



In vitro gastrointestinal digestion of bovine α_{S1} - and α_{S2} -casein variants gives rise to different IgE-binding epitopes



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ABSTRACT

The occurrence and differences of resistant regions containing IgE-binding epitopes of α_{S1} -casein (α_{S1} -CN) variants B and C, as well as α_{S2} -CN A and B, after in vitro gastrointestinal digestion was investigated using mass spectrometry. The amino acid substitutions characterising the genetic variants affected the peptide pattern arising from the caseins and thus modifications in their allergenic epitopes occurred. Peptides f174–193 in α_{S1} -CN B and f179–198 in α_{S1} -CN C correspond to the IgE-binding epitope f173–194, which has been reported as one of the major epitopes in α_{S1} -CN B. Within α_{S2} -CN, the two variant-specific peptides, f7–29 from variant A and f1–22 from variant B, contain the previously identified IgE-binding epitope f1–20. These peptides, and in consequence the protein variants, may exhibit different immunoreactions, which could be significant in the production of milk with improved nutritional properties, such as hypoallergenic quality, by selection and breeding of cows with particular milk protein genotypes.

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1. Introduction

Milk is one of the most commonly consumed foods worldwide primarily because of its high nutritional value and health benefits (Hilpert et al., 2009; Pereira et al., 2002). Nevertheless, cows' milk allergy (CMA) is one of the most common causes of food allergy in early childhood, affecting around 2–3% of infants (Saarinen, Pelkonen, Mäkelä, & Savilahti, 2005). In adults, a prevalence of less than 0.5% is reported (Osterballe, Mortz, Hansen, Andersen, & Bindeslev-Jensen, 2009). Usually, the majority of children outgrow their allergy by the age of 3 years, but recent studies have found an increasing number of patients remaining allergic to cows' milk for a longer period (Saarinen et al., 2005; Skripak, Matsui, Mudd, & Wood, 2007). Symptoms of CMA range from mild to severe reactions and involve an inflammation of the skin and respiratory tract, as well as the gastrointestinal tract. CMA is defined as an adverse immunological reaction to one or more milk proteins, which is mainly mediated by milk-protein-specific immunoglobulin E (IgE) antibodies.

Whole casein (CN), which comprises the 4 proteins α_{S1} -, α_{S2} -, β - and κ -casein, belongs to the major allergenic protein in bovine milk (Bernard, Créminon, Yvon, & Wal, 1998; Docena, Fernandez, Chirido,

& Fossati, 1996). Of these, α_{S1} -CN, the most abundant protein, and α_{S2} -CN constitute 40% and 10% of the whole casein, respectively. Both are phosphoproteins with a varying degree of phosphorylation and a high content of proline residues distributed throughout the molecule (Farrell et al., 2004). Due to this high proportion of proline residues, the two proteins exhibit no defined tertiary structure but an open flexible structure with significant amounts of secondary structure (α -helices or β -sheets) (Farrell et al., 2004; Swaisgood, 1993). Therefore, they are very susceptible to proteases and peptidases and undergo rapid breakdown during digestion (Astwood, Leach, & Fuchs, 1996; Swaisgood, 2003).

Within these milk proteins, a wide range of genetic variants have been described resulting from substitutions and deletions of amino acids and leading to different genotypes in milk (Caroli, Chessa, & Erhardt, 2009; Erhardt, 1993a, 1993b). A total of 9 α_{S1} -CN variants (A, B, C, D, E, F, G, H, I) and 4 α_{S2} -CN variants (A, B, C, D) could be demonstrated for *Bos* genus with important differences in occurrence and frequency of the single variants between species and breeds (Caroli et al., 2009).

Milk protein variants are important in animal breeding due to their influence on milk performance traits (Heck et al., 2009; Martin, Szymanowska, Zwierzchowski, & Leroux, 2002). They have also potential significance for human nutrition, which has not yet been adequately exploited. Effects on the functional quality of milk and of dairy products concerning bioactive peptides, which are considered as ingredients for so-called functional foods, have

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been described; it has been demonstrated that biologically active peptides resulting from various casein variants show differences in their antihypertensive, opioid, dipeptidyl peptidase-4 inhibitory and osteoblast mineralisation activities (Jinsmaa & Yoshikawa, 1999; Tulipano et al., 2010; Tulipano, Sibilia, Caroli, & Cocchi, 2011; Weimann, Meisel, & Erhardt, 2009).

Until now, the knowledge about the influence of the genetic polymorphism on the allergenicity is low. However, it can be assumed that the milk protein variants differ in their allergenic properties, which has already been shown for caprine milk (Ballabio et al., 2011). It is believed that one prerequisite for a food allergen is its resistance to digestion during the passage through the different stages of the digestive tract (Bredehorst & David, 2001; Breiteneder & Mills, 2005). Indeed, despite the excellent digestibility of the caseins, they act as potent allergens (Bernard et al., 1998; Docena et al., 1996). A possible explanation can be that sufficient immunologically active protein fragments, which retain IgE-binding or T-cell stimulation capacities, survive the degradative environment of the gastrointestinal tract, are taken up by the gut, and are then presented to the mucosal immune system to sensitise an individual or elicit an allergic response in a sensitised individual (Eiwegger et al., 2006; Moreno, Mellon, Wickham, Bottrill, & Mills, 2005).

The immunoreactive structures, known as IgE-binding epitopes, are responsible for the allergenicity of a protein. They comprise groups of amino acids within the protein sequence that are bound by specific IgE antibodies. Several IgE-binding epitopes of bovine α_{S1} - and α_{S2} -CN with a length of 9–24 amino acids have been identified by SPOT membrane- and microarray immunoassay with sera from CMA patients (Busse, Järvinen, Vila, Beyer, & Sampson, 2002; Ceredo et al., 2008; Chatchatee, Järvinen, Bardina, Beyer, & Sampson, 2001; Lin et al., 2009). Previous studies have reported the persistence of some of these IgE-binding epitopes after simulated gastrointestinal digestion, suggesting that digested low molecular mass peptides can retain an allergenic potential (Dupont et al., 2010a, 2010b; Picariello et al., 2010). However, these studies didn't take into account the influence of the genetic polymorphism of milk proteins on the IgE-binding epitope structure.

