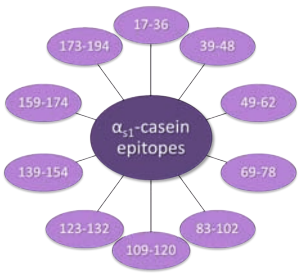
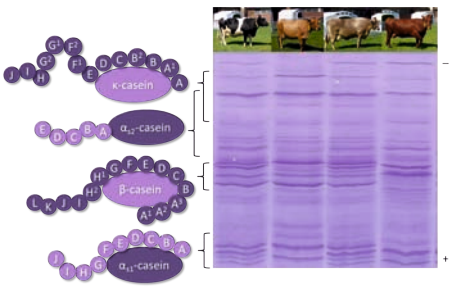
Appearance of Epitopes in Bovine Milk Protein Variants, Their Allergenicity and Potential Use in Human Nutrition

MARIA LISSON





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Meinen Eltern für ihre beispiellose Unterstützung

in all meinen Lebenslagen

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List of abbreviations

AA	Amino acid
AAF	Amino acid-based formula
ACE	Angiotensin I converting enzyme
α_{S1}-CN	α_{s1}-casein
α_{S2}-CN	α_{s2}-casein
α-LA	α-lactalbumin
BSA	Bovine serum albumin
β-CN	β-casein
β-LG	β-lactoglobulin
κ-CN	κ-casein
CMA	Cow milk allergy
CMI	Cow milk intolerance
EHF	Extensively hydrolyzed formula
ESFA	European food safety authority
IEF	Isoelectric focusing
IgE	Immunoglobulin E
Igs	Immunoglobulins
LF	Lactoferrin
MALDI-TOF-MS	Matrix assisted laser desorption/ionization-time of flight-mass spectrometry
OIT	Oral immunotherapy
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
RAST	Radio allergo sorbent test
SLIT	Sublingual immunotherapy
SPT	Skin prick test

1. General introduction

1.1. Importance of cow milk

1.1.1. Economic importance of cow milk

The use of milk for nutritional purposes took place in the seventh millennium BC, early in the evolution of animal domestication (Evershed et al., 2008). Today, it is not only a substantial food within human nutrition but also one of the most important agricultural products in the world. The demand for milk contributes to rise, which is due to an increasing population and income, economic growth, changed dietary habits, as well as the growing popularity of dairy products, particularly in developing countries (OECD/FAO, 2012). Worldwide cow milk production in 2011, representing 83% of total milk (the rest coming from buffalo, goat, sheep, and camel), amounted to 605,644,740 tonnes with the top 5 producing countries (USA: 14.69%, India: 8.66%, China: 6.09%, Brazil: 5.29%, and Russia: 5.18%) accounting for 39.9% of production (FAOstat, 2013). The worldwide average milk consumption (liquid milk and processed products) increased to 107.3 kg per capita per year in 2011. Asia represents the largest consuming region with 39% of global milk consumption, followed by Europe at 28% and North America at 13% (IDF, 2012). However, milk consumption per capita varies widely among and even within regions from highs in Europe (277 kg) to lows in Asia (67 kg). The countries Estonia, Ireland, and Finland in Northern Europe show the highest consumption per capita of liquid milk, all in excess of 130 kg, whereas per capita butter and cheese consumption dominate in Western Europe with France (butter: 7.5 kg, cheese: > 25 kg) and Luxembourg (cheese: > 25 kg) at the top (IDF, 2012).

Germany is the sixth largest producer in the world generating 30,685,200 tonnes milk in 2012 and accounting for 5% of world production (BLE, 2013a,b; FAOstat, 2013). In 2012, 4,190,485 dairy cows were kept by 82,865 German dairy cow farmers, with a milk yield of 7,323 kg per cow per year (4.13% fat and 3.41% protein) and with Holstein Friesian and Simmental as the most important cattle breeds (BLE, 2013a,b; DeStatis, 2013). Within the last 12 years, per capita milk consumption in Germany has varied only slightly and has strongly depended on both product and price. Thus, per capita consumption of fresh milk products decreased from 92.88 kg in 2000 to 90.42 kg in 2012, whereas for cheese a continuous increase from 21.6 kg to 23.7 kg was recorded. The per capita consumption of milk products in 2012 amounted to 54.64 kg for drinking milk, 18.1 kg for yogurt, 5.64 kg for cream products, and 6.18 kg for butter (BLE, 2013a,b).

1.1.2. Dietary importance of cow milk

Cow milk and dairy products are food staples in most Western societies and their consumption is recommended by many nutrition organizations. Cow milk can be described as one of the most nutritionally complete foods being rich in essential nutrients such as protein, fat, vitamins, and minerals (Table 1). The main constituent of milk is water at ca. 87-88%, with the remainder being ca. 3-6% fat, 4.7-4.8% carbohydrate (lactose), 3.2-3.6% protein, and 0.7-0.8% ash representing minerals and vitamins (Töpel, 2004).

The protein of milk is characterized by a high biological value as it comprises all essential amino acids (AA) in beneficial combination (Buttriss, 2003). Due to the high content of the essential AA lysine, milk can be used as valuable supplement for plant proteins that are limited in this AA (Tomé et al., 2004). Cow milk is also rich in the branched chain AA valine, leucine, and isoleucine, which promote protein synthesis, suppress protein catabolism, serve as substrates for gluconeogenesis, and trigger muscle protein synthesis (Etzel, 2004; Haug et al., 2007). Furthermore, cow milk contains a wide range of proteins acting as enzymes and enzymes inhibitors, metal and vitamin binding proteins, growth factors, hormones, as well as antibodies (Claeys et al., 2013). It is also an important source of biologically active peptides that have beneficial effects on human health (Korhonen & Pihlanto, 2006).

Cow milk possesses small amounts of essential omega-6- (linoleic acid, 18:2) and omega-3- (α-linolenic acid, 18:3) fatty acids as well as conjugated linoleic acid (CLA). The later one has been shown to have anticarcinogenic properties with an average content in milk varying between 0.3 and 0.6% of total fatty acids (Dhiman et al., 2000; Haug et al., 2007). The content of these fatty acids is greatly influenced by farming of animals and feeding regime (grain, fiber and energy intake, dietary fats, seasonal and regional effects) (Claeys et al., 2013).

Cow milk makes a contribution for the provision of nearly all minerals, trace elements, and vitamins needed for human health. The greatest amount of calcium is obtained from milk and dairy products and, therefore, it is difficult to achieve dietary calcium recommendations without consuming these foods. Further relevant minerals in milk are phosphor, potassium, and magnesium, which are essential for bone metabolism (Caroli et al., 2011). Together with zinc, they are present in a highly bioavailable form being well absorbed and utilized by the body.

Table 1. Composition of some important nutrients (per 100 g) in whole milk with a standardized fat content of 3.5% (Dhiman et al., 2000; DEBInet, 2013).

Milk component	Concentration in 100 g whole milk
Energy (kcal)	65
(kJ)	272
Fat (g)	3.569
Saturates (g)	2.401
Monounsaturates (g)	0.882
Polyunsaturates (g)	0.074
α-linolenic acid 18:3 (g)	0.025
Linoleic acid, 18:2 (g)	0.046
Protein (g)	3.382
Casein (g)	2.706
Whey proteins (g)	0.676
Carbohydrates	4.7
Lactose (g)	4.685
Glucose (g)	0.006
Galactose (g)	0.009
Oligosaccharides (g)	< 0.1
Water-soluble vitamins	
Thiamin (vit B_1) (mg)	0.037
Riboflavin (vit B_2) (mg)	0.18
Pyridoxine (vit B_6) (mg)	0.039
Cobalamin (vit B_{12}) (µg)	0.4
Pantothenic acid (mg)	0.35
Folate (µg)	9
Fat-soluble vitamins	
Retinol (vit A) (µg)	28
Cholecalciferol (vit D) (µg)	0.09
Tocopherol (vit E) (mg)	0.07
Minerals	
Calcium (mg)	120
Potassium (mg)	140
Magnesium (mg)	12
Zinc (mg)	0.4
Phosphor (mg)	92
Sodium (mg)	45
Chloride (mg)	102
Essential amino acids	
Lysine (mg)	283
Valine (mg)	194
Leucine (mg)	328
Isoleucine (mg)	147
Proline (mg)	322

Concerning vitamins, all known vitamins are available in whole milk, although with varying quantities and with vitamin B_{12}, vitamin B_2, and vitamin A as the most important ones. Milk

content of several vitamins and minerals such as vitamin A and E, as well as selenium and iodine are influenced by the cows' diet (Haug et al., 2007; Claeys et al., 2013).

1.2. Cow milk allergy

1.2.1. Definition, epidemiology, and etiology

Despite the numerous health benefits of cow milk, its consumption can lead to nutritional and immunological problems such as cow milk allergy (CMA) or cow milk intolerance (CMI). CMA has to be differentiated from CMI as they are distinct disorders that need separate methods of diagnosis and different strategies for treatment and management (Crittenden & Bennett, 2005). CMA is clinically an immunologically mediated adverse reaction to single or multiple proteins found in cow milk (Figure 1).

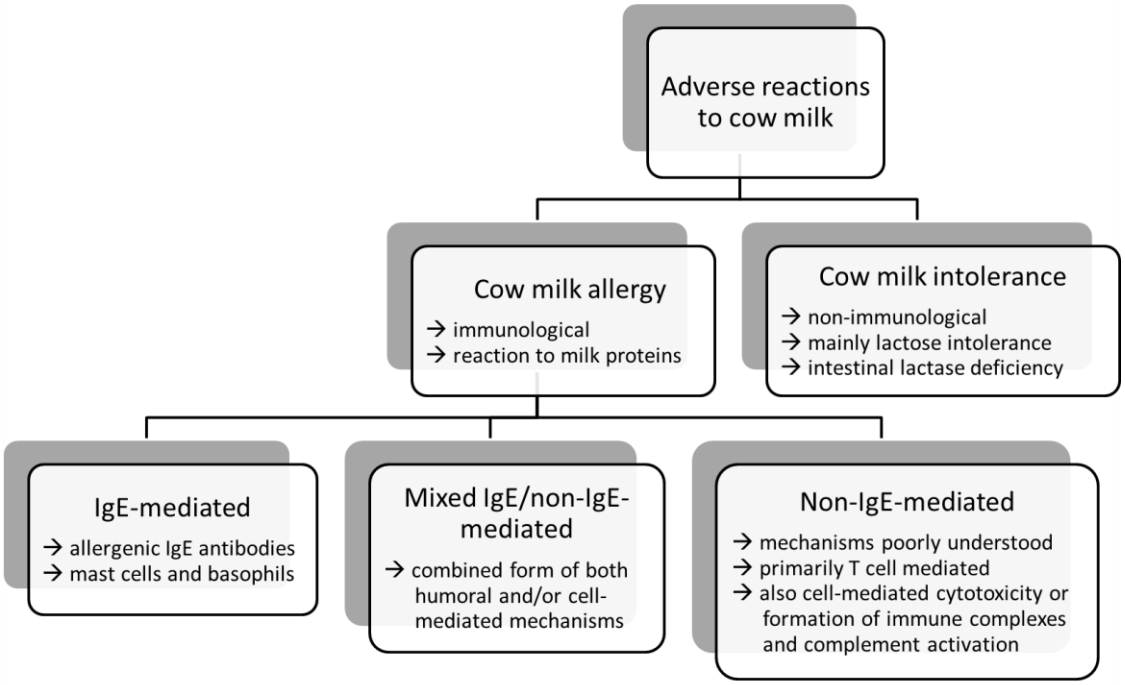

Figure 1. Classification of adverse reactions of cow milk (adapted from Bahna, 2002; Crittenden & Bennett, 2005; Hochwallner et al., 2011).

In contrast, CMI refers to non immunologic reactions to cow milk such as disorders of digestion, absorption, or metabolism of certain cow milk components, mainly lactose (Bahna, 2002; Monaci et al., 2006).

Cow milk protein is often the first foreign food antigen infants are orally exposed to. That is why CMA is the most common food allergy in infants and young children with an incidence

of 2-3% in the general population (Skripak et al., 2007). CMA in adults is rare and affects 0.1-0.5% of the grown persons (Lam et al., 2008; Osterballe et al., 2009). Usually, CMA occurs during the first year of life after introduction of cow milk or cow milk based-formula in the infant´s diet (Benhamou et al., 2009). It may also appear via breast milk, when the maternal diet contains cow milk (Huang & Kim, 2012). The majority of children outgrow their allergy at school age, but tolerance can even develop later (Savilahti & Savilahti, 2013). Skripak et al. (2007) found rates of tolerances of 19% at the age of 4 years, 42% at the age of 8 years, 64% at the age of 12 years, and 79% at the age of 16 years.

The etiology of CMA seems to be multifactorial and has not been clearly defined. It is probably based on the interaction between genetic predisposition of the individual and environmental factors (Cochrane et al., 2009; Savilahti & Savilahti, 2013).

