPFGPI H NSLPQ ⁷²	A ¹ , B
	1,865.93	1,865.93	110–125 ²	¹¹⁰ PFPKYPVEPFTE S QSL ¹²⁵	A ¹ , A ²
	1,865.93	1,865.93	109–123	¹⁰⁹ MPFPKYPVEPFTE R Q ¹²³	B
	2,390.28	2,390.28	59–80	⁵⁹ VYFPFGPI P NSLPQNIPPLTQT ⁸⁰	A ²
	2,430.28	2,430.28	59–80	⁵⁹ VYFPFGPI H NSLPQNIPPLTQT ⁸⁰	A ¹ , B
	2,503.36	2,503.36	58–80 ²	⁵⁸ LVYFPFGPI P NSLPQNIPPLTQT ⁸⁰	A ²
	2,543.36	2,543.37	58–80	⁵⁸ LVYFPFGPI H NSLPQNIPPLTQT ⁸⁰	A ¹ , B
	2,556.18	2,556.22	108–129	¹⁰⁸ EMFPKYPVEPFTE S QSLTLD ¹²⁹	A ¹ , A ²
	2,556.15	2,556.31	103–123	¹⁰³ APKHKEMFPKYPVEPFTE R Q ¹²³	B

¹Monoisotopic mass values.²Peptides also identified by Dupont et al. (2010a,b).

AA 114 to 124 was solely found in the digests of β -CN A¹ and A². A corresponding peptide (AA 118 to 127) was already present in β -CN B after gastric digestion (Table 1). Probably, this peptide was further hydrolyzed to smaller fragments than AA 114 to 124 and was, therefore, not detectable in variant B during duodenal digestion. The peptides AA 109 to 123 in β -CN B, AA 110 to 125 in β -CN A¹ and A², as well as AA 103 to 123 in β -CN B and AA 108 to 129 in β -CN A¹ and A², occurred with identical masses but due to the mutation with modifications in their sequences.

Gastric Digestion of κ -CN (Phase 1)

With the gastric digestion of κ -CN A, B and E 66 congruent peptides with molecular masses ranging from 400 to 3,001 Da could be determined. As reported in Figure 4a, 64 of the 66 peptides were clearly assignable. Peptides were observed in almost all regions of the protein, especially in area AA 40 to 66. However, the regions AA 1 to 17 and AA 130 to 169 gave a low number of peptides. The AA substitutions at positions 136, 148, and 155 resulted in the formation of 8 different peptides in the individual variants (Table 2). Peptides AA 130 to 137 and AA 125 to 137 originated from the digests of all κ -CN variants, with a mass shift of about 12 Da, which relies on the replacement of a threonine (T) by an isoleucine (I) at position 136 in variant B. This casein variant is characterized by a further substitution at position 148, where an aspartic acid (D) is located instead of an alanine (A) as compared with variant A and E. Thus, peptides AA 140 to 162 and

AA 127 to 151 were only found in κ -CN B. These larger fragments contained some missed theoretical cleavage sites of pepsin, maybe caused by steric hindrance, limiting the access of pepsin to these areas. Additionally, a more degraded peptide (AA 135 to 145) was detected in κ -CN B but a similar peptide (AA 136 to 149) occurred also in the samples of κ -CN A and E. κ -Casein E differs from κ -CN A and B by a glycine (G) instead of a serine (S) at position 155. Obviously, the mutation has no influence on the arising peptide pattern, as no peptides comprising the substitution appeared. However, glycine and serine seemed to be an unspecific substrate for pepsin because peptide AA 156 to 161 could be identified in all variants (Figure 3a). Presumably, this is due to the decreasing substrate specificity of pepsin at pH >2.

Duodenal Digestion of κ -CN (Phase 2)

The MALDI-TOF-MS analysis of the duodenal digests from κ -CN A, B, and E showed the presence of 69 common peptides with molecular masses between 402 and 3,123 Da, covering most of the whole protein sequence. A precise identification was made for 62 of the 69 peptides (Figure 3b). Although almost the same number of peptides existed after gastric and duodenal digestion, more fragments in the range from 402 to 1,000 Da were observed at the end of this phase, which indicated further degradation of the individual casein variants. In contrast, 29 peptides originating from gastric digestion survived the duodenal digestion unaltered and did not undergo progressed hydrolysis by the pancreatic enzymes. Comparable with gastric digestion,

Figure 4. Identification of peptides derived from gastric (a) and duodenal (b) digestion of κ -casein (CN) A, B and E by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; continuous lines). Peptides with AA substitutions occurring in all κ -CN variants are represented as small dashed lines (-----), peptides only present in κ -CN B are indicated with dotted lines (.....), and peptides detected solely in κ -CN A and E are shown with large dashed lines (---). The positions of the amino acid substitutions and IgE-binding epitopes (Figure 4b) are highlighted in bold.