Therefore, the objective of this study was the characterisation of peptides resulting from *in vitro* gastrointestinal digestion of different bovine α_{S1} - and α_{S2} -CN variants using mass spectrometry, with the aim of identifying resistant regions containing IgE-binding epitopes and to determine possible differences between the casein variants.

2. Materials and methods

2.1. Milk samples

A total of 979 individual milk samples were collected from the 3 bovine breeds, German Holstein Friesian ($n = 421$), Pinzgauer ($n = 362$) and German Gelbvieh ($n = 196$), derived from associations and private dairy farms in Germany and Austria.

2.2. Screening of different bovine breeds by isoelectric focussing of milk samples

Milk protein variants of skimmed milk samples and isolated casein fractions from anion-exchange chromatography were determined by isoelectric focussing (IEF) according to Erhardt (1989) carried out on 0.3-mm thin polyacrylamide gels using carrier ampholytes. The gels ($T = 5.01$; $C = 3.75$; 7.09 M urea) contained 0.644 mL of the following mixture of carrier ampholytes: 15.5% (v/v) Pharmalyte pH 2.5–5.0 (GE Healthcare Europe GmbH, Freiburg, Germany), 37.9% (v/v) Pharmalyte pH 4.2–4.9 (GE Healthcare

Europe GmbH) and 46.6% (v/v) Servalyte pH 5.0–7.0 (Serva Electrophoresis, Heidelberg, Germany). After prefocusing for 14 min (20 mA, 3000 V), milk samples were separated for 67 min (40 mA, 3000 V). The gels were fixed in 20% (w/v) trichloroacetic acid and stained with a solution of 0.1% (w/v) Coomassie brilliant blue R 250 (Serva Electrophoresis), 45% (v/v) ethanol and 10% acetic acid (v/v). Analysis and identification of the genetic variants were done manually using cow milk samples with known α_{S1} -, α_{S2} -, β - and κ -CN variants as reference samples. Allele and genotype frequencies were calculated with the program PopGene V 1.32 (Yeh, Yang, & Boyle, 1999).

2.3. Preparation of caseins from milk

Whole casein was obtained by acid precipitation at pH 4.6 after skimming of milk samples from cows with defined casein phenotypes (α_{S1} -CN B, C and α_{S2} -CN A, B). Briefly, milk samples were acidified with 50% (v/v) acetic acid to pH 4.6 and centrifuged at $1620 \times g$ for 10 min at 4 °C. The casein precipitate was dissolved in distilled water with 1.0 M NaOH. Steps of precipitation and washing were repeated three times. Whole casein was finally lyophilised and stored at -20 °C until analysis.

2.4. Purification of caseins by anion-exchange chromatography

Separation of α_{S1} - and α_{S2} -CN variants from the whole casein fraction was performed by anion-exchange chromatography according to a modified method of Andrews and Alichanidis (1983). A column (46.5×2.5 cm) was packed with diethylaminoethyl (DEAE) 52-cellulose resin (Serva Electrophoresis) to reach a column volume of 50 mL and equilibrated with 0.01 M Tris/imidazol, pH 7.0, 3.3 M urea, 0.01 M 2- β -mercaptoethanol and 3.1 mM sodium azide as equilibration (EQ) buffer. An amount of 1.0–1.5 mg lyophilised whole casein was dissolved in 30–40 mL EQ buffer and applied onto the DEAE-cellulose column. Stepwise elution of the caseins was performed with a NaCl gradient (0.075–0.17 M) using a peristaltic pump (Ismatec Ip-4, Zürich, Switzerland). The absorbance of the column effluents were monitored at 280 nm (Uvicord SII, LKB, Bromma, Sweden) and fractions (7 mL) collected. The main peak fractions of the resulting chromatogram corresponding to the different purified caseins were analysed by IEF as described above, then separately pooled and dialysed against 3×5 L distilled water for 24 h. After lyophilisation, the purity and identity of the isolated casein fractions were assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix assisted laser desorption ionisation-time of flight-mass spectrometry (MALDI-TOF-MS). Samples were frozen (-20 °C) for storage until used.

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to Laemmli (1970) with 1.5-mm thin polyacrylamide gels (16×14 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, New York, USA). Sample preparations were made for electrophoresis containing 11.5% (v/v) 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. The electrophoretic run was done in running buffer comprising 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3, at 400 V and 25 mA for the stacking gel and at 400 V and 40 mA for the running gel. The gels were fixed with 50% trichloroacetic acid, stained with 0.1% (w/v) Coomassie brilliant blue R 250 and destained with a solution of 30% ethanol (v/v) and 10% (v/v) acetic acid. A molecular marker (Protein Test

Mixture 6, Serva Electrophoresis) added to each gel was used to estimate the molecular weight of the casein fractions.

2.6. In vitro gastrointestinal digestion of casein variants

The casein variants (α_{S1} -CN B, C and α_{S2} -CN A, B) were digested in duplicate by the procedure described by Moreno et al. (2005). Each digestion was performed at least twice and control incubations in the absence of proteases were done.

2.6.1. In vitro gastric digestion (phase 1)

A sample of 10–15 mg purified casein was dissolved in 1 mL of simulated gastric fluid (SGF; 0.15 M NaCl, pH 2.5) and adjusted to pH 2.5 with 1.0 M HCl. After incubation at 37 °C for 15 min, a solution of 0.32% (w/v) porcine pepsin (activity: 3300 U mg⁻¹ of protein using haemoglobin as substrate, Sigma–Aldrich, Steinheim, Germany) in SGF, pH 2.5, was added at an approximately physiological ratio of enzyme:casein (1:20, w/w), giving 165 U of pepsin mg⁻¹ protein. Samples and a control with no enzyme addition were incubated at 37 °C, 150 rpm for 60 min. The digestion was stopped by raising the pH to 7.5 using 1.0 M NaOH. Aliquots (40 μ L) in triplicate were removed for MALDI-TOF-MS analysis.