1.2.2. Pathogenesis and manifestation of cow milk allergy

Although the exact site of food absorption and allergy induction remains unclear, most of the food allergens are thought to sensitize an individual via the gastrointestinal tract (Moreno, 2007; Schnell & Herman, 2009). However, also the skin has been shown to be an important route of sensitization to food allergens (Dunkin et al., 2011; Berin & Sampson, 2013). The gastrointestinal tract represents the largest surface area in the human body with the main function being the digestion and absorption of food, as well as the prevention of pathogen, toxin, and food antigen penetration. It is comprised of a single layer of epithelial cells that establish a selective barrier separating the external environment from the internal milieu and allowing the transport of nutrients, while retaining larger molecules and harmful pathogens within the lumen (Perrier & Corhésy, 2011). This mucosal barrier is armed with complex physiological and immunological protective mechanisms. The physiologic part is composed of the single layer of epithelial cells that are joined together by tight junctions and covered with a thick mucus layer secreting a number of factors such as mucins, antimicrobial peptides, and trefoil factors into the mucosal lumen. Moreover, luminal and brush border enzymes, bile salts, and extremes of pH contribute to the protection against the invasion of pathogens and antigens. The immunologic barrier includes the innate immune cells and factors, represented by polymorphonuclear neutrophils, macrophages, dendritic cells, natural killer cells, and Toll-like receptors, as well as the adaptive immune cells and factors, comprising intraepithelial and lamina propria lymphocytes, Peyer patches, secretory IgA, and

cytokines that play a key role in defending and elimination of invading pathogenic bacteria and foreign antigens (Sicherer & Sampson, 2010; Berin & Sampson, 2013). Consequently, an abrogation or alteration of this mucosal barrier through e.g. stomach pH neutralization, immaturity of the infants gut barrier and immune system, low stomach pepsin activity at birth, raised intragastric pH in infants or patients taking antacids, as well as malfunction of pancreatic and intestinal enzymes might promote CMA in infants and adults (Cochrane et al., 2009; Sicherer & Sampson, 2010). In addition, an enhanced intestinal permeability can probably contribute to CMA by increasing the exposure and passage of intact allergens (Sicherer & Sampson, 2010; Perrier & Corhésy, 2011). In most people, the intestinal immune system recognizes milk proteins as harmless and suppresses immunoreactivity leading to oral tolerance. The development of oral tolerance is supposed to be obtained through the production of regulatory T-cells or by anergy and deletion of allergen specific T-cells. It is believed that in CMA the process of oral tolerance is abrogated or broken down (Fiocchi et al., 2010; Sicherer & Sampson, 2010; Perrier & Corhésy, 2011). Also, several factors such as genetic predisposition, infections, intestinal microflora modification, age at fist exposure, maternal diet, antigen transfer through breast milk, as well as the nature, timing, dose, quantity, and frequency of antigen load, influence oral tolerance induction or sensitization to cow milk (Cochrane et al., 2009; Fiocchi et al., 2010; Savilahti & Savilahti, 2013). The immune mechanisms involved in CMA are classified in Immunoglobulin E (IgE)-mediated and non-IgE-mediated, as well as mixed IgE-/non-IgE-mediated forms (Figure 1) (Fiocchi et al., 2010).

1.2.2.1. IgE-mediated cow milk allergy

IgE-mediated CMA is considered to be the most common form in infants and children (Vanto et al., 2004). The mechanisms of IgE-mediated CMA are well understood and consist of 2 phases: First sensitization followed by memory induction and second immediate reaction (Figure 2a and b).

a) Sensitization and memory induction

After primary exposure, cow milk protein is absorbed by the gut mucosa and presented to the immune cells (Figure 2a). The allergen is taken up by antigen presenting cells, especially dendritic cells, and degraded to peptide fragments, which are presented via major histocompatibility compelx class-II (MHC-II) to the T-cell receptor of naïve CD4+ T cells.

These CD4+ T cells differentiate into helper 2 (Th2) cells that secrete cytokines IL-4 and IL-13. Both cytokines induce the B cell activation signal and class switching of B cells to IgE. The specific IgE antibodies circulate in the organism and attach with the high affinity IgE-receptor FcεR1 of mast cells or basophiles (Kumar et al., 2012).

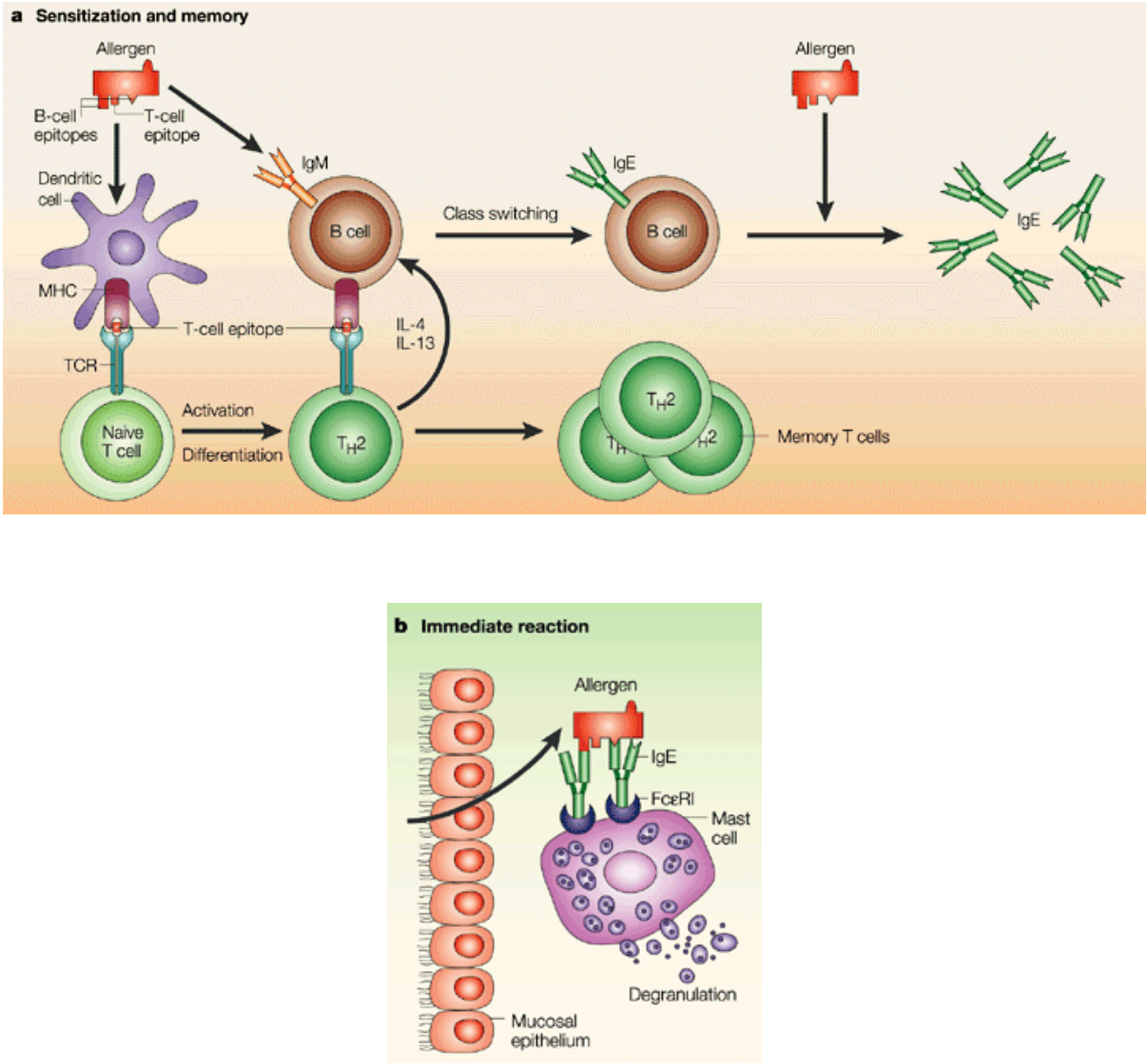

Figure 2. a) Sensitization and memory induction of IgE-mediated allergic reaction and b) Immediate phase of IgE-mediated allergic reaction (Valenta, 2002).

Sensitization leads to the establishment of allergen-specific memory T cells and of IgE^+ memory B cells, which produce increased levels of allergen-specific IgE antibodies after repeated contact with the allergen (Valenta, 2002).

b) Immediate reaction

After a subsequent contact with cow milk protein, the allergen binds via the immunoreactive structures (epitopes) to the specific IgE antibodies on mast cells and basophiles. Cross-linking of high affinity IgE-receptor FcεR1 by allergen-bound IgE results in the degranulation of mast cells and basophiles as well as the release of inflammatory mediators such as histamine, prostaglandins, leuktotriens, and chemokines (Figure 2b). These mediators are able to cause smooth muscle contraction, vasodilatation, mucous secretion, local swelling, and other allergic symptoms (Valenta, 2002; Kumar et al., 2012).

IgE-mediated CMA is characterized by acute onset, with symptoms arising within a few minutes to hours. Clinical manifestation can involve one or more body systems such as the skin, the respiratory system, and the gastrointestinal tract (Figure 3). The majority of CMA patients show more than 2 symptoms and the severity varies from mild (e.g. angioedema) to severe and life-threatening anaphylaxis (cardiovascular collapse or syncope) (Du Toit et al., 2010; Fiocchi et al., 2010). The patients typically show high levels of cow milk protein-specific IgE antibodies in serum or have a positive skin prick test (SPT), or both (Savilahti & Savilahti, 2013).

1.2.2.2. Non-IgE-mediated cow milk allergy

Non-IgE-mediated CMA appears in a significant proportion of infants but it is more common in adults (Crittenden & Bennett, 2005). In contrast to IgE-mediated CMA, the immunological mechanisms of non-IgE-mediated CMA are poorly understood. However, reactions are probably due to a cellular immune response and primarily mediated through T cells (Ho et al., 2012; Savilahti & Savilahti, 2013). It is thought that T cells, activated by the allergen, proliferate and produce different cytokines (e.g. IL-5, IFN-γ), which lead to the activation of e.g. eosinophils or macrophages and, hence, to the release of various inflammatory mediators (Shek et al., 2005; Benhamou et al., 2009; Ho et al., 2012). Non-IgE-mediated mechanisms may also involve antibody-dependent cell-mediated cytotoxicity or formation of immune complexes and complement activation (Hochwallner et al., 2011). The reactions of non-IgE-mediated CMA are of delayed onset and of subacute or chronic nature. Symptoms usually occur from 1 hour to several days after ingestion of cow milk protein and mainly affect the gastrointestinal tract or the skin (Figure 3). The diagnosis of non-IgE-mediated CMA is more difficult than IgE-mediated CMA because the patients do not have circulating

milk protein-specific IgE antibodies in serum and their SPT result is negative (Crittenden & Bennett, 2005; Fiocchi et al., 2010).

1.2.2.3. Mixed IgE-/non-IgE-mediated cow milk allergy

The mixed IgE-/non-IgE-mediated CMA is a combined form of both humoral and/or cell-mediated mechanisms and is usually characterized by a delayed or chronic onset (Figure 3). The symptoms may appear as atopic dermatitis and eosinophilic gastroenteropathies (Fiocchi et al., 2010).

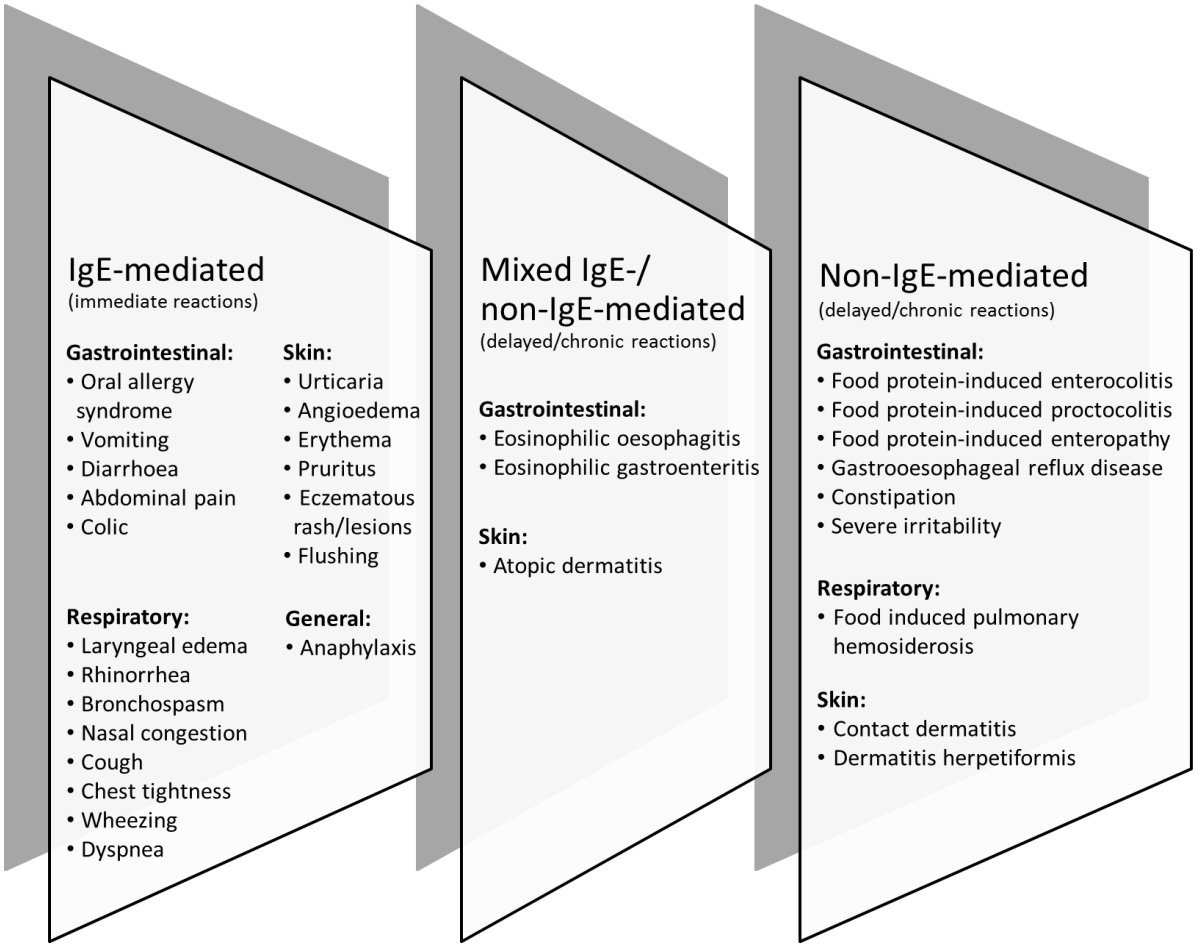

Figure 3. Clinical manifestations of cow milk allergy (Benhamou et al., 2009; Fiocchi et al., 2010; Waserman & Watson, 2011).