Table 2. Identification of peptides with AA substitutions (bold letters) after gastric and duodenal digestion of κ-CN A, B, and E by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Phase	Experimental mass [M + H] ¹	Theoretical mass [M + H] ¹	Position	Sequence	κ-CN variant
Gastric digestion (phase 1)	833.38	833.39	130–137	¹³⁰ PTSTPT T E ¹³⁷	A, E
	845.44	845.43	130–137	¹³⁰ PTSTPT I E ¹³⁷	B
	1,120.52	1,120.57	135–145	¹³⁵ T IEAVESTVAT ¹⁴⁵	B
	1,290.60	1,290.61	125–137	¹²⁵ IASGEPTSTPT T E ¹³⁷	A, E
	1,302.64	1,302.64	125–137	¹²⁵ IASGEPTSTPT I E ¹³⁷	B
	1,451.74	1,451.68	136–149	¹³⁶ T EAVESTVATLE D S ¹⁴⁹	A, E
	2,412.21	2,412.20	140–162	¹⁴⁰ ESTVATLEASPEVIESPPEINTV ¹⁶²	B
	2,503.23	2,503.19	127–151	¹²⁷ SGEPTSTPT I EAVESTVATLEASPE ¹⁵¹	B
Duodenal digestion (phase 2)	1,290.60	1,290.61	125–137	¹²⁵ IASGEPTSTPT T E ¹³⁷	A, E
	1,302.64	1,302.64	125–137	¹²⁵ IASGEPTSTPT I E ¹³⁷	B
	1,451.74	1,451.68	136–149	¹³⁶ T EAVESTVATLE D S ¹⁴⁹	A, E
	1,714.81	1,714.87	134–150	¹³⁴ PTIEAVESTVATLEASP ¹⁵⁰	B

¹Monoisotopic mass values.

the C-terminal region AA 130 to 169 only generated a few peptides and was characterized by variant-specific enzyme cleavages, as the positions of the AA substitutions were located there (Table 2). Peptides AA 125 to 137 and AA 136 to 149 already arose after gastric digestion and, according to that, they were not further broken down, probably due to the missing typical trypsin and chymotrypsin cleavage sites. However, in the digests of κ-CN B, peptide AA 134 to 150, corresponding to peptide AA 136 to 149 of κ-CN A and E, was found and presumably displays a degradation product of the gastric peptide AA 127 to 151 (Table 2).

DISCUSSION

The aim of the present study was to investigate the occurrence and difference of fragments containing IgE-binding epitopes after in vitro gastrointestinal digestion of several β- and κ-CN variants. As reported for the caseins (Astwood et al., 1996; Fu et al., 2002; Dupont et al., 2010a), all β- and κ-CN variants were rapidly broken down by pepsin during gastric digestion and no intact protein could be detected at the end of this phase. The addition of the pancreatic enzymes trypsin and chymotrypsin resulted in further degradation of the casein variants and gave a similar but more complex peptide mixture than in phase 1. This is consistent with the fact that β- and κ-CN share an open and flexible structure with no defined tertiary structure, which makes them very susceptible to all proteases, such as pepsin, trypsin, and chymotrypsin (Swaigood, 1993, 2003).

During duodenal digestion, β-CN A¹, A², and B were digested to 70 peptide fragments, and κ-CN A, B, and E were digested to 69 peptide fragments of sizes <3,200 Da, with the longest peptides composed of 28 AA. Using a modified model of gastrointestinal digestion, Dupont et al. (2010a) also identified 70 peptides after

duodenal digestion of β-CN A², but in the range from 561 to 1,730 Da. Almost half of these peptides were consistent with the peptides found in the present study. Variations in the peptide pattern of both studies can be explained by using a modified form of digestion and a different MS technique in the study of Dupont et al. (2010a). In another study about digested raw, pasteurized, and sterilized milk, as well as yogurt, only a few peptides were detected for κ-CN, especially in area AA 106 to 169 compared with the 69 peptides observed in the present study (Dupont et al., 2010b). However, this could be due to the fact that Dupont et al. (2010b) used whole milk as well as heated and processed dairy products instead of isolated κ-CN fractions applied here. The authors suspected that κ-CN from whole or heated milk probably generates heat-induced aggregates with whey proteins through the formation of disulfide bridges, which was already described by others (Lowe et al., 2004; Patel et al., 2006). This aggregation may be the reason for protection of κ-CN from digestion, which was not given in the present study.