2.6.2. In vitro duodenal digestion (phase 2)

The in vitro duodenal digestion took place with the 60 min gastric digests (phase 1) as starting material. Before duodenal proteolysis, the pH of the digests was adjusted to 6.5 with 0.1 M NaOH and then following solutions were added to simulate a duodenal environment: a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate and sodium glyco-deoxycholic acid, 1.0 M CaCl₂ and 0.25 M bis-Tris, pH 6.5. Then, solutions of 0.1% (w/v) porcine trypsin (activity: 13,800 U mg⁻¹ protein using benzoylarginine ethyl ester as substrate, Sigma–Aldrich) and 0.4% (w/v) bovine α -chymotrypsin (activity: 40 U mg⁻¹ protein using benzoyltyrosine ethyl ether as substrate, Sigma–Aldrich) in water were added in approximately physiological enzyme:protein ratios of 1:400 (w/w) for trypsin and 1:100 (w/w) for chymotrypsin, to give 34.5 U of trypsin mg⁻¹ of protein, as well as 0.40 U of chymotrypsin mg⁻¹ of protein. The duodenal digestion was carried out in a shaking incubator (37 °C, 150 rpm) for 60 min and stopped by addition of a solution of soybean Bowman–Birk trypsin–chymotrypsin inhibitor (Sigma–Aldrich) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix. At the end aliquots (40 μ L) in triplicate were taken for MALDI-TOF-MS analysis.

2.7. Matrix assisted laser desorption ionisation-time of flight-mass spectrometry

To identify the peptides remaining after gastric (phase 1) and duodenal (phase 2) digestion, samples collected at the end of both phases of digestion, as well as isolated casein fractions from anion-exchange chromatography, were analysed by MALDI-TOF-MS. Prior to analysis, casein digests were desalted using ZipTip C₁₈ columns (Millipore, Eschborn, Germany). The samples were crystallised by the dried droplet method using 2,5-dihydroxy benzoic acid as matrix. The matrix solution was prepared by dissolving 2,5-dihydroxy benzoic acid and methylene-diphosphonic acid (Fluka, Neu-Ulm, Germany) at concentrations of 5 mg mL⁻¹ in water, respectively. MALDI-TOF-MS analyses were performed using an Ultraflex TOF/TOF I mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a UV nitrogen laser (λ = 337 nm, 3 ns pulse width) to desorb/ionise the matrix/analyte material. Mass spectra were obtained in positive reflectron ion mode over the m/z range 400–4000. Approximately 250 spectra were accumulated.

For external calibration, a peptide standard mixture (Bruker Daltonics) was used.

2.8. Data analysis

MALDI-TOF-MS data analysis was performed with the Compass 1.2 software (Bruker Daltonics). An assignment of observed ions to the corresponding amino acid sequences was done, which was based on the known sequences of the different caseins by using the protein database Swiss-Prot and TrEMBL, as well as the tools FindPept and Peptide Mass (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) with cysteines treated with nothing and methionines oxidised; (c) mass tolerance set to 50 ppm, with a maximum of 75 ppm; (d) pepsin, trypsin and chymotrypsin selected as enzymes.

3. Results and discussion

3.1. Screening of different bovine breeds

With the screening of the 3 bovine breeds by IEF, the known genetic variants α_{S1} -CN B and C, as well as α_{S2} -CN A, B and D, could be demonstrated with different frequencies (Fig. 1 and Table 1). In agreement with previous studies (Caroli, Rizzi, Lühken, & Erhardt, 2010; Erhardt, 1993b, 1996) α_{S1} -CN B showed the highest frequency in all breeds analysed. According to Caroli et al. (2010) and Erhardt (1996), α_{S1} -CN C is a common variant in Pinzgauer, whereas it could not be found in Gelbvieh, which is in contrast to the finding of Erhardt (1993b).

As reported by others (Caroli et al., 2010; Erhardt, 1993b, 1996), α_{S2} -CN A was the predominant variant in the 3 bovine breeds investigated. A relatively high frequency of variant B could be detected in Pinzgauer. α_{S2} -CN B is usually present in *Bos indicus*

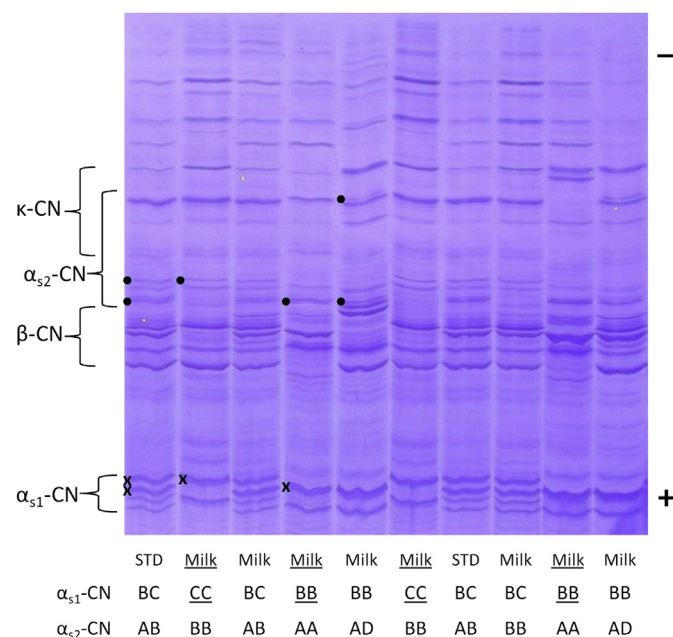


Fig. 1. Isoelectric focussing pattern of milk samples from cows with different α_{S1} - and α_{S2} -casein (CN) genotypes, which are indicated in capitals at the bottom of the illustration. Reference samples (STD) were used for identification of the casein variants. The major bands of the homozygous and heterozygous forms of α_{S1} -CN (BB, BC, CC) are marked by a cross, while the α_{S2} -CN genotypes (AA, AB, AD, BB) are denoted with a dot. Milk samples used for isolation and digestion of casein variants are underlined.