1.2.3. Diagnosis of cow milk allergy

The diagnosis of CMA is based on a comprehensive history and careful physical examination to gain information about the nature and course of symptoms, the feeding history, and the relationship of symptoms to milk ingestion or to other factors such as exercise or drugs

(Bahna, 2002). In a next step, SPT and/or serum-specific IgE tests, such as Radio allegro sorbent test (RAST) or ImmunoCAP® (Phadia, Uppsala, Sweden), can be performed. For SPT, fresh cow milk or whole cow milk protein extracts are applied to the skin and pricked. The test is considered positive if the diameter of wheal reaction is at least 3 mm larger than in the negative control (Savilahti & Savilahti, 2013). RAST or ImmunoCAP® are quantitative immunoassays for the measurement of milk protein-specific IgE antibodies in serum. Patient sera are incubated with a solid phase matrix carrying the immobilized allergen, which captures specific IgE antibodies of the patients' serum. The detection of allergen-specific IgE antibodies takes place with a labeled antibody specific for the Fc portion of human IgE (Asero et al., 2007; Benhamou et al., 2009). In clinical practice, 0.35 kU_A/L is commonly used as the cut-off value for cow milk-specific IgE levels (Fiocchi et al., 2010). However, these diagnostic tests alone do not provide sufficient evidence for food allergy as they only indicate sensitization to the food and, thus, a supportive clinical history is needed.

If diagnosis of CMA remains uncertain, elimination diet and oral food challenges (open or double-blind) with cow milk are an important tool. After avoidance for usually 2-4 weeks, gradual increasing amounts of cow milk or formula are given to the patient at time intervals. The challenge is stopped when clinical symptoms are observed or after a specified quantity has been ingested without positive reactions. The double-blind, placebo-controlled oral food challenge (DBPCFC) is considered as the gold standard for diagnosis of CMA (Asero et al., 2007; Benhamou et al., 2009). Nevertheless, oral challenges are expensive, time-consuming, difficult to perform, and accompanied by the risk of anaphylaxis (Fiocchi et al., 2010).

1.2.4. Management and treatment of cow milk allergy

1.2.4.1. Avoidance of cow milk

Currently, the only effective treatment for CMA is a consequent avoidance of cow milk and cow milk based products. In breast-fed infants with CMA, a maternal cow milk elimination diet with calcium supplements is recommended as long as breastfeeding persists. Afterwards, the infant should receive a hypoallergenic formula. If infants with CMA are not breastfed or the mother cannot or no longer whishes to breastfeed, the use of safe, affordable, and nutritionally adequate formulas are required. A hypoallergenic formula contains < 1% of immunoreactive proteins and is tolerated by at least 90% of infants with CMA (Dupont et al., 2011). These criteria are met by extensively hydrolyzed cow milk whey

and/or casein formulas (EHF) and by amino acid-based formulas (AAF). In EHF, the allergenic epitopes of cow milk proteins are destroyed by extensive hydrolysis, which mainly generates peptides of < 1500 Da as well as free AA (Dupont et al., 2011). The main drawbacks are the bitter taste of the peptides and the loss of physical and biologic functionality of the milk proteins (Lee, 1992). EHF are tolerated by the majority of infants with CMA but they can induce adverse reactions in some allergic patients (Boissieu et al., 1997). Residual allergenicity of EHF may be due to short peptides still containing IgE-binding epitopes or allergenic higher-molecular-weight particles that arise through aggregation of smaller peptides during production (Beyer, 2007). In case of allergic reactions to EHF or for infants with severe CMA or multiple food allergies, AAF are required. AAF are regarded as non-allergenic, since they are pure chemically made formulas, which are free of intact proteins and peptides consisting of isolated AA only (Greef et al., 2012).

The use of a soya-based formula in CMA is not recommended before the age of 6 month, because of the high levels of phytates, aluminum, and phyto-oestrogens present in soya protein. Moreover, soya is a potent allergen itself and 10-14% of infants with CMA have concomitant soya allergy (Dupont et al., 2011). Milk of other animal source such as goat, sheep, buffalo, horse, or donkey are not suitable as general substitute in CMA because these milks are not nutritionally adequate to meet the infants' needs. Furthermore, a high degree of cross-reactivity exists between milk proteins from cow, goat, sheep, as well as buffalo, which is based on the close homology between these proteins (Du Toit et al., 2010; Dupont et al., 2011).

1.2.4.2. Prevention

Exclusive breastfeeding for 4-6 months is regarded as the best preventative for CMA. Human milk is low in allergens by nature, provides antigens for the neonatal intestinal immune system, and contains bioactive factors (e.g. IgA, growth factors, cytokines, lactoferrin, lysozyme, and oligosaccharides) that modulate immune development, defense against pathogens, as well as promote beneficial colonization by commensal bacteria (Tooley et al., 2009; Ballard & Morrow, 2013).

There is some evidence that probiotics (e. g. lactobacillus GG) and prebiotics (oligosaccharides) have protective effect against allergic manifestation and infections (Arslanoglu et al., 2008). It has also been demonstrated that probiotics accelerate the

development of tolerance in infants (Berni Canani et al., 2012). Pro and prebiotics are frequently added to infant formulas, as they can induce the development of an intestinal microbiota similar to that found in breastfed infants (Arslanoglu et al., 2008). However, further research is needed before routine use of prebiotics as data from several studies yielded contradictory results (Osborn & Sinn, 2013).

1.2.4.3. Immunotherapy

Oral immunotherapy (OIT) is a promising approach for treatment of CMA but yet it is not considered to be ready for use in clinical practice due to the relatively high frequency and unpredictability of adverse reactions (Huang & Kim, 2012). OIT aims to induce desensitization and optimally tolerance to the implicated food. During an induction phase, small increasing amounts of cow milk are administrated to the patient. Afterwards, maintenance of desensitization is based on regular consumption of a maximum tolerated dose of cow milk (Dupont et al., 2011; Savilahti & Savilahti, 2013). Several studies on cow milk OIT have been published and recently reviewed (Brożek et al., 2012; Savilahti & Savilahti, 2013). The majority of patients with CMA were successfully desensitized to cow milk by OIT. Yet, to date there is no evidence whether permanent tolerance can be achieved by OIT.

Another possibility for treatment of CMA is called sublingual immunotherapy (SLIT). SLIT also involves small increasing amounts of cow milk but they are placed under the tongue. In SLIT, the typical dosage amounts are low but there is less potential to provoke adverse effects (Huang & Kim, 2012). It has been demonstrated that SLIT is able to desensitize CMA patients to varying degrees (Boissieu & Dupont, 2006; Keet et al., 2012). More research is needed to ascertain which form of immunotherapy is more effective in inducing longterm tolerance with the lowest risk of severe adverse reactions (Narisety & Keet, 2012).

1.3. Cow milk allergens

Cow milk contains 30-35 g of protein per liter comprising more than 25 different proteins. However, only some of them are known to be potential allergens (Hochwallner et al., 2013). Based on the behavior by acidification of milk at pH 4.6, 20 °C, milk proteins are divided into 2 main groups. These are the insoluble casein and the soluble whey protein fraction (Table 2). Caseins (designation: Bos d 8 according to the WHO-IUIS allergen nomenclature sub-

committee) and the whey proteins α-lactalbumin (α-LA, Bos d 4) and β-lactoglobulin (β-LG, Bos d 5) are the most important allergens in cow milk, whereas the whey proteins bovine serum albumin (BSA, Bos d 6), immunoglobulins (Igs, Bos d 7) and lactoferrin (Lf, Bos d lactoferrin) are defined as minor milk allergens (Järvinen & Chatchatee, 2009). Most patients with CMA are sensitized to several cow milk proteins with a great variability of the IgE response in specificity and intensity (Wal, 2002; Hochwallner et al., 2010a). Thus, no single protein can be held responsible for the major part of allergenicity in milk. There are controversial reports about the prevalence of IgE reactivity to certain cow milk proteins. The majority of studies found the caseins being more allergenic than the whey proteins (Docena et al., 1996; Shek et al., 2005; Gaudin et al., 2008; D'Urbano et al., 2010) but Hochwallner et al. (2010a) detected the whey proteins β-LG and α-LA as the most frequently recognized milk allergens.

1.3.1. Caseins

The casein fraction accounts for 80% of total milk protein and comprises the 4 different proteins α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN), β-casein (β-CN), and κ-casein (κ-CN) (Table 2) (Wal, 2002; Farrell et al., 2004). They are coded by members of a multigene family and tightly linked within a 250-kb cluster on chromosome 6 in the order α_{S1}-, β-, α_{S2}-, κ-CN (Threadgill & Womack, 1990; Hayes et al., 1993).

The caseins show little sequence homology but they share some common features such as heat stability, high susceptibility to all proteases and exopeptidases, dipolar structure with amphiphatic properties, and calcium sensitivity of α_{S1}-, α_{S2}- and β-CN (Wal, 2002). The caseins are phosphoproteins with a varying degree of phosphorylation occurring at serine or threonine residues. The calcium-sensitive α_{S1}-, α_{S2}-, and β-CN contain cluster of phosphoseryl residues with the motif SerP-SerP-SerP-Glu-Glu in their polar domains. These polyanionic phosphate groups bind metal ions, including calcium, zinc, and magnesium with high affinity, which influence the physical-chemically, functional, and alimentary characteristics of the caseins (Fox, 2003). In addition, caseins own a high proportion of the structure breaking AA proline, which contributes to their open, flexible, and mobile conformation. This is why the caseins lack on stable secondary and tertiary structures and have been referred to as rheomorphic (Swaisgood, 1993; Fox & McSweeney, 1998).

Table 2. Characterization of major and minor allergenic proteins in cow milk (Wal, 2002; Farrell et al., 2004).

	Caseins (80%) ~30 g/L				**Whey proteins (20%) ~5 g/L**				
	α_{S1}-CN	α_{S2}-CN	β-CN	κ-CN	α-LA	β-LG	Igs	BSA	Lf
Allergen name[1]	Bos d	Bos d	Bos d	Bos d	Bos d	Bos d	Bos d	Bos d	Bos d
	9	10	11	12	4	5	7	6	Lf
Concentration %	32	10	28	10	5	10	3	1	traces
Concentration g/L	12-15	3-4	9-11	3-4	1-1.5	3-4	0.6-1.0	0.1-0.4	0.009
Size (kD)	23.6	25.2	24.0	19.0	14.2	18.3	150	66.3	80
Residues/molecule									
Amino acids (AA)	199	207	209	169	123	162	-	583	689
Proline	17	10	35	20	2	8	-	28	30
Cysteine	0	2	0	2	8	5	-	35	34
Disulphide bridges	0	1	0	1	4	2	-	17	17
Phosphate	8	11	5	1	0	0	-	0	0
Isoionic point (pI)	4.9-5	5.2-5.4	5.1-5.4	5.4-5.6	4.8	5.3	-	4.9-5	8.7

[1] according to the WHO-IUIS allergen nomenclature sub-committee.

The different caseins associate with each other in solution to form aggregates termed micelles, where the calcium-sensitive α_{S1}-, α_{S2}- and β-CN are stabilized within the micelle by κ-CN, which is located on the surface. These complexes bind essential minerals such as calcium phosphate (Fox & McSweeney, 1998; Wal, 2002).

Among the 4 caseins, α_{S1}-CN has been identified as the most immunogenic protein inducing strong immediate or delayed allergic reactions, and many subjects with CMA show a high titer of IgE specific for this protein (Shek et al., 2005; Ruiter et al., 2006, Gaudin et al., 2008; Schulmeister et al., 2009).

1.3.2. Whey proteins

Whey proteins represent 20% of total milk protein with β-LG and α-LA as the main components followed by the minor constituents BSA, Igs, LF, and the proteose-peptone fraction (Table 2) (Farrell et al., 2004).

The 2 major whey proteins and allergens, β-LG and α-LA, are well characterized and localized on chromosome 11 and 5, respectively (Hayes et al., 1993; Hayes & Petit, 1993). In contrast to the caseins, typical features are solubility at pH 4.6 and heat instability, as well as a hydrophilic surface with a homogeneous distribution of hydrophobic and hydrophilic side chains. Because of the low content of proline, β-LG and α-LA are highly structured proteins and show stable secondary, tertiary, and in most cases, quaternary structures. They have no phosphorylated AA but are rich in sulfur, which mainly occurs in form of cysteine and

methonine. The high content in cysteine allows the formation of disulfide bridges that contribute to the compact and stable structure of these proteins and enable the intermolecular formation of complexes (Fox & McSweeney, 1998; Fox, 2003).

Due to the fact that β-LG is not found in human milk and is relatively stable to digestion, it has long been considered as the major allergen in cow milk. A high degree of homology between the AA sequences of α-LA from cow and human exists being 74% identical and another 6% similar (Wal, 2002). Nevertheless, frequencies in IgE recognition of up to 75.8% are found for this protein and recent data suggest that α-LA is more important in allergenicity than β-LG (Hochwallner et al., 2010a).

1.3.3. Genetic polymorphisms of milk proteins

Within the 6 main milk proteins a marked heterogeneity exists, which arises from genetic polymorphisms or other factors such as the variability in the degree of phosphorylation and glycosylation and the presence of disulfide bonds (Fox & McSweeney, 1998). Based on these heterogeneities, differences in the molecular weight, electrical charges, and hydrophobicity of the proteins occur (Farrell et al., 2004). The genetic polymorphism of milk proteins is a consequence of either substitutions of AA or deletions of a certain AA sequence along the peptide chain and result in several variants for each protein. These protein variants are controlled by autosomal codominant genes and are inherited in a mendelian manner leading to different genotypes in milk (Ng-Kwai-Hang et al., 1984). Over the last years, a total of 54 genetic variants could be demonstrated in the 6 main milk proteins in *Bos genus* at protein and DNA level by different electrophoretic [Isoelectric focusing (IEF), Polyacrylamide gel electrophoresis] and DNA based [Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP), sequencing, allele-specific PCR, single strand conformation polymporphism] techniques (Prinzenberg et al., 1996, 1999; Barroso et al., 1999; Caroli et al., 2009). Recently, 5 new casein DNA sequence variants with a predicted effect on the protein sequence and with the designations α_{S1}-CN J, α_{S2}-CN E, and β-CN J, K, and L were discovered especially in *Bos indicus* breeds by direct sequencing on DNA-level (Gallinat et al., 2013). However, investigations on the expression of these 5 new variants on protein level still remain to be carried out. By using DNA based techniques, protein variants can be identified, which are not detectable at protein level with IEF because the AA exchange does

not alter the isoelectric point (silent protein variant) or the nucleotide substitutions within the codon do not modify the correspondent AA (synonymous variants) (Caroli et al., 2009).