β-Casein contains serine-linked phosphate groups at positions 15, 17, 18, and 19, which are clustered and flanked by glutamate residues, whereas κ-CN possesses only 1 site of phosphorylation at position 149 but, on the other hand, some sites of glycosylation (121, 131, 133, 135, 136, 142, and 165; Farrell et al., 2004). It has been shown that peptides carrying phosphorylated serine residues can resist in vivo and in vitro gastrointestinal digestion (Meisel et al., 2003; Miquel et al., 2005). Conceivably, this may also apply to the glycosylated threonine residues as posttranslational modifications, explaining the low number of peptides found in area AA 125 to 169, which is in accordance to other studies, where peptide AA 106 to 169 particularly resisted digestion (Ledoux et al., 1999; Dupont et al., 2010b). In the present study, the phosphoserine cluster of β-CN survived within peptide AA 15 to 19, although

without the flanking glutamate residues. However, this peptide resulted from unspecific cleavage of chymotrypsin, which may elucidate why previous studies found the phosphoserine cluster of β -CN in complete form after *in vitro* digestion (Dupont et al., 2010a,b; Picariello et al., 2010). This is of special interest, as dephosphorylation of β -CN led to a decreased recognition by IgE antibodies of human sera. Therefore, it has been assumed that the major phosphorylation sites act as important allergenic epitopes in the caseins (Bernard et al., 2000). Nevertheless, further investigations are needed as epitope mapping studies were performed with synthetic peptides lacking a phosphorylated form.

Digested protein fragments must be of sufficient size, being able to sensitize, elicit an allergic reaction, and react with IgE antibodies, which requires the presence of intact IgE-binding epitopes. It is generally accepted that peptides have to be approximately 3,500 Da to possess allergenic potential (Huby et al., 2000; FAO/WHO, 2001). However, a study of sensitizing capacity of digested *Ara h 1* has shown peptide fragments smaller than 2,000 Da inducing a significant antibody response (Bøgh et al., 2009). This can also be expected for the caseins because, despite their excellent digestibility, they act as potent allergens (Docena et al., 1996; Gaudin et al., 2008). Given the fact that fragments up to 28 AA in length survived the gastrointestinal digestion of β - and κ -CN variants, a comparison of these peptides with the previously identified IgE-binding epitopes of β - and κ -CN was performed (Chatchatee et al., 2001; Cerecedo et al., 2008; Lin et al., 2009). The 7 β -CN peptides AA 44 to 60, AA 46 to 58, AA 69 to 93, AA 78 to 93, AA 106 to 119, AA 144 to 163, and AA 164 to 185 exhibited the important IgE-binding epitopes AA 45 to 54, AA 83 to 92, AA 107 to 120, AA 149 to 164, and AA 167 to 184 (Figure 3b; Chatchatee et al., 2001). These findings are partially consistent with the results of Dupont et al. (2010a,b), which demonstrated solely fragments AA 81 to 93 and AA 76 to 93, to be long enough to contain the epitope AA 83 to 92 after gastrointestinal digestion of β -CN. For κ -CN, the IgE-binding epitopes AA 21 to 44, AA 67 to 78, AA 95 to 116, and AA 111 to 126 (Figure 4b) survived gastrointestinal digestion within the 4 peptides AA 18 to 42, AA 65 to 81, AA 95 to 120, and AA 106 to 124 (Chatchatee et al., 2001). In contrast, the κ -CN peptides identified by Dupont et al. (2010b) were too short to harbor entire IgE-binding epitopes. Such results were also obtained for other food allergens such as the easily digestible peanut allergen *Ara h 1*, some of whose epitopes were able to resist the digestion process (Eiwegger et al., 2006; Bøgh et al., 2009, 2012). Furthermore, it has been shown that this allergen retains its allergenic potential when digested to smaller

fragments. The authors attributed this to the peptides forming defined aggregates or to co-immunization with mixtures of the peptides (Bøgh et al., 2012). This could be equally probable for casein peptides and would imply an allergenic activity of shorter peptides containing no intact IgE-binding epitopes anymore. Among others, this may explain the high sensitization rate to the very well and rapidly digestible caseins.

Although several studies have been done on the *in vitro* digestion of caseins, none has taken into account the genetic polymorphism of milk proteins until now. It had been anticipated that the genetic polymorphism, characterized by substitutions and deletions of AA, affect the IgE-binding epitope structure of the caseins and thus milk from cows with distinct genotypes might show different allergenic properties (Chessa et al., 2008; Caroli et al., 2009; Ballabio et al., 2011).