Table 1

Allele frequencies of α_{S1} - and α_{S2} -casein in different cattle breeds identified by isoelectric focussing.

Breed	n	α_{S1} -casein		α_{S2} -casein		
		B	C	A	B	D
German Holstein Friesian	421	0.994	0.006	1.0	—	—
Pinzgauer	362	0.778	0.222	0.899	0.101	—
German Gelbvieh	196	1.0	—	0.906	—	0.094

breeds, and the occurrence of this variant in Pinzgauer can be explained by an introgression of *B. indicus*, as already discussed by Caroli et al. (2010). The rare variant α_{S2} -CN D was present in Gelbvieh with low frequencies and in the heterozygous genotype AD. Only the homozygous forms α_{S1} -CN BB and CC, as well as α_{S2} -CN AA and BB, were included in further analyses.

3.2. Purification of α_{S1} - and α_{S2} -casein variants

After precipitation and anion-exchange chromatography, α_{S1} -CN B and C, as well as one sample of α_{S2} -CN, were recovered in pure forms from the other caseins, as shown by SDS-PAGE and MALDI-TOF-MS. A significant contamination with α_{S1} -CN could only be detected in samples of α_{S2} -CN A and B. On SDS-PAGE (Fig. 2), the α_{S1} -CN variants (lanes 1–3) displayed one band at molecular mass of around 32,000 Da, whereas the position of α_{S1} -CN C (lane 3) was slightly below α_{S1} -CN B. The α_{S2} -CN variants (lanes 4–6) showed one main band and had almost the same electrophoretic mobility on SDS-PAGE as α_{S1} -CN (Fig. 2). However, α_{S2} -CN A (lane 5) migrated slightly faster. On SDS-PAGE gels, the molecular masses for α_{S1} - and α_{S2} -CN exceeded the theoretically calculated masses of 23,614 Da and 25,229 Da, respectively (Farrell et al., 2004). This is accordance with other studies, where molecular masses of approximately 30,000–32,400 Da were observed for α -CN (Almaas et al., 2006; Docena et al., 1996; Pardo & Natalucci, 2002).

In addition, mass spectrometry analyses were performed to confirm the purity of the isolated casein fractions. For this purpose, the samples were digested with trypsin and subjected to MALDI-TOF-MS. Then, the purity of the samples was determined on the

basis of the intensity coverage (%) of the matched tryptic peptides. According to SDS-PAGE, no contamination with other caseins could be demonstrated in the three α_{S1} -CN samples.

Two of the 3 α_{S2} -CN samples contained some traces of α_{S1} -CN, which confirms the observations from other studies that it is difficult to get a pure separation of α_{S2} -CN with ion-exchange chromatography (Holland, Rahimi Yazdi, Ion Titapiccolo, & Corredig, 2010; Hollar, Law, Dalglish, & Brown, 1991; Turhan, Barbano, & Etzel, 2003). The α_{S2} -CN B fraction (lane 4 in Fig. 2) exhibited the highest content of α_{S1} -CN. The signal intensity of α_{S1} -CN peptides in α_{S2} -CN B accounted for 20.6%, whereas in α_{S2} -CN A (lane 6 in Fig. 2) this was 5.5%, as calculated by BioTools (Bruker Daltonics).

3.3. In vitro gastrointestinal digestion of α_{S1} - and α_{S2} -casein variants

As reported for the caseins (Astwood et al., 1996; Dupont et al., 2010a; Fu, Abbott, & Hatzos, 2002), the genetic variants α_{S1} -CN B and C, as well as α_{S2} -CN A and B, were rapidly hydrolysed by pepsin during gastric digestion, and no measurable amount of intact protein could be found at the end of this phase. By continuing the digestion with duodenal enzymes, the casein peptides were further broken down, leading to a more complex peptide mixture than in phase 1. This is expected as α_{S1} - and α_{S2} -CN are poorly structured proteins with no defined tertiary structure, which are therefore very susceptible to proteases such as pepsin, trypsin and chymotrypsin (Astwood et al., 1996; Swaisgood, 2003).

Using MALDI-TOF-MS, it was possible to identify the remaining peptides after gastrointestinal digestion. This method enables the mapping of the complex peptide mixture occurring in the digests of several casein variants. Nevertheless, in both phases of the digestion, for some masses, more than one peptide was assigned during the FindPept database search, not allowing unambiguous determination of these peptides. In consequence, these were not considered in further evaluations.

3.3.1. Gastric digestion (phase 1) of α_{S1} -casein

After gastric digestion, a total of 37 common peptides were found for α_{S1} -CN B and C with molecular masses between 415 and 3017 Da. Among these, 29 peptides were unambiguously identified (Fig. 3a). As indicated in Fig. 3a, almost no breakdown could be observed in region 54–89 of α_{S1} -CN, while more pepsin-generated peptides were detected in the rest of the protein sequence. The region 54–89 is a highly phosphorylated area with phosphoserine residues in positions 64, 66, 67, 68 and 75. Post-translational modifications such as phosphorylations are one reason why parts of a sequence cannot be identified by MS, because they lead to low abundance and suppression effects of peptides (Boersema, Mohammed, & Heck, 2009). Furthermore, phosphopeptides have a poor ionisation and fragmentation efficiency, and therefore limit the analysis with MS (Asara & Allison, 1999). Moreover, a comparison with the theoretical cleavage sites of pepsin (pH > 2) in the region 54–89 confirmed that no typical and specific enzyme cleavage occurs in this part of α_{S1} -CN sequence.