1.3.3.1. Casein polymorphisms

1.3.3.1.1. α_{S1}-casein

α_{S1}-CN represents up to 40% of the casein fraction in bovine milk. It exists as one major and one minor form, which share the same AA sequence but differ in their degree of phosphorylation (8-9). The reference protein α_{S1}-CN B is a single-chain protein consisting of 199 AA with 8 phosphorylated serine residues and no cysteine (Table 2). Four of the 8 phosphorylated serine residues are arranged with glutamyl residues as an anionic cluster at position AA 63-70 (Farrell et al., 2004).

So far, 10 genetic variants have been identified on protein and DNA level (Table 3) (Caroli et al., 2009; Gallinat et al., 2013).

Table 3. Characterization and distribution of the α_{S1}-CN variants[1] (adapted from Caroli et al., 2009; Gallinat et al., 2013).

α_{S1}-CN variant	**Variation with respect to α_{S1}-CN B**	**Diffusion**	**Species[2]**	**Breed**
A	Deletion AA 14-26	Rather common	T	Holstein-Friesian, Red Danish, German Red, Kostroma, other Friesian
B	Reference protein	Most common	T, I, G	All breeds
C	192 Glu → Gly	Common	All	All breeds, very common in *Bos indicus* breeds
D	53 Ala → ThrP	Rather common	T	Flamande, Red Danish, Red Polish, Jersey, Italian Brown, German Holstein, etc.
E	59 Gln → Lys 192 Glu → Gly	Common	G	
F	66 SerP → Leu	Rare	T	German Black and White, Italian Brown, Ayrshire, etc.
G	Lower protein expression	Rather common	T	Italian Brown, Podolian, other Italian breeds, Carora
H	Deletion 51-58	Rare	T	Kuri
I	84 Glu → Asp	Rather common	T, I	Carora, Turkish Red Steppe, Banyo Gudali
J	167 Val → Phe[3]	Rather common	T, I	Sarabi, Gir

[1]The reference protein is shown in bold.

[2]T = *Bos taurus*; I = *Bos indicus*; G = *Bos grunniens*

[3]Predicted effect on protein sequence.

The reference protein α_{S1}-CN B is the most common variant in *Bos taurus* cattle. The α_{S1}-CN variants C, D, E, F, I, and J are characterized by single AA substitutions, whereas in α_{S1}-CN A and H deletions occur. A special case is given with α_{S1}-CN G as the mature protein is not different from the B variant but it is synthesized with a lower amount due to an insertion of 371 bp in the 19th exon (Rando et al., 1998).

1.3.3.1.2. α_{S2}-casein

α_{S2}-CN accounts for 10% of the casein fraction in bovine milk and consists of 2 major and several minor forms. These components show varying levels of post-translational phosphorylations (10-13) and minor degrees of intermolecular disulfide bonding. The reference protein α_{S2}-CN A is comprised of 207 AA and contains 11 phosphorylated serine residues as well as 2 cysteins (Table 2) at positions AA 36 and 40, which form an intramolecular disulfide bond. Due to 3 anionic clusters of phosphoseryl and glutamyl residues at positions AA 8-12, AA 56-61, and AA 129-133, α_{S2}-CN is the most hydrophilic protein among the caseins (Farrell et al., 2004).

Within α_{S2}-CN, the 5 genetic variants A, B, C, D, and E have been described on protein and DNA level. Variants A, B, C, and E differ from each other by single AA substitutions and variant D by a deletion of 9 AA (Table 4) (Caroli et al., 2009; Gallinat et al., 2013).

Table 4. Characterization and distribution of the α_{S2}-CN variants[1] (adapted from Caroli et al., 2009; Gallinat et al., 2013).

α_{S2}-CN variant	**Variation with respect to α_{S2}-CN A**	**Diffusion**	**Species[2]**	**Breed**
A	Reference protein	Most common	All	All breeds
B	8 SerP → Phe	Common	I, T, G	All *Bos indicus* breeds, Turkish Gray Steppe, Namchi, Anatolian Black, Pinzgauer, etc.
C	33 Glu → Gly 47 Ala → Thr 130 Thr → Ile	Rare	G	Mongolia breeds
D	Deletion AA 51-59	Rather common	T	Montbéliarde, Vosgienne, German Yellow and Simmental, Menorquina, Ayrshire
E	7 Val → Ile[3]	Rare	I	Sarabi

[1]The reference protein is shown in bold.

[2]T = *Bos taurus*; I = *Bos indicus*; G = *Bos grunniens*

[3]Predicted effect on protein sequence.

1.3.3.1.3. β-casein

Up to 45% of the casein fraction in bovine milk is assigned to β-CN (Farrell et al., 2004). The most common variant used as reference protein is β-CN A[2]. It is composed of 207 AA with no cysteine and 5 phosphoseryl residues (Table 2), of which 4 are clustered with glutamyl residues at position AA 14-21. Due to the large hydrophobic C-terminal sequence, β-CN is the most hydrophobic of the caseins. The action of the native milk protease plasmin at 3 different positions gives rise to the formation of γ_1-, γ_2-, and γ_3-CN, as well as the complementary proteose peptones (Farrell et al., 2004).

Table 5. Characterization and distribution of the β-CN variants[1] (adapted from Caroli et al., 2009; Gallinat et al., 2013).

β-CN variant	Variation with respect to β-CN A[2]	Diffusion	Species[2]	Breed
A[1]	67 Pro → His	Common	T, I	Most breeds
A[2]	Reference protein	Most common	All	All breeds
A[3]	106 His → Gln	Rather common	T, I	Holstein Friesian, Jersey, Simmental, Sahiwal, German Red Pied, etc.
B	67 Pro → His 122 Ser → Arg	Common	T, I	Most *taurus* breeds, Hariana, Choa
C	35 SerP → Ser 37 Glu → Lys 67 Pro → His	Rather common	T	Guernsey, Reggiana, Pinzgauer, Italian Brown, Piemontese, etc.
D	18 SerP → Lys	Rare	I	Indian Deshi, East African Boran
E	36 Glu → Lys	Rare	T	Piemontese
F	67 Pro → His 152 Pro → Leu	Rare	T	Meuse-Rhine-Yssel
G	67 Pro → His 137-138[3] Pro → Leu	Rare	T	Holstein-Friesian
H[1]	25 Arg → Cys 67 Pro → His 88 Leu → Ile	Rare	T	Korean cattle
H[2]	72 Gln → Glu 93 Met → Leu	Rare	T	Normande
I	93 Met → Leu	Rather common	T	Italian Red Pied, Italian Holstein, German Holstein, Belgian Blue, Jersey, etc.
J	20 Glu → Lys[4]	Rare	I	Golpayegani, Sistani
K	179 Pro → Ala[4]	Rather common	I	Gir
L	197 Val → Ala[4]	Rare	I	Gir

[1]The reference protein is shown in bold.

[2]T = *Bos taurus*; I = *Bos indicus*; G = *Bos grunniens*

[3]Depending on the sequences, the Pro-Leu inversion at position 137 or 138 is controversial.

[4]Predicted effect on the protein sequence.

For β-CN, the 15 genetic variants A^1, A^2, A^3, B, C, D, E, F, G, H^1, H^2, I, J, K, and L have been reported, which are all based on AA substitutions (Table 5) (Caroli et al., 2009; Gallinat et al., 2013). Variant I represents a silent protein variant because the exchanged AA are both neutral and do not modify the electric charge or the isoelectric point. In consequence, it is not identifiable at protein level by IEF (Jann et al., 2002).

1.3.3.1.4. κ-casein

κ-CN accounts for 10% of the casein fraction in bovine milk and comprise a major carbohydrate-free component and a minimum of 6 minor components showing varying degrees of phosphorylation and glycosylation. The major carbohydrate-free component κ-CN A is considered as the reference protein. It is characterized by 169 AA, 1 phosphorylated serine residue, and 2 cysteins (Table 2) at positions AA 11 and 88, which enable the formation of inter- and intramolecular disulfide bonds leading to several polymeric forms (Farrell et al., 2004). On the basis of these disulfide bonds, κ-CN can participate in thiol-disulfid bond interchange reactions with the heat-denaturable whey proteins upon heat treatment (Lowe et al., 2004). κ-CN is the only casein that possesses solely 1 phosphat group (Table 2) and, thus, no anionic cluster of phosphoseryl residues occurs in this protein. Therefore, it is soluble in the presence of calcium and in contrast to the other caseins not calcium sensitive (Swaisgood, 1993; Farrell et al., 2004). κ-CN is the specific substrate of chymosin, which cleavages the peptide bound between Phe (105) and Met (106) leading to the hydrophobic para-κ-CN (AA 1-105) and the highly charged and glycosylated caseinomacropeptide (AA 106-171). This cleavage initiates the clotting of milk, the first step of cheesemaking (Fox & McSweeney, 1998; Caroli et al., 2009).

A total of 14 genetic variants of κ-CN (A, A^1, B, B^2, C, D, E, F^1, F^2, G^1, G^2, H, I, and J) are known (Table 6) (Caroli et al., 2009). A silent protein variant is given with κ-CN H. The AA substitution has no influence on the isoelectric point of the protein as the involved AA are both neutral. κ-CN A^1 is a synonymous variant meaning that the nucleotide substitution within the codon does not alter the corresponding AA. Thus, κ-CN A^1 share the same protein sequence as the reference protein κ-CN A. κ-CN A^1 and H cannot be demonstrated at protein level by IEF and, therefore, typing at DNA level is required for their detection (Prinzenberg et al., 1999; Caroli et al. 2009).

Table 6. Characterization and distribution of the κ-CN variants[1] (adapted from Caroli et al., 2009).

κ-CN variant	Variation with respect to κ-CN A	Diffusion	Species[2]	Breed
A	Reference protein	Most common	T, I	All breeds
A[1]	Codon CCA → CCG 150 Pro → Pro Synonymous variant	Rather common[3]	I, T T x I[4]	Boran, Brahman, Nelore, Santa Gertrudis, etc.
B	136 Thr → Ile 148 Asp → Ala	Most common	I, T	All breeds
B[2]	136 Thr → Ile 148 Asp → Ala 153 Ile → Thr	Rare	T	Russian cattle
C	97 Arg → His 136 Thr → Ile 148 Asp → Ala	Rather common	T	Grey Alpine, Italian Brown, German Simmental, Menorquina, etc.
D	97 Arg → His	Rare	T	Italian Brown
E	155 Ser → Gly	Rather common	T	Angler, German Black and White, Holstein-Friesian, Ayrshire
F[1]	148 Asp → Val	Rare	T	Yakut
F[2]	10 Arg → His 136 Thr → Ile 148 Asp → Ala	Rare	T	Finnish Ayrshire
G[1]	97 Arg → Cys 136 Thr → Ile 148 Asp → Ala	Rare	T	Pinzgauer
G[2]	148 Asp → Ala	Common	G	
H	135 Thr → Ile	Common[3]	I, T T x I[4]	Madagascar zebu, White Fulani, Wadara, Red Bororo, N´Dama, Pinzgauer
I	104 Ser → Ala	Rare	T x I[4]	Namibia
J	136 Thr → Ile 148 Asp → Ala 155 Ser → Arg	Rare	T	Ivory Coast, Burkina Faso

[1]The reference protein is shown in bold.
[2]T = *Bos taurus*; I = *Bos indicus*; G = *Bos grunniens*
[3]Mainly in *Bos indicus* breeds.
[4]T x I = *Bos taurus* and *Bos indicus* crossings.

1.3.3.2. Whey protein polymorphisms

1.3.3.2.1. α-lactalbumin

α-LA is a monomeric globular calcium binding protein and represents 20% of total whey protein in bovine milk. It plays an important role in the biosynthesis of lactose as it is a regulatory component of the lactose-synthetase complex. α-LA modifies the substrate specificity of galactosyltransferase to form lactose from glucose and UDP-galactose (Fox, 2003).

The reference protein α-LA B is made of 123 AA with a very high content of essential AA accounting for 63.2% of total AA in this protein (Farrell et al., 2004). α-LA possesses 8 cysteine residues allowing the formation of 4 disulfide bonds (Table 2) at positions AA 6 and 120, AA 61 and 77, AA 73 and 91, as well as AA 28 and 111, which stabilize the structure of the protein. Native α-LA consists of a large α-helical domain and a small β-sheet domain. Both domains are connected by a calcium binding loop that contains a single strong calcium binding site to which also magnesium, manganese, sodium or potassium attaches. The binding of these cations increases the stability of α-LA against heat or several denaturing agents (Permyakov & Berliner, 2000).

As shown in Table 7, 4 α-LA variants (A, B, C, and D) have been demonstrated (Caroli et al., 2009; Visker et al., 2012).

Table 7. Characterization and distribution of the α-LA variants[1] (adapted from Caroli et al., 2009; Visker et al., 2012).

α-LA variant	**Variation with respect to α-LA B**	**Diffusion**	**Species[2]**	**Breed**
A	10 Arg → Gln	Rather common	I, T	All *indicus* and some *taurus* breeds
B	Reference protein	Most common		All breeds
C	Asp → Asn Position unknown	Rare	J	Australia breeds
D	65 Gln → His	Rare	T	Holstein-Friesian

[1]The reference protein is shown in bold.

[2]T = *Bos taurus*; I = *Bos indicus*; G = *Bos grunniens*; J = *Bos javanicus*

1.3.3.2.2. β-lactoglobulin

β-LG is a dimeric protein and amounts for 65% of total whey protein in bovine milk. The reference protein β-LG B is composed of 162 AA and contains 5 cysteine residues (Table 2). Two disulfide bonds at positions AA 66 and 160 and AA 106 and 119, as well as 1 free thiol group at position AA 121 are present in this protein. Due to the reactive free thiol group, interactions and complex formation with k-CN during heat treatment occur (Sawyer, 1969; Lowe et al., 2004). β-LG belongs to the lipocalin family that bind and carry a variety of hydrophobic ligands such as retinol. All members of this family show a common tertiary structure characterized by a single 8-stranded antiparallel β-barrel with an internal ligand-binding site (Zsila et al., 2002). The structure of β-LG consist of 9 β-strands (A-I) and 1 major

α-helix at the C-terminal end of the molecule. The 8 β-strands A-H form a β-barrel, which encloses a hydrophobic cavity where retinol or fatty acids bind (Sakai et al., 2000).
Several AA substitutions give rise to the existence of the 11 genetic variants A, B, C, D, E, F, G, H, I, J, and W (Table 8) (Caroli et al., 2009).