The present study demonstrated an influence of the genetic polymorphism on the arising peptide pattern and the allergenic epitopes of β - and κ -CN variants. For β -CN A¹, A², and B, MALDI-TOF-MS analysis enabled the detection of 13 different peptides, which contained the variant specific AA substitution. Five of the 7 peptides identified for β -CN A² were also found in the studies of Dupont et al. (2010a,b; Table 1). Gastrointestinal digestion of κ -CN A, B, and E showed the presence of 4 distinct peptides comprising the characterizing AA substitution. On the other hand, Dupont et al. (2010b) detected only the κ -CN peptides AA 138 to 146 and AA 139 to 146 in yogurt and sterilized and pasteurized milk but not in whole raw milk, which is presumably attributable to the variations in the conditions of both studies described above.

In addition to these observations, peptides AA 108 to 129 of β -CN A¹ and A², AA 103 to 123 of β -CN B, as well as AA 59 to 72, AA 59 to 80, and AA 58 to 80 of all 3 variants correspond to the IgE-binding epitopes AA 107 to 120 and AA 55 to 70 (Chatchatee et al., 2001). In κ -CN, the variant-specific peptides AA 136 to 149 (κ -CN A and E) and AA 134 to 150 (κ -CN B) are congruent with the IgE-binding epitope AA 137 to 148 (Chatchatee et al., 2001). It is quite possible that these mutations have an effect on the immunoreactivity of the epitopes, as reported by previous studies (Cocco et al., 2003, 2007; Han et al., 2008). It has been shown that single or double AA substitutions within peptides representing the core epitopes of β - and κ -CN resulted in an elimination or reduction of IgE-binding by pooled sera from CMA patients. For epitope AA 55 to 70 of β -CN, IgE binding was eliminated by the substitution of 1 AA, whereas exchanges at 2 positions were necessary in epitope AA 107 to 120 of β -CN (Cocco et al., 2007). This was also demonstrated for epitope AA 137 to 148 of κ -CN, which showed a loss of IgE binding

after introduction of single-peptide mutations (Han et al., 2008). However, in all studies, only the small, neutral and uncharged AA alanine was used for the substitutions at the single positions in the distinct epitopes of β - and κ -CN. Nevertheless, the genetic variants are characterized by substitutions of different AA, which could give other immunoreactions than those observed by Cocco et al. (2003, 2007) and Han et al. (2008). Moreover, most AA, which induced a loss of IgE binding, were hydrophobic and located in the center of the epitope (Cocco et al., 2003, 2007; Han et al., 2008). According to this, the substitution of the hydrophobic proline by the hydrophilic histidine in peptides AA 59 to 72, AA 59 to 80, and AA 58 to 80 of β -CN A¹ and B might give reduced immunoreactivity compared with β -CN A². The exchange of a hydrophilic AA at position 122 in peptides AA 108 to 129 of β -CN A¹ and A² as well as AA 103 to 123 of β -CN B occurs out of the core epitope AA 107 to 120. Due to the larger fragments and consequently differences in the sequences, modifications in the IgE binding to both peptides can be expected. In contrast to peptide AA 136 to 149 of κ -CN A and E, a reduction in IgE binding might be conceivable for peptide AA 134 to 150 of κ -CN B, where the hydrophilic threonine and aspartic acid at positions 136 and 148 of variant A and E, respectively, are replaced by the hydrophobic isoleucine and the neutral alanine. Thus, investigations are needed to gain knowledge about the effect of the mutations on the allergenic potential of these epitopes and, as a consequence, of the whole protein. Moreover, a large reservoir of genetic variants exists within β - and κ -CN, as further AA substitutions than included in this study occur in both proteins. In this context, it would be of great importance to consider cattle breeds threatened by extinction because they can possess rare variants, such as κ -CN G, which are not represented in the main breeds. This variant was first discovered in the endangered breed Pinzgauer and could not be found in another population until now (Erhardt, 1996). Such rare genetic variations are of significant physiological relevance, as they may differ in their allergenic properties from the main variants. However, several unknown factors exist in the protein chemistry as well as the immune response and regulation. Taken together with the high number of epitopes occurring in the caseins, hypoallergenic activity of genetic variants cannot be assumed in general.

CONCLUSIONS

It has been demonstrated that in vitro gastrointestinal digestion of several β - and κ -CN variants gave rise to a considerable number of peptides, some of which containing intact IgE-binding epitopes. As a

consequence of the genetic polymorphism, differences between specific peptides and hence epitopes of the single variants could be identified, which may influence the allergenicity of these proteins. Thus, the next step will be to investigate the allergenic potential of these peptides in a future study by using protein microarray technology. The results of the present study showed that the genetic polymorphism needs to be taken into account when evaluating the allergenic potential of milk proteins.

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