In addition to the peptides found together in both α_{S1} -CN variants, MALDI-TOF analysis also revealed that the digestion gave rise to peptides differing from each other due to the amino acid substitution at position 192, which distinguishes variant B (glutamic acid, E) from C (glycine, G) (Table 2). Thus, peptides f177–192 and f193–199 occurred only in the digests of variant B, because the glutamic acid at position 192 is a preferential cleavage site for pepsin (Sweeney & Walker, 1993). However, a corresponding peptide (f176–192) was found in samples of α_{S1} -CN C despite a glycine being at position 192, which can be explained by the decreasing

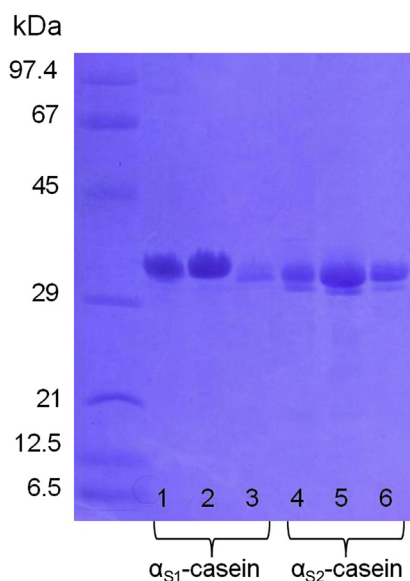


Fig. 2. SDS-PAGE patterns of isolated α_{S1} - and α_{S2} -casein (CN) fractions obtained after anion exchange chromatography: lanes 1 and 2, α_{S1} -CN B; lane 3, α_{S1} -CN C; lane 4, α_{S2} -CN B; lanes 5 and 6, α_{S2} -CN A.

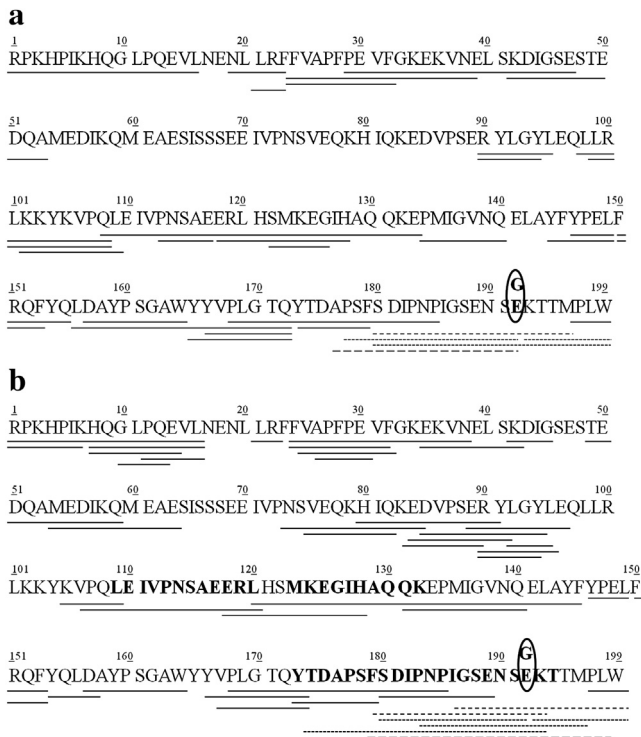


Fig. 3. Peptides identified by MALDI-TOF-MS after (a) gastric and (b) duodenal digestion of α_{S1} -casein (CN) variants B and C. Peptides that were unambiguously identified are represented by black continuous lines. Peptides containing the amino acid substitutions and occurring in all α_{S1} -CN variants are indicated with short dashed lines (---), peptides found only in α_{S1} -CN B are shown with very short dashed lines (----) and peptides detected solely in α_{S1} -CN C are marked by long dashed lines (-----). The positions of the amino acid substitutions and IgE-binding epitopes are highlighted in bold.

substrate specificity of pepsin at pH > 2. It can be assumed that the rate of hydrolysis for this peptide is low, since the related peptide f193–199 could not be identified in the digests of α_{S1} -CN C, presumably because of its low concentration, not allowing detection by MALDI-TOF-MS. Peptide f180–196 was identical in both variants, with a mass difference of about 72 Da, resulting from the varying molecular weights of glutamic acid (147.13 Da) and glycine

(75.07 Da). Furthermore, the larger fragment f180–199 was solely observed in variant B (Table 2).

3.3.2. Duodenal digestion (phase 2) of α_{S1} -casein

Overall, 58 identical peptides could be identified in the digests of α_{S1} -CN B and C by MALDI-TOF-MS, with molecular masses between 414 and 2998 Da. Of these, 43 peptides were exactly assigned (Fig. 3b). The higher number of shorter fragments, as well as the cleavage of larger peptides from the gastric digest in areas 1–16, 25–45 and 73–96, indicated a further breakdown of the α_{S1} -CN variants. Moreover, 17 peptides observed in phase 1 digestion were also found in phase 2, suggesting that these peptides did not undergo a progressive degradation by trypsin and chymotrypsin. Similar to gastric digestion, different peptides could be demonstrated after duodenal digestion, and these contained the variant specific amino acid substitutions (Table 2). Peptide f180–193 was identical in both variants and probably displays a breakdown product of peptide f180–196 arising from gastric digestion. Peptides f180–192 and f193–199 occurred exclusively in α_{S1} -CN B, because glutamic acid is not only a specific cleavage site of pepsin but also of chymotrypsin, even if it is cleaved with a low hydrolysis rate (Kellermann, 2006). Together with peptide f184–196, they might originate from peptide f180–199, which was solely present in the gastric digest of variant B (Table 2).