Table 8. Characterization and distribution of the β-LG variants[1] (adapted from Caroli et al., 2009).

β-LG variant	Variation with respect to β-LG	Diffusion	Species[2]	Breed
A	64 Gly → Asp 118 Ala → Val	Most common	T, I, G	All breeds
B	Reference protein	Most common	T, I, G	All breeds
C	59 Gln → His	Rather common	T	Jersey
D	45 Glu → Gln	Rather common	T	Montbéliarde, German Holstein, German Simmental, etc.
E	158 Glu → Gly	Most common	G, J	Nepal *grunniens*, Australia *javanicus*
F	50 Pro → Ser 129 Asp → Tyr 158 Glu → Gly	Rare	J	Australia *javanicus*
G	78 Ile → Met 158 Glu → Gly	Rare	J	Australia *javanicus*
H	64 Gly → Asp 70 Lys → Asn 118 Ala → Val	Rare	T	Italian Friesian
I	108 Glu → Gly	Rare	T	Polish Red
J	126 Pro → Leu	Rare	T	Hungarian Grey
W	56 Ile → Leu	Rare	T	Murnau-Werdenfelser, Jersey, Red Holstein x Simmental

[1]The reference protein is shown in bold.
[2]T = *Bos taurus*; I = *Bos indicus*; G = *Bos grunniens*; J = *Bos javanicus*

1.3.3.3. Importance of milk protein polymorphisms

The importance of genetic polymorphisms in animal breeding is due to their associations to milk composition, productions traits, and technological properties of milk (Bovenhuis et al., 1992; Martin et al., 2002; Heck et al., 2009). A greater concentration of β-LG is given in milk with the AA genotype than with AB or BB but associated with lower casein content (Lundén et al., 1997; Heck et al., 2009). Milk containing the κ-CN genotype BB shows a higher concentration of κ-CN, improved milk clotting properties, and a higher cheese yield, whereas unfavorable effects on cheese making have been reported for the variants E and G of κ-CN (Wedholm et al., 2006; Caroli et al., 2009). Genetic polymorphisms of milk proteins are used in phylogenetic studies by investigating the evolution of animal resources and milk protein

genes (Jann et al., 2004; Ibeagha-Awemu et al., 2007). In addition, milk protein polymorphisms enable the species identification in milk and dairy products. Thus, admixtures of milk from different species in products of protected designation of origin such as mozzarella cheese from buffalo and feta cheese from sheep can be detected (Otaviano et al., 2008; Reale et al., 2008). Furthermore, milk protein polymorphisms play a notable role in human nutrition because they influence the nutritional value of milk and milk products determined by bioactive peptides that manifest a wide range of effects including antihypertensive, opioid, dipeptidyl peptidase-4 inhibitory, and mineral-binding activities. Therefore, they are considered as ingredients of functional foods, dietary supplements or nutraceuticals (Kamiński et al., 2007; Weimann et al., 2009; Tulipano et al., 2010, 2011).

1.3.4. Allergenic structures in milk proteins

Milk allergy is caused by an accumulation of potential antigenic sites or specific areas (molecular sections) within the protein, against which the immune system forms antibodies. These AA sequences within the allergenic proteins that are bound by IgE antibodies are termed IgE-binding epitopes. There are 2 general categories of IgE-binding epitopes, linear (sequential) and conformational (Figure 4). Linear epitopes are formed by a sequence of contiguous AA on the peptide chain of the protein. They refer to the primary structure of the protein. In contrast, in conformational epitopes, the AA are from different regions of the protein and must be brought together by folding of the polypeptide chain. This is the reason why their allergenicity is associated with the secondary and tertiary structure of the protein (Lin & Sampson, 2009; Nowak-Wegrzyn & Fiocchi, 2009).

Linear epitopes are prevalent and more important in food allergens as food proteins usually undergo several processes such as cooking and digestion. Linear epitopes are mainly located in the hydrophobic parts of the protein, buried within the tertiary structure and, therefore, not accessible for the IgE antibodies in the native form of the protein. During food processing and cooking, leading to heat denaturation and alteration of the native structure, as well as during digestion in the gastrointestinal tract, resulting in further modification and breakup of the tertiary structure, the conformational epitopes are destroyed and the linear epitopes are exposed and presented to the immune system (Figure 4) (Hartmann et al., 2007; Lin & Sampson, 2009).

The IgE-binding epitopes in the caseins are generally suggested to be linear. This is due to the fact that caseins are not significantly affected by heating, lack on stable and defined secondary and tertiary structures, and are rapidly and extensively degraded by proteolytic enzymes during digestion (Figure 4) (Vila et al., 2001; Wal, 2002). Nevertheless, α_{S1}-CN was found to contain both linear and conformational epitopes (Schulmeister et al., 2009). In the whey proteins β-LG and α-LA, mainly conformational but also linear epitopes are relevant for their allergenicity as they are characterized by stabile secondary and tertiary structures, a higher resistance to proteolytic enzymes, and instability against heat (Figure 4) (Järvinen et al., 2001, 2002; Wal, 2002; Hochwallner et al., 2010b).

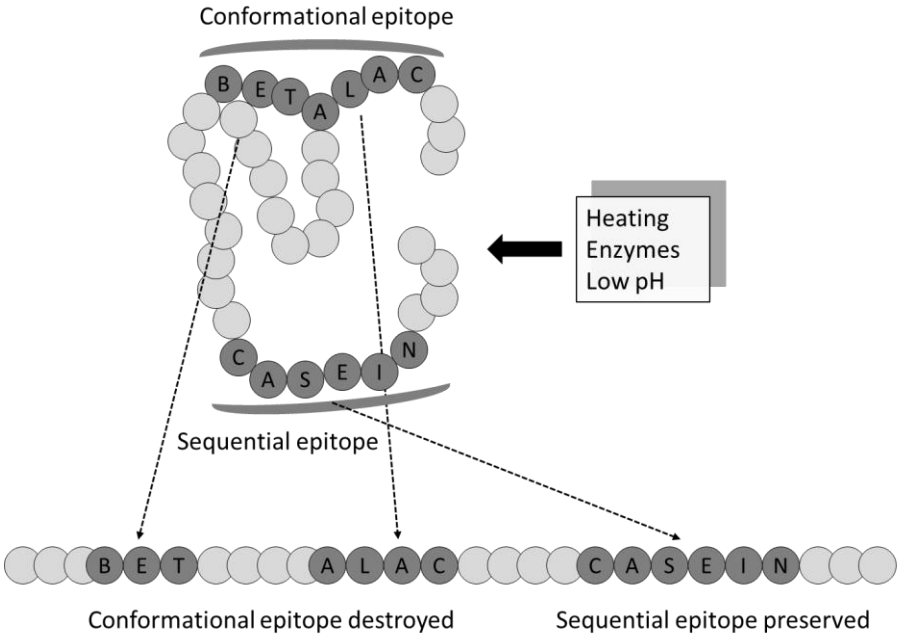

Figure 4. Conformational and linear (sequential) epitopes in milk proteins. Conformational epitopes are altered or destroyed by food processing such as heat treatment, enzymatic digestion, or low pH, while linear epitopes are generally not affected. ALAC, α-lactalbumin; BETALAC, β-lactoglobulin (adapted from Nowak-Wegrzyn & Fiocchi, 2009).

Linear eptiopes have some prognostic value in the natural history of CMA as associations between linear epitopes and persistent CMA have been demonstrated. Patients with persistent CMA were found to have higher levels of IgE antibodies against linear epitopes as opposed to conformational epitopes of milk proteins (Vila et al., 2001). In addition, IgE recognition of certain linear epitopes of caseins dominated in patients with persistent CMA (Vila et al., 2001; Järvinen et al., 2002). Furthermore, patients with persistent CMA show an

increased epitope diversity and higher affinity of IgE binding than those with transient CMA (Wang et al., 2010).

The linear IgE-binding epitopes of the caseins and whey proteins have been identified by several studies using SPOT membrane and microarray immunoassays (Chatchatee et al., 2001a,b; Järvinen et al., 2001; Busse et al., 2002; Cerecedo et al., 2008; Lin et al., 2009; Hochwallner et al., 2010b). The results obtained from these 2 systems are mostly quite consistent (Table 9).

Table 9. Linear IgE-binding epitopes of the caseins and whey proteins identified by SPOT membrane or microarray immunoassay.

Protein	IgE-binding epitopes (AA position)	Technique	Reference
α_{S1}-CN	17-36, 39-48, 69-78, 83-102, 109-120, 123-132, 139-154, 159-174, 173-194*	SPOT membrane and Microarray	Chatchatee et al. (2001a)
	28-50	Microarray	Cerecedo et al. (2008)
	49-62	Microarray	Lin et al. (2009)
	1-31, 65-100, 134-167	Microarray	Schulmeister et al. (2009)
α_{S2}-CN	31-44, 43-56, 83-100, 93-108, 105-114, 117-128, 143-158, 157-172, 165-188, 191-200*	SPOT membrane and Microarray	Busse et al. (2002)
	1-20, 13-32, 67-86, 181-207	Microarray	Cerecedo et al. (2008)
	69-77	Microarray	Lin et al. (2009)
β-CN	1-16, 45-54, 55-70, 83-92, 107-120, 135-144, 149-164, 167-184, 185-208*	SPOT membrane and Microarray	Chatchatee et al. (2001b)
	25-50, 52-74, 154-173	Microarray	Cerecedo et al. (2008)
	16-39	Microarray	Lin et al. (2009)
κ-CN	9-26, 21-44, 47-68, 67-78, 95-116, 111-126, 137-148, 149-166*	SPOT membrane and Microarray	Chatchatee et al. (2001a)
	34-53	Microarray	Cerecedo et al. (2008)
α-LA	1-16, 13-26, 47-58, 93-102	SPOT membrane	Järvinen et al. (2001)
	1-19, 15-34, 105-123, 45-64, 60-79, 90-109	Microarray	Hochwallner et al. (2010b)
β-LG	1-16, 31-48, 47-60, 67-78, 75-86, 127-144, 141-152*	SPOT membrane and Microarray	Järvinen et al. (2001)
	58-77	Microarray	Cerecedo et al. (2008)
	106-119	Microarray	Lin et al. (2009)

*Peptides with AA sequences of at least 90% overlapped with the epitopes identified by Lin et al. (2009) using microarray immunoassay.

The microarray technology provides many advantages (e.g. assay thousands of target peptides in parallel, low volume of serum, robust replication, statistical analysis, and simultaneously testing of several immunoglobulin subclasses) and is nowadays mainly applied in epitope profiling of food allergens (Lin et al., 2009; Lin & Sampson, 2009).

1.3.5. Allergy to other mammalian milk

In the recent years, there has been an increasing interest in the search for an alternative protein source for CMA patients with the focus on the use of proteins from non-bovine milk. The mammals cow, water buffalo, goat, sheep, pig, camel, horse, donkey, and human are phylogenetically either close or far related (Figure 5).

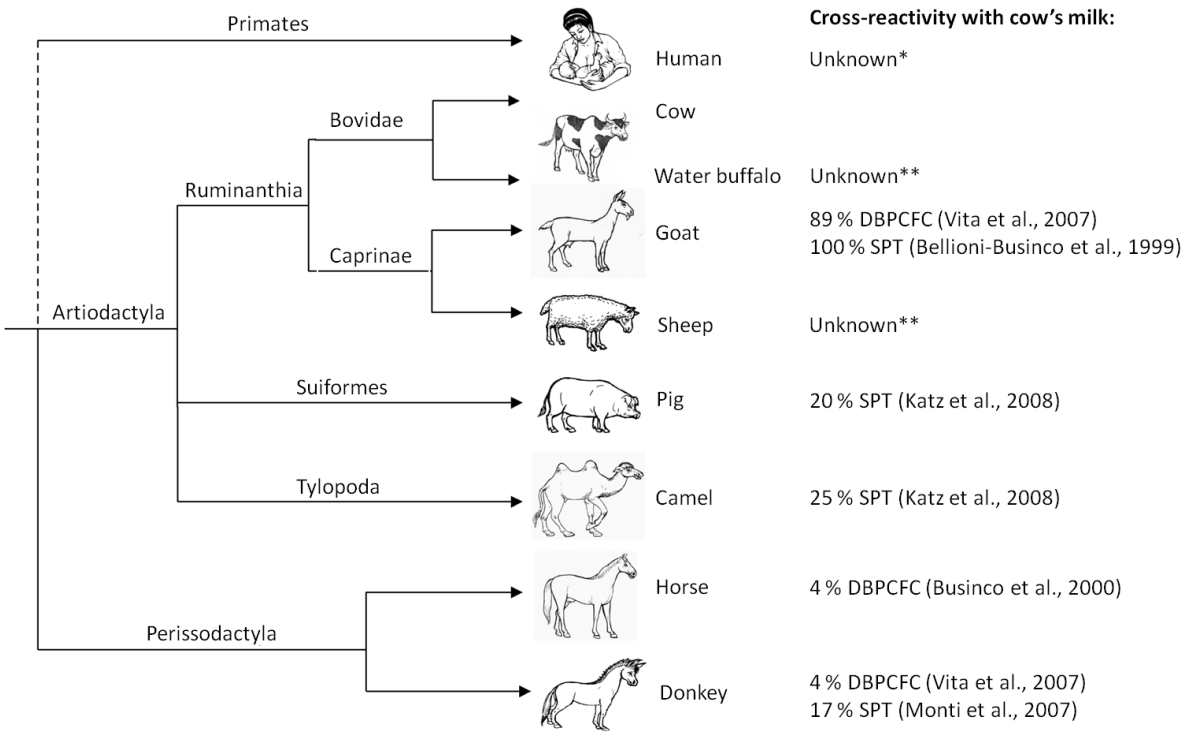

Figure 5. Phylogenetic relationship and clinical cross-reactivity between milk from cow and other mammalian species. DBPCFC, double-blind, placebo-controlled food challenge; SPT, skin prick test. *clinical cross-reactivity suggested; **no clinical studies available (adapted from Järvinen & Chatchatee, 2009).