The mass difference of 88 Da instead of 72 Da between the peptide f187–199 found common in α_{S1} -CN B and C is due to the oxidation of a methionine residue to methionine sulphoxide (+16 Da) in the peptide of variant B. Oxidation of methionine residues in peptide sequences often takes place in mass spectrometry and arises from light or oxygen exposure of the sample rather than from biological oxidation during digestion (Gobom et al., 2001). Peptide f174–193 in α_{S1} -CN B and the similar peptide f179–198 in α_{S1} -CN C had identical masses, explaining the divergence in their sequences (Table 2). However, peptide f179–198 of α_{S1} -CN C resulted from unspecific chymotrypsin cleavage at position 179(F)–180(S) and contained 1 missed trypsin cleavage at position 193(K)–194(T), whereas peptide f174–193 of α_{S1} -CN B was a specific cleavage product of both enzymes with no missed cleavage. One explanation for the distinct peptides occurring between the individual variants might be that the amino acid substitution changing the peptide sequence contributes to steric hindrance, which limits the access to this region for the enzymes (Dupont et al., 2010a).

Table 2

Identification of peptides with amino acid substitutions after gastric (phase 1) and duodenal (phase 2) digestion of α_{S1} -casein variants B and C by MALDI-TOF-MS.

Experimental mass [M + H] ^a	Theoretical mass [M + H] ^a	Position	Sequence ^b	α_{S1} -casein variant
<i>Gastric digestion</i>				
876.48	876.47	193–199	(E) ¹⁹³ KTTMPLW ¹⁹⁹	B
1689.79	1689.76	177–192	¹⁷⁷ PSFSDIPNPIGSENSE ¹⁹²	B
1688.78	1688.78	176–192	¹⁷⁶ APSFSDIPNPIGSENSE ¹⁹²	C
1819.83	1819.84	180–196	¹⁸⁰ SDIPNPIGSENSEKTTM ¹⁹⁶	B
1747.79	1747.83	180–196	¹⁸⁰ SDIPNPIGSENSGKTTM ¹⁹⁶	C
2216.06	2216.05	180–199	¹⁸⁰ SDIPNPIGSENSEKTTMPLW ¹⁹⁹	B
<i>Duodenal digestion</i>				
876.48	876.47	193–199	(E) ¹⁹³ KTTMPLW ¹⁹⁹	B
1358.62	1358.61	180–192	¹⁸⁰ SDIPNPIGSENSE ¹⁹²	B
1407.63	1407.64	184–196	¹⁸⁴ NPIGSENSEKTTM ¹⁹⁶	B
1407.61	1407.66	187–199	¹⁸⁷ GSENSEKTTMPLW ¹⁹⁹	C
1495.75	1495.63	187–199	¹⁸⁷ GSENSEKTTMPLW ¹⁹⁹	B
1486.71	1486.70	180–193	¹⁸⁰ SDIPNPIGSENSEK ¹⁹³	B
1414.67	1414.68	180–193	¹⁸⁰ SDIPNPIGSENSGK ¹⁹³	C
2105.01	2104.97	174–193	¹⁷⁴ TDAPSFSDIPNPIGSENSEK ¹⁹³	B
2105.04	2105.02	179–198	¹⁷⁹ FSDIPNPIGSENSGKTTMPL ¹⁹⁸	C

^a Monoisotopic mass values.

^b Bold letters represent the position of the amino acid substitution differentiating α_{S1} -casein variants B and C.

It is interesting to note that, with the exception of peptide f180–199, such large fragments were not observed after gastric digestion. Conceivably, the gastric phase gave fragments larger than 4000 Da, which were not detectable with MALDI-TOF-MS in this study. Dupont et al. (2010b) reported the existence of peptides f179–198, f173–198 and f180–199 derived from α_{S1} -CN after gastrointestinal digestion of yoghurt and sterilised milk. In contrast to the results of this study, Dupont et al. (2010b) identified no corresponding peptides in raw milk, and the authors attributed this to a higher degree of proteolysis as compared with heat-treated milk, which seemed to be more resistant to digestion. Nevertheless, a comparison of those results with the present study is difficult, because Dupont et al. (2010b) used a modified form of in vitro digestion adapted to infants. Furthermore, whole milk samples were digested instead of isolated casein fractions as studied here.

3.3.3. Gastric digestion (phase 1) of α_{S2} -casein

The gastric digestion of α_{S2} -CN A and B generated 44 identical peptides with molecular masses between 402 and 3130 Da; among these, 32 peptides were unambiguously identified (Fig. 4a). With the exception of regions 34–59 and 198–207, which barely provided any fragments, the peptides were distributed along the whole protein sequence. It can be suggested that some of the intact sequences were extensively hydrolysed and therefore too small for detection by MALDI-TOF-MS. In addition, several peptides may

have a low ionisation efficiency, be of low intensity, or be completely suppressed, and thus not measurable by MALDI-TOF-MS (Lochnit & Geyer, 2004). Due to a substitution of the phosphorylated serine (S) in variant A by phenylalanine (F) in variant B at position 8, the 3 peptides f7–12, f8–14 and f5–20 could only be found in α_{S2} -CN A and peptide f9–18 solely in α_{S2} -CN B, as phenylalanine is a specific cleavage site for pepsin (Table 3) (Sweeney & Walker, 1993). However, a similar peptide to the α_{S2} -CN A fragment f5–20 could be demonstrated in α_{S2} -CN B in form of peptide f4–21.

3.3.4. Duodenal digestion (phase 2) of α_{S2} -casein

MALDI-TOF-MS analysis of the duodenal digests from α_{S2} -CN A and B showed the presence of 67 identical peptides with molecular masses from 400 to 2556 Da, covering most of the protein sequence. A precise assignment was possible for 54 of the 67 peptides (Fig. 4b). Compared with phase 1 digestion, the increase in shorter fragments, especially in regions 20–30, 89–110, 115–130, as well as 151–170, indicated further breakdown of both casein variants. Moreover, new peptides arose from the almost uncut areas 35–66 and 190–207. Thirteen fragments from phase 1 were also found in phase 2 and did not undergo further hydrolysis by trypsin and chymotrypsin. Due to the preferential cleavage by chymotrypsin at phenylalanine, the gastric peptide f4–21 of α_{S2} -CN B was cut at position 8 during duodenal digestion and thus the resulting peptide f9–21 was specific for this variant (Table 3) (Kellermann, 2006; Sweeney & Walker, 1993). A replacement of the phosphorylated serine by a phenylalanine in variant B played a decisive role concerning the phosphoserine cluster comprising the specific motif Ser(P)₃–Glu₂. Previously, it has been demonstrated that peptides carrying phosphorylated serine residues resist in vivo and in vitro gastro-duodenal digestion (Dupont et al., 2010b; Meisel et al., 2003; Miquel et al., 2005). This is of significance as it has been assumed that the major phosphorylation sites act as important allergenic epitopes, because dephosphorylation of caseins led to a lower immunoreactivity (Bernard, Meisel, Creminon, & Wal, 2000). In this study, gastrointestinal digestion had no effect on the phosphoserine clusters of α_{S1} -CN B and C at position 64–70, since they were present in an intact form. In contrast, the main phosphoserine clusters of α_{S2} -CN A and B at positions 8–12 and 56–60 were particularly resistant to gastrointestinal digestion. Two cleavage sites at positions 11 and 56 could be identified in both α_{S2} -CN variants, with a further one at position 8 in α_{S2} -CN B caused by the mutation (Fig. 4b). Consistent with these findings, Su, Qi, He, Yuan, and Zhang (2007) also showed that multi-phosphorylated α_{S1} - and α_{S2} -CN peptides were not stable against hydrolysis and did not survive pancreatic digestion. However, the role of the major phosphorylated sequences as crucial epitopes in caseins remains unclear, since epitope mapping studies were performed with synthetic peptides lacking a phosphorylated form (Busse et al., 2002; Cerecedo et al., 2008; Chatchatee et al., 2001; Lin et al., 2009).

As indicated in Table 3, peptide f7–29, showing one oxidised methionine, occurred only in variant A and peptide f1–22 solely in variant B with slight variations in their masses. These two peptides contained 4, respectively, 5 missed theoretical cleavages of the pancreatic enzymes. The phosphoserine cluster might also be responsible for this observation, as phosphorylation may change the structure of a protein so that its substrate properties can be impaired (Winter, Kugelstadt, Seidler, Kappes, & Lehmann, 2009). Furthermore, it has been reported that phosphorylations located close to peptide bonds, which are a target of a specific proteolytic enzyme, are able to inhibit such cleavage (Hynek, Zuzalkova, Sikac, & Kas, 2002). Dupont et al. (2010b) also demonstrated that large fragments can resist digestion but, in contrast to this study, they identified no peptides in α_{S2} -CN region 1–70 either in

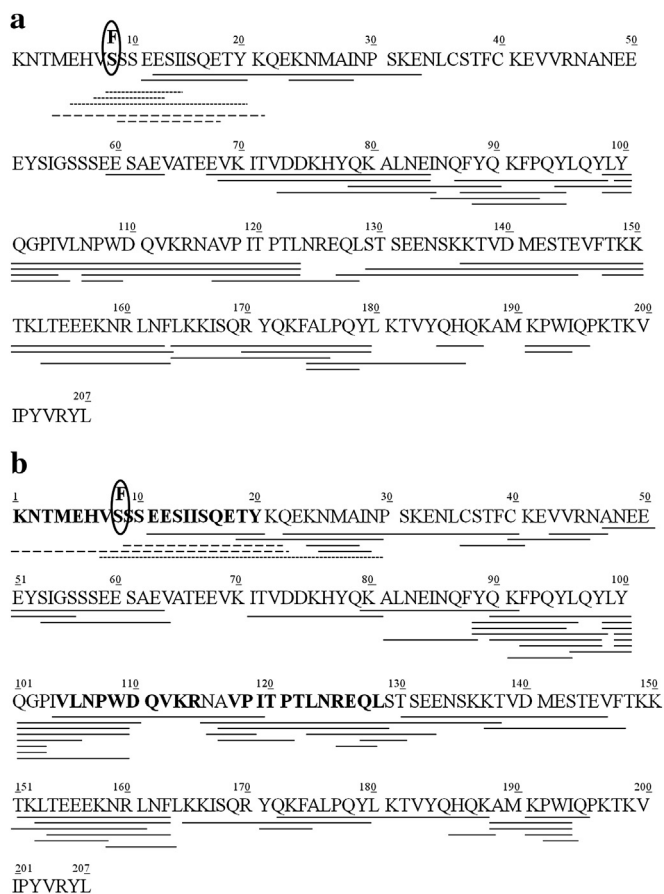


Fig. 4. Peptides identified by MALDI-TOF-MS after (a) gastric and (b) duodenal digestion of α_{S2} -casein (CN) variants A and B. Peptides that were unambiguously identified are represented by black continuous lines. Peptides with amino acid substitutions found only in α_{S2} -CN A are shown with short dashed lines (---). Peptides detected solely in α_{S2} -CN B are marked by large dashed lines (---). The positions of the amino acid substitutions and IgE-binding epitopes are highlighted in bold.

Table 3Identification of peptides with amino acid substitutions after gastric (phase 1) and duodenal (phase 2) digestion of α_{S2} -casein variants A and B by MALDI-TOF-MS.