Thus, they are characterized by a quite similar milk protein expression, although with some differences in the protein composition and distribution (Table 10). Based on the high homology of the milk proteins, cross-reactivity between the proteins from different mammals occurs. The milk proteins of cow and buffalo represent the highest homologies with 96.1 %, followed by cow and sheep with 91.1%, as well as cow and goat with 87.6%. Lower sequence similarities are found between the proteins of cow and pig (64.2%), horse (62.4%), donkey (62.8%), dromedary (60%), as well as humans (58.4%) (Järvinen & Chatchatee, 2009; Restani et al., 2009).

Goat milk has been proposed as an alternative to cow milk because of the lower quantity of α-CN. However, several studies demonstrated that most patients with CMA are also

sensitized to goat milk (Bellioni-Businco et al., 1999; Restani, 2004; Vita et al., 2007). Clinical studies on the allergenicity to sheep and buffalo milk in patients with CMA are lacking, probably due to the high homology with goat, respectively, cow milk making a better tolerance to sheep or buffalo milk unlikely. Moreover, a number of case reports indicated selective allergies to goat, sheep, buffalo, or ewe milk or to all but without any reactivity to cow milk (Restani et al., 1999; Ah-Leung et al., 2006; Broekaert et al., 2008; Mori et al., 2013).

Table 10. Mammalian taxonomy and homolgoy between each cow milk protein and the milk proteins from other animal species (adapted from Restani et al., 2009).

	Cow	**Water buffalo**	**Sheep**	**Goat**	**Pig**	**Dromedary**	**Horse**	**Donkey**	**Human**
Family	Bovidae	Bovidae	Bovidae	Bovidae	Suidae	Camelidae	Equidae	Equidae	Primates
Genus	*Bos*	*Bubalus*	*Ovis*	*Capra*	*Sus*	*Camelus*	*Equus*	*Equus*	*Homo*
Species	B. domesticus	B. bubalis	O. aries	C. aegagrus	S. domestica	C. dromedarius	E. f. caballus	E. asinus	H. sapiens
Protein (g%)	3.3	4.5	4.9	4.3	4.8	3.6	2.14	2.2	1.25
Caseins (%)	80	82	84	84	58	74	56	58	40
Whey proteins (%)	20	18	16	16	42	26	44	42	60
Homology									
α_{S1}-casein	100	95.3	88.3	87.9	47.2	44.2	43.3	-[1]	31.9
α_{S2}-casein	100	95.0	89.2	88.3	62.8	58.3	-[1]	60.0	-[1]
β-casein	100	97.8	92.0	91.1	67.0	69.2	60.5	-[1]	56.5
κ-casein	100	92.6	84.9	84.9	54.3	58.4	57.4	-[1]	53.2
α-lactalbumin	100	99.3	97.2	95.1	74.6	69.7	72.4 (A) 69.1 (B/C)	71.5	73.9
β-lactoglobulin	100	96.7	93.9	94.4	63.9	absent	59.4 (1)	56.9 (1) 51.6 (2)	absent
Serum albumin	100	-[1]	92.4	71.2	79.9	-[1]	74.5	74.1	76.6
Average	100	96.1	91.1	87.6	64.2	60.0	62.4	62.8	58.4

[1] - Not known

The protein variants of α-LA and β-LG are shown in parentheses

The greater phylogenetical distance from Bovidae and, in consequence, the lower level of homology between the proteins of cow and horse (62.4%), cow and donkey (62.8%), as well as especially cow and dromedary (60%) offer these milks as a more suitable protein source for CMA subjects. Similar to human milk, the protein content and the ratio of casein to whey protein in milk from Equidae (horse and donkey) is lower than from milk of Bovidae. It has been shown that donkey's milk featured a high clinical tolerability, palatability, and nutritional adequacy in subjects with CMA (Vita et al., 2007; Monti et al., 2012). Furthermore, a high tolerability was also found for mare's milk (Businco et al., 2000). Finally, in common with human milk, camel/dromedary milk is characterized by the absence of β-LG leading to a good tolerance in patients with CMA (Shabo et al., 2005; Restani et al., 2009;

Ehlayel et al., 2011a,b). Nevertheless, due to the more or less similarity in the AA sequence between the proteins of the different ruminants, all these milks may exhibit a certain rate of clinical cross-reactivity and, thus, in general, milk of other animal source cannot be recommended as substitute for cow milk. Instead, the best alternative diet for patients with CMA remains to be defined individually (Restani et al., 2009). In fact, a significant sequence homology between the known IgE-binding epitopes of cow and the corresponding AA sequences of human milk proteins exist. That is why cross-reactivity between conserved regions of bovine and human milk proteins could be identified explaining why exclusively breastfed infants with CMA occasionally continued to manifest symptoms despite strict maternal milk avoidance (Järvinen et al., 2012).

1.4. Aims and design of the study

The current treatment for CMA is the elimination of cow milk proteins and dairy products from the daily nutrition. This is often hardly to achieve because milk proteins play a very important role not only in the production of infant formula but also as processing aids. Therefore, they are widely used in many dairy and food products. Within the caseins and whey proteins, a wide range of genetic variants has been described for cow but also for other species such as sheep, goat, or camel. Nevertheless, their importance regarding human nutrition and in particular the allergenic potential has not yet been adequately exploited.

Epitope mapping studies of α_{S1}-, α_{S2}-, β-, and κ-CN as well as α-LA and β-LG identified a large number of linear IgE-binding epitopes within these proteins. Some of the AA substitutions and deletions characterizing the genetic variants are located within the epitope sequence but the effects on their immunoreactivity are still unknown. Before milk proteins induce an allergenic reaction, several processes such as digestion, absorption, and interaction with the mucosal immune system occur. Despite the caseins are readily and easily digestible proteins, it has been proven that the digestion products of β-CN and β-LG still contain IgE-binding epitopes and, thus, retain an allergenic potential (Dupont et al., 2010a,b; Picariello et al., 2010; Benedé et al., 2014). However, the effect of the genetic polymorphisms on the digestion of casein variants in association with their IgE-binding epitopes has not yet been considered. Therefore, the main focus of this study was the investigation of the influence of the genetic polymorphisms on the digestibility and allergenicity of casein variants, in order

to assess if they should be considered in the search for a safe and alternative protein source for CMA patients.

For analysis of the digestibility, in vitro hydrolysis of several α_{S1}-, α_{S2}-, β-, and κ-CN variants detected after a screening of productive and endangered cattle breeds was compared using a gastrointestinal digestion model system, mimicking the passage of the milk proteins from the stomach into the duodenum, and mass spectrometry to identify differences in the peptide profile between individual casein variants and to determine resistant fragments containing IgE-binding epitopes. In vitro gastrointestinal digestion of the genetic variants α_{S1}-CN B and C as well as α_{S2}-CN A and B is described in **publication 1** and of β-CN A^1, A^2, and B, as well as κ-CN A, B, and E in **publication 2.**

To study the allergenicity of milk protein variants, the influence of the AA substitutions and deletions occurring within IgE-binding epitopes of all known casein variants were analyzed by microarray immunoassays with sera from patients with CMA. Moreover, the IgE-binding properties of the variant-specific peptide products resulting from digestion of the α_{S1}-, α_{S2}-, β-, and κ-CN variants were evaluated. As there is an increasing interest in the use of milk from other species, caseins from goat and water buffalo were also included in the microarray immunoassays to determine variations in the allergenicity between these species. **Publication 3** mainly focuses on the differences in IgE-binding between epitopes of α_{S1}-, α_{S2}-, β-, and κ-CN variants and delineates the allergenic potential of corresponding epitopes in caseins from goat and water buffalo.

2. 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.

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, & Bindslev-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, Chirdo, & 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 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; Cerecedo 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 focusing of milk samples

Milk protein variants of skimmed milk samples and isolated casein fractions from anion-exchange chromatography were determined by isoelectric focusing (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 x *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 x 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 lyophilized 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 x 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 sulfate polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to Laemmli (1970) with 1.5-mm thin polyacrylamide gels (16 x 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 µL) 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 glycodeoxycholic acid, 1.0 M $CaCl_2$ and 0.25 M bis-Tris, pH 6.5. Then, solutions of 0.1% (w/v) porcine trypsin (activity: 13,800 U mg^{-1} of protein using benzoylarginine ethyl ester as substrate, Sigma-Aldrich) and 0.4% (w/v) bovine α-chymotrypsin (activity: 40 U mg^{-1} of 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 Bowmann-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 µL) 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 C_{18} 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).

Table 1
Allele frequencies of α_{S1}- and α_{S2}-casein in different cattle breeds identified by isoelectric focusing.

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

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* 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.

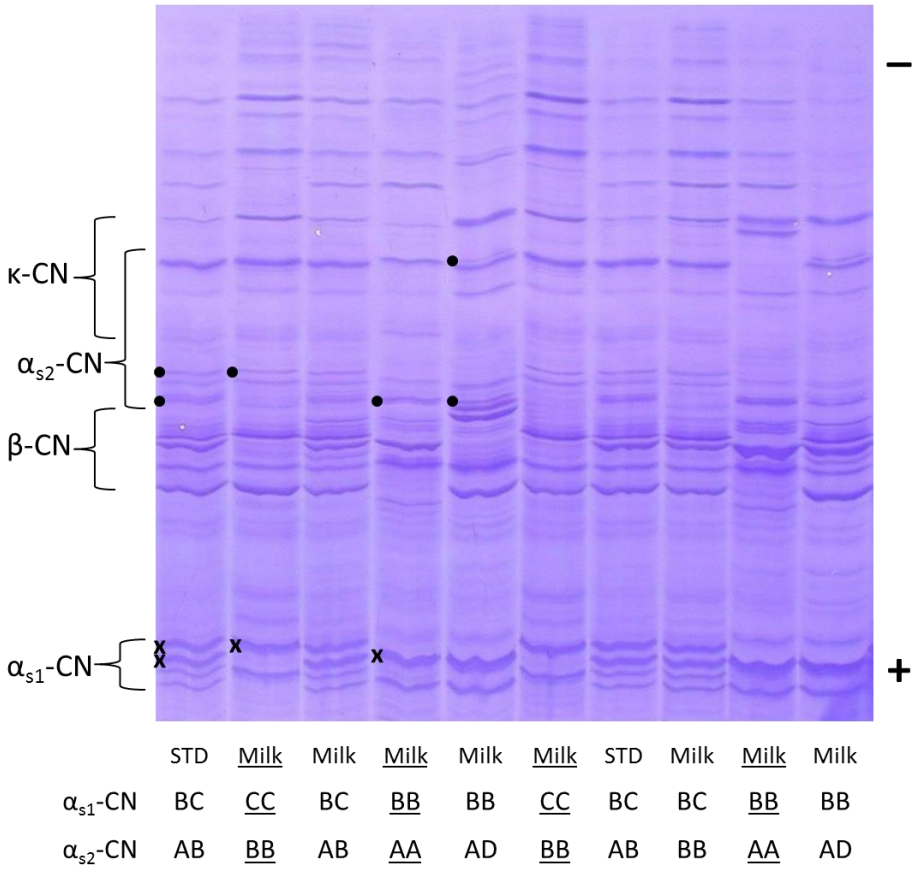

Fig. 1. Isoelectric focusing pattern of milk samples from cows with different α_{S1}- and α_{S2}-casein (CN) genotypes, which are indicated in capitals at the bottom of the illustration. Reference samples (STD) were used for identification of the casein variants. The major bands of the homozygous and 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.

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.

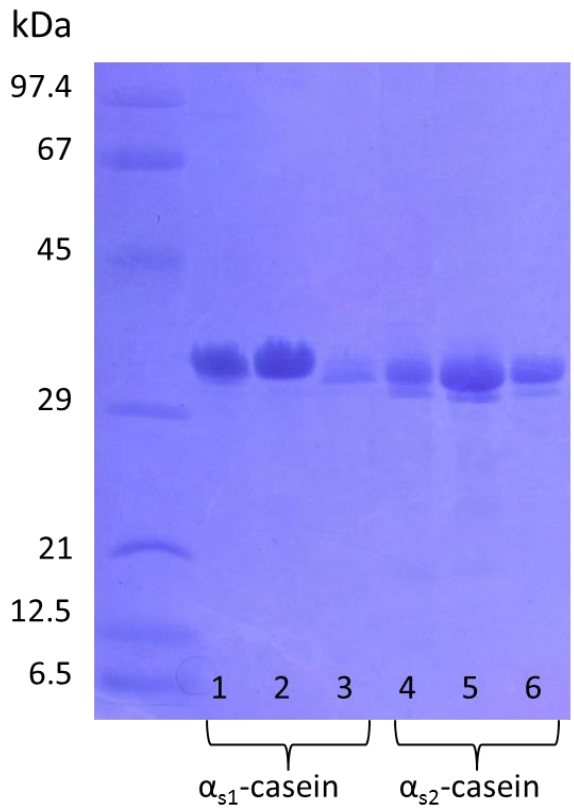

Fig. 2. SDS-PAGE patterns of isolated α_{S1}- and α_{S2}-casein (CN) fractions obtained after anion exchange chromatography: lanes 1 and 2, α_{S1}-CN B; lane 3, α_{S1}-CN C; lane 4, α_{S2}-CN B; lanes 5 and 6, α_{S2}-CN A.

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, Dalgleish, & 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.

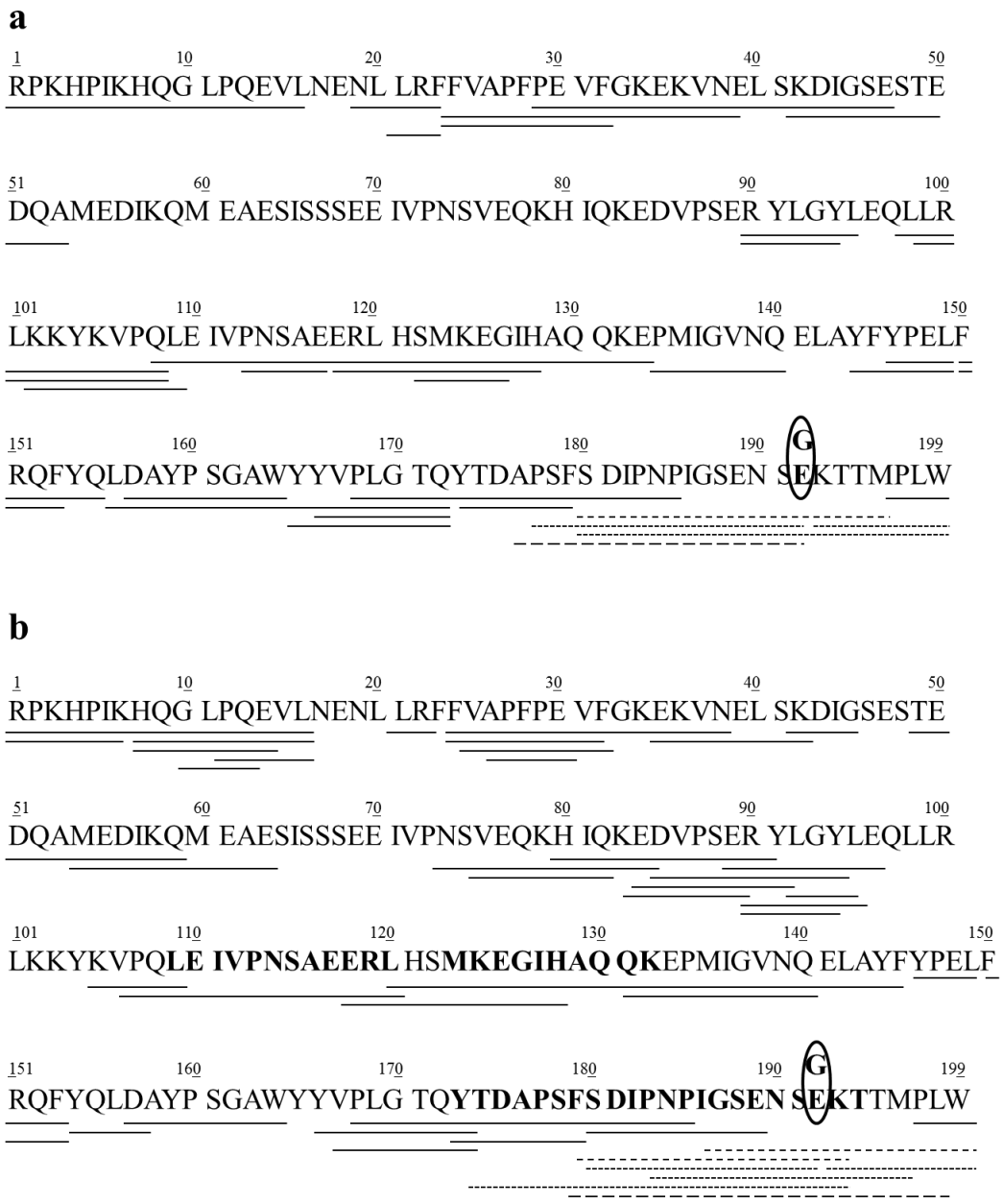

Fig. 3. Peptides identified by MALDI-TOF-MS after (a) gastric and (b) duodenal digestion of α_{S1}-casein (CN) variants B and C. Peptides that were unambiguously identified are represented by black continuous lines. Peptides containing the amino acid substitutions and occurring in all α_{S1}- CN variants 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.

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).

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
Gastric digestion				
876.48	876.47	193-199	(**E**)/^{193}KTTMPLW^{199}	B
1689.79	1689.76	177-192	^{177}PSFSDIPNPIGSENS**E**^{192}	B
1688.78	1688.78	176-192	^{176}APSFSDIPNPIGSENS**G**^{192}	C
1819.83	1819.84	180-196	^{180}SDIPNPIGSENS**E**KTTM^{196}	B
1747.79	1747.83	180-196	^{180}SDIPNPIGSENS**G**KTTM^{196}	C
2216.06	2216.05	180-199	^{180}SDIPNPIGSENS**E**KTTMPLW^{199}	B
Duodenal digestion				
876.48	876.47	193-199	(**E**)/^{193}KTTMPLW^{199}	B
1358.62	1358.61	180-192	^{180}SDIPNPIGSENS**E**^{192}	B
1407.63	1407.64	184-196	^{184}NPIGSENS**E**KTTM^{196}	B
1407.61	1407.66	187-199	^{187}GSENS**G**KTTMPLW^{199}	C
1495.75	1495.63	187-199	^{187}GSENS**E**KTTMPLW^{199}	B
1486.71	1486.70	180-193	^{180}SDIPNPIGSENS**E**K^{193}	B
1414.67	1414.68	180-193	^{180}SDIPNPIGSENS**G**K^{193}	C
2105.01	2104.97	174-193	^{174}TDAPSFSDIPNPIGSENS**E**K^{193}	B
2105.04	2105.02	179-198	^{179}FSDIPNPIGSENS**G**KTTMPL^{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.

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 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).

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.

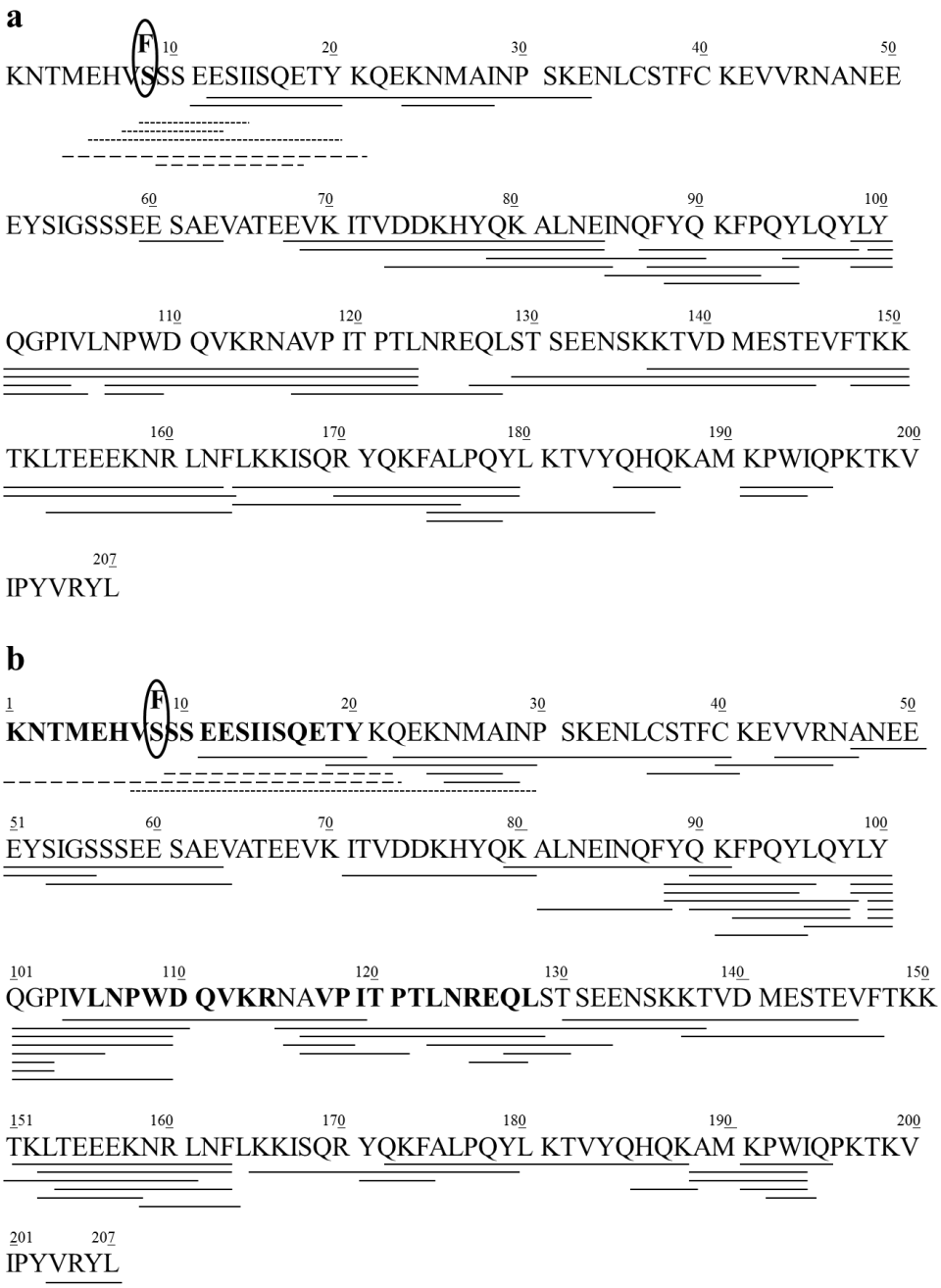

Fig. 4. Peptides identified by MALDI-TOF-MS after (a) gastric and (b) duodenal digestion of α_{S2}-casein (CN) variants A and B. Peptides that were unambiguously identified are represented by black continuous lines. Peptides with amino acid substitutions found only in α_{S2}-CN A are shown with short 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.

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).

Table 3

Identification 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
Gastric digestion				
637.28	637.27	7-12	^{7}V**S**SSEE^{12}	A
738.34	738.32	8-14	^{8}**S**SSEESI^{14}	A
1108.55	1108.51	9-18	(**F**)/^{9}SSEESIISQE^{18}	B
1824.77	1824.81	5-20	^{5}EHV**S**SSEESIISQETY^{20}	A
2143.98	2159.92	4-21	^{4}MEHV**F**SSEESIISQETYK^{21}	B
Duodenal digestion				
1500.71	1500.71	9-21	**(F)**/^{9}SSEESIISQETYK^{21}	B
2615.27	2615.23	1-22	^{1}KNTMEHV**F**SSEESIISQETYKQ^{22}	B
2631.20	2631.25	7-29	^{7}V**S**SSEESIISQETYKQEKNMAIN^{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.

A replacement of the phosphorylated serine by a phenylalanine in variant B played a decisive role concerning the phosphoseryl cluster comprising the specific motif $Ser(P)_3$-Glu_2. 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 phosphoseryl clusters of α_{S1}-CN B and C at position 64-70, since they were present in an intact form. In contrast, the main phosphoseryl 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 oxidized 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 phosphoseryl 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 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 (Swaisgood, 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 phosphoseryl 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 emphasizes 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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3. 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 A^1, A^2, 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 A^1 and A^2, 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 allergenicity. 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 (Skripak 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 phosphoseryl 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 phosphoseryl 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 (Swaisgood, 1993; Farrell et al., 2004). Therefore, they are very susceptible to hydrolysis by all proteases (Astwood et al., 1996; Swaisgood, 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 (A^1, A^2, A^3, B, C, D, E, F, G, H^1, H^2, and I) and 12 κ-CN variants (A, B, C, D, E, F^1, F^2, G^1, G^2, H, I, and J) were identified in the *Bos* genus, 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 A^1, A^2, and B are widely distributed in all breeds, with β-CN A^2 showing the highest frequency. β-Casein A^3, 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 G^2 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 $0_1 0_1$ and 0_1F 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 allergenic 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 (≥95% by thin-layer chromatography), sodium glycodeoxycholate (≥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 carrier 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 A^1, A^2, and B, and κ-CN A, B, and E) were skimmed by centrifugation at 1,620 × *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 × *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 × 2.5 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 m*M* 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 × 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, 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 m*M* Tris, 192 m*M* 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 A^1, A^2, 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 µL) 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 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 µL) 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 A^1, A^2, 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.

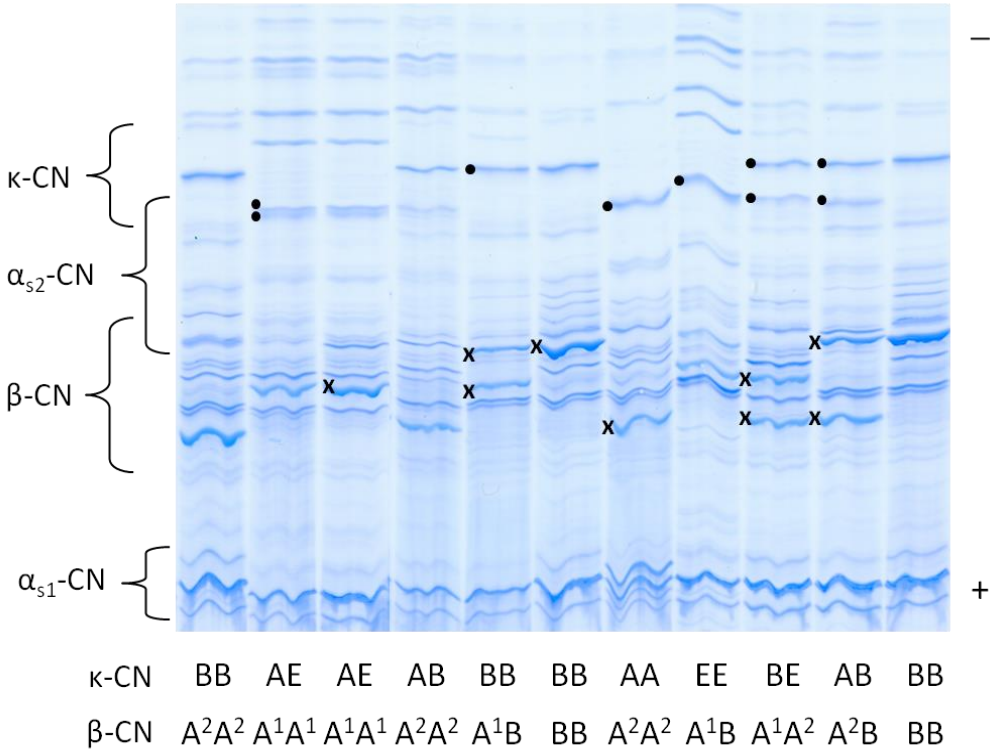

Figure 1. Separation of bovine milk samples with different β- and κ-CN genotypes by isoelectric focusing. The positions of the major bands of the β-CN variants are indicated by an x and the main fractions of the κ-CN variants are marked by a dot.