Experimental mass [M + H] ^a	Theoretical mass [M + H] ^a	Position	Sequence ^b	α_{S2} -casein variant
<i>Gastric digestion</i>				
637.28	637.27	7–12	⁷ VSSEE ¹²	A
738.34	738.32	8–14	⁸ SSSESI ¹⁴	A
1108.55	1108.51	9–18	(F) ⁹ SSSESIISQE ¹⁸	B
1824.77	1824.81	5–20	⁵ EHVSSESIISQETY ²⁰	A
2143.98	2159.92	4–21	⁴ MEHVSSESIISQETYK ²¹	B
<i>Duodenal digestion</i>				
1500.71	1500.71	9–21	(F) ⁹ SSSESIISQETYK ²¹	B
2615.27	2615.23	1–22	¹ KNTMEHVSSESIISQETYKQ ²²	B
2631.20	2631.25	7–29	⁷ VSSESIISQETYKQEKMAIN ²⁹	A

^a Monoisotopic mass values.^b Bold letters represent the position of the amino acid substitution differentiating α_{S2} -casein variants A and B.

raw milk or in pasteurised and sterilised milk. The authors attributed this to the fact that α_{S2} -CN contains two cysteine residues at positions 36 and 40 (Swaigood, 2003), which probably generate heat-induced aggregates with whey proteins through the formation of disulphide bridges in whole milk, resulting in a protection of some larger parts of the protein against digestion (Dupont et al., 2010b).

3.4. Comparison of identified peptides with IgE-binding epitopes

Most food allergens are thought to sensitise an individual via the gastrointestinal tract (GIT). Therefore, these proteins need to survive the digestion in an immunologically active form to cross the gastrointestinal mucosa to interact with the immune system (Mills, Jenkins, Alcocer, & Shewry, 2004). Thus, digested protein fragments must be of sufficient size, being able to sensitise, elicit an allergic reaction, and react with IgE antibodies, which requires the presence of intact IgE-binding epitopes. It is generally assumed that peptides have to be approximately 3500 Da in size to possess an allergenic potential (FAO/WHO, 2001; Huby, Dearman, & Kimber, 2000). However, a study of sensitising capacity of the digested main peanut allergen Ara h 1 demonstrated that peptide fragments smaller than 2000 Da can induce 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 (Bernard et al., 1998; Docena et al., 1996). A comparison of the identified peptides in α_{S1} - and α_{S2} -CN after gastrointestinal digestion with the IgE-binding epitopes described in literature showed that peptides f106–120 and f120–145 in α_{S1} -CN B and C correspond to the known epitopes f109–120, as well as f123–132 (Fig. 3b) (Chatchatee et al., 2001).

For α_{S2} -CN A and B, the epitopes f105–114 and f117–128 were found to resist digestion within peptides f104–119 and f116–128 (Fig. 4b) (Busse et al., 2002). This was also reported for the milk proteins β -CN and β -lactoglobulin (Dupont et al., 2010a; Picariello et al., 2010), as well as Ara h 1 (Bøgh et al., 2009), the epitopes of which are also able to survive the digestion process.

Furthermore, it had been anticipated that the amino acid substitutions or deletions characterising the genetic milk protein variants could influence their IgE-binding epitope structure. Hence, it might be that milk produced from distinct genotypes could exhibit different allergenic properties (Ballabio et al., 2011; Caroli et al., 2009). The present study revealed that the amino acid substitutions affect the peptide pattern arising from the α_{S1} - and α_{S2} -CN variants, as modifications in their allergenic epitopes occurred. Peptides f174–193 in α_{S1} -CN B and f179–198 in α_{S1} -CN C correspond to fragment f173–194, which has been reported as one of the major IgE-binding epitopes in α_{S1} -CN B (Fig. 3b) (Chatchatee et al., 2001).

In α_{S2} -CN, the variant specific peptides f7–29 (α_{S2} -CN A) and f1–22 (α_{S2} -CN B) are congruent with the known IgE-binding epitope f1–20 (Fig. 4b) (Cerecedo et al., 2008). Whether these modifications change the allergenicity of the different epitopes still remains unclear, because investigations are lacking. However, previous studies indicated that single or multiple amino acid substitutions within IgE-binding epitopes of some caseins resulted in an elimination or reduction of IgE-binding by pooled sera from cows' milk-allergic patients (Cocco, Järvinen, Han, Beyer, & Sampson, 2007; Cocco, Järvinen, Sampson, & Beyer, 2003). Cocco et al. (2003) demonstrated that an exchange of the glutamic acid with alanine at position 192 in the IgE-binding epitope f179–192 of α_{S1} -CN led to a slightly decreased binding to this epitope. In α_{S1} -CN C, a glycine is located at position 192. Glycine and alanine have similar features; both are small, neutral and uncharged amino acids. Taking into account the results of Cocco et al. (2003), it can be assumed that peptide f179–198 in α_{S1} -CN C possesses a lower immunoreactivity than peptide f174–193 identified in the digests of α_{S1} -CN B.

For the different peptides detected in α_{S2} -CN A (f7–29) and α_{S2} -CN B (f1–22) modifications in their IgE-binding are conceivable. The missing phosphorylated serine residue in variant B, changing the sequence of the phosphoserine cluster, may result in a diminished IgE-binding to this epitope, as described for dephosphorylated milk proteins (Bernard et al., 2000). The absence of binding to one of the main epitopes in α_{S1} - and α_{S2} -CN could induce a reduced allergenicity of the whole protein. However, further investigations are needed to confirm this.

4. Conclusions

The current study showed that in-vitro gastrointestinal digestion of α_{S1} -CN and α_{S2} -CN variants resulted in a wide range of peptides, which are long enough to contain IgE-binding epitopes. Due to the amino acid substitution characterising the genetic variants, some peptides, and in consequence epitopes, differ from each other. As the described epitopes refer to the reference proteins α_{S1} -CN B and α_{S2} -CN A and differences between the epitopes of the single variants could be demonstrated, further research is needed to examine the allergenic potential of these peptides. In this context, endangered breeds should be taken into consideration, because they play an important role concerning their preservation as gene reserves. Cattle breeds threatened by extinction can exhibit rare variants which are not present in the main breeds, as it was shown for α_{S1} -CN F (Erhardt, 1993a). Such rare variants could possess a lower allergenic potential than the main variants and should therefore be investigated in further studies, as they could be useful for the production of milk with improved nutritional properties such as hypoallergenic qualities. Moreover, this emphasises the importance of genetic polymorphisms of milk proteins not only

in the field of animal breeding but also within the scope of human nutrition.

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