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 A^1 and A^2 (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 with κ- or α_{S2}-CN. As already assumed by SDS-PAGE,

impurities with κ-CN were measurable in β-CN A^1 and β-CN A^2 (lanes 1 and 4, Figure 2). The intensity coverage of the matched κ-CN peptides in the samples were 15.1% for β-CN A^1 and 6.3% for β-CN A^2. In contrast, β-CN A^1 (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 A^2 and β-CN B (lanes 3 and 5, Figure 2).

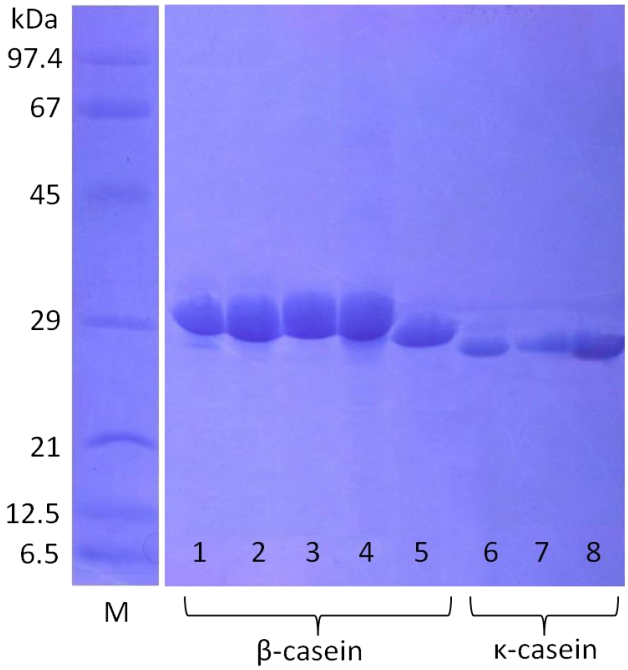

Figure 2. Sodium dodecyl sulfate PAGE analysis of different β- and κ-CN fractions obtained from anion-exchange chromatography. M= molecular weight marker. Lanes 1 and 2 = β-CN A^1; lanes 3 and 4 = β-CN A^2; lane 5 = β-CN B; lane 6 = κ-CN E; lane 7 = κ-CN A; lane 8 = κ-CN B.

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 A^1, A^2, 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 A^1, A^2, 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 A^1 and A^2 by an arginine (R) in β-CN B, has no negative effect on the cleavage of the enzyme. A further substitution distinguishes variant A^1 and B (histidine) from A^2 (proline) at position 67.

Table 1. Identification of peptides with AA substitutions (bold letters) after gastric and duodenal digestion of β-CN A^1, A^2, 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 (phase1)	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}PFTE**R**QSLTL^{127}	B
	2,390.19	2,390.28	59-80	^{59}VYPFPGPI**P**NSLPQNIPPLTQT^{80}	A^2
	2,430.18	2,430.28	59-80	^{59}VYPFPGPI**H**NSLPQNIPPLTQT^{80}	A^1, B
Duodenal digestion (phase 2)	1,100.57	1,100.57	59-68[2]	^{59}VYPFPGPI**P**N^{68}	A^2
	1,140.57	1,140.58	59-68	^{59}VYPFPGPI**H**N^{68}	A^1, B
	1,283.58	1,283.58	114-124[2]	^{114}YPVEPFTE**S**QS^{124}	A^1, A^2
	1,525.77	1,525.81	59-72[2]	^{59}VYPFPGPI**P**NSLPQ^{72}	A^2
	1,565.78	1,565.81	59-72	^{59}VYPFPGPI**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}VYPFPGPI**P**NSLPQNIPPLTQT^{80}	A^2
	2,430.28	2,430.28	59-80	^{59}VYPFPGPI**H**NSLPQNIPPLTQT^{80}	A^1, B
	2,503.36	2,503.36	58-80[2]	^{58}LVYPFPGPI**P**NSLPQNIPPLTQT^{80}	A^2
	2,543.36	2,543.37	58-80	^{58}LVYPFPGPI**H**NSLPQNIPPLTQT^{80}	A^1, B
	2,556.18	2,556.22	108-129	^{108}EMPFPKYPVEPFTE**S**QSLTLTD^{129}	A^1, A^2
	2,556.15	2,556.31	103-123	^{103}APKHKEMPFPKYPVEPFTE**R**Q^{123}	B

[1]Monoisotopic mass values.

[2]Peptides also identified by Dupont et al. (2010a,b).

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-Casein (Phase 2)

Duodenal digestion of β-CN A^1, A^2, 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 A^1, B, and A^2 and with peptide AA 59 to 80 originating from gastric digestion. Peptid AA 114 to 124 was solely found in the digests of β-CN A^1 and A^2. 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 A^1 and A^2, as well as AA 103 to 123 in β-CN B and AA 108 to 129 in β-CN A^1 and A^2, occurred with identical masses but due to the mutation with modifications in their sequences.

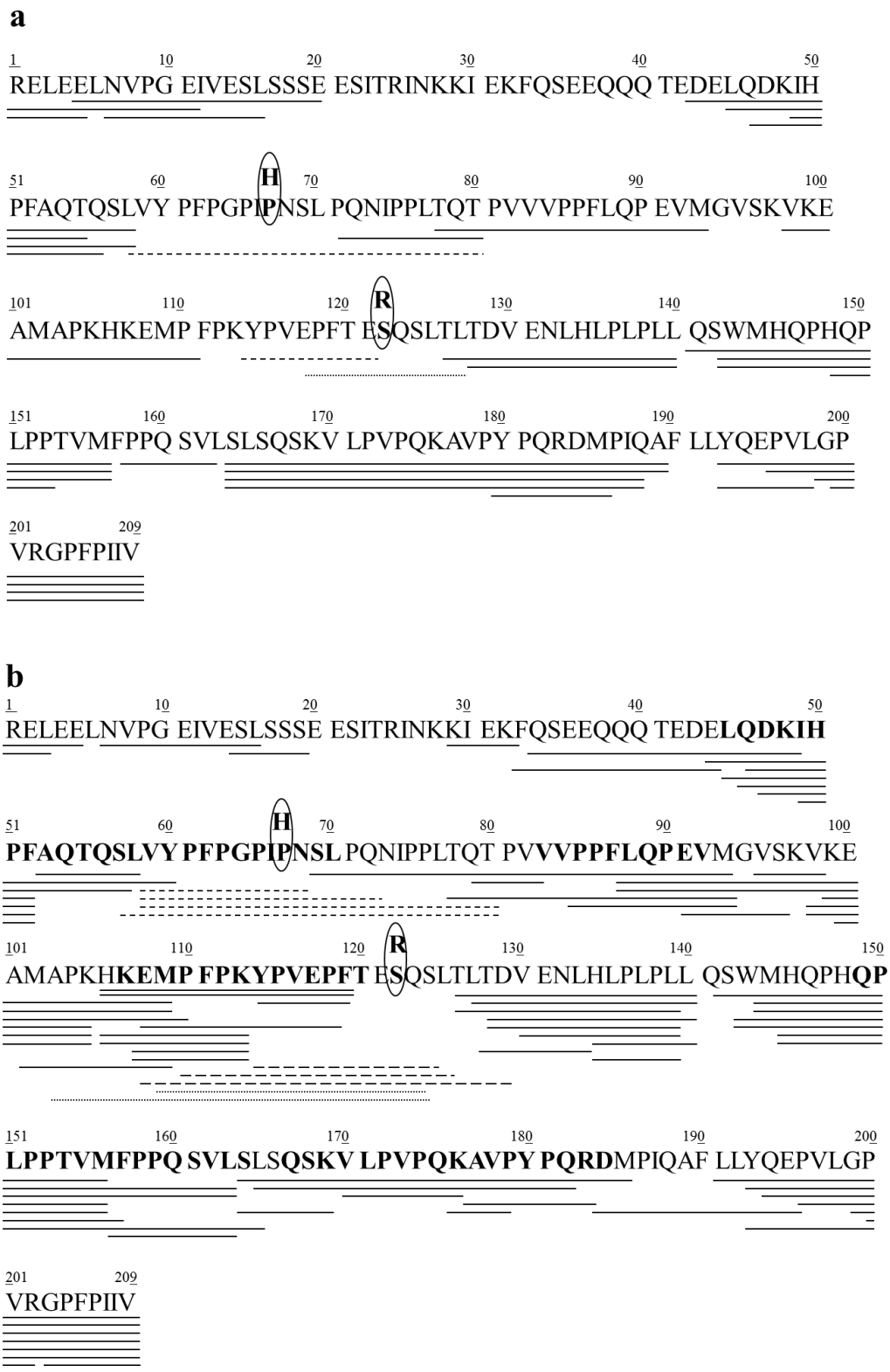

Figure 3. Identification of peptides derived from gastric (a) and duodenal (b) digestion of β-CN A^1, A^2, and B by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; continuous lines). Peptides with AA substitutions occurring in all β-CN variants are represented as small dashed lines (-----), peptides only present in β-CN B are indicated with dotted lines (……) and peptides detected solely in β-CN A^1 and A^2 are shown with large dashed lines (- - - -). The positions of the amino acid substitutions and IgE-binding epitopes (Figure 3b) are highlighted in bold.

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.

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**I**EAVESTVAT^{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}ESTVATLE**A**SPEVIE**S**PPEINTV^{162}	B
	2,503.23	2,503.19	127-151	^{127}SGEPTSTPT**I**EAVESTVATLE**A**SPE^{151}	B
Duodenal digsestion (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}PT**I**EAVESTVATLE**A**SP^{150}	B

[1]Monoisotopic mass values.

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, 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).

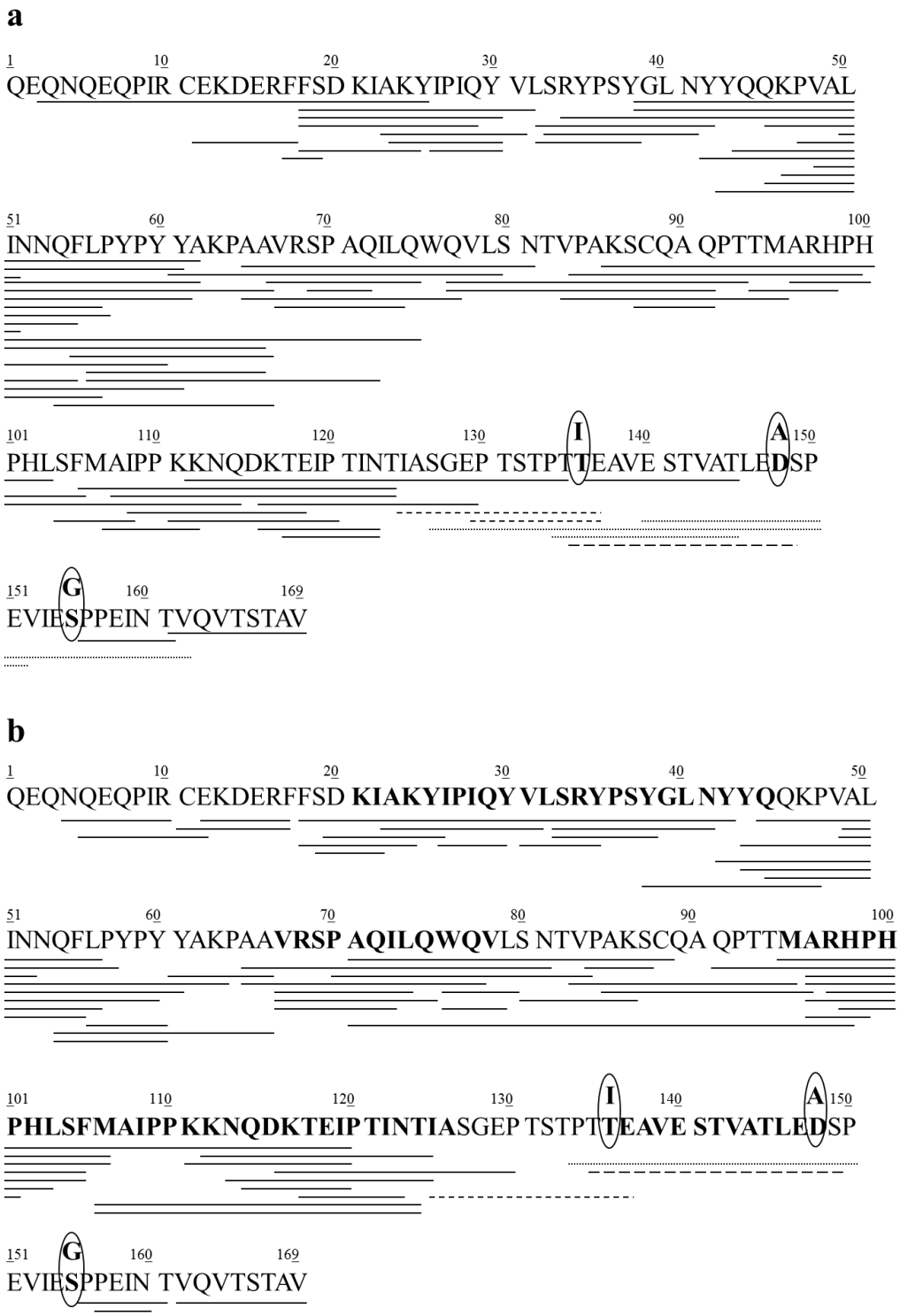

Figure 4. Identification of peptides derived from gastric (a) and duodenal (b) digestion of κ-casein (CN) A, B, and E by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; continuous lines). Peptides with AA substitutions occurring in all κ-CN variants are represented 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.

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 (Swaisgood, 1993, 2003).

During duodenal digestion, β-CN A^1, A^2, 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 A^2, 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 gastroduodenal

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 phosphoseryl 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 phosphoseryl 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 (Bogh 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 IgEbinding 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; Bogh 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 (Bogh 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 A^1, A^2, 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 A^2 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 A^1 and A^2, 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 A^1 and B might give reduced immunoreactivity compared with β-CN A^2. The exchange of a hydrophilic AA at position 122 in peptides AA 108 to 129 of β-CN A^1 and A^2 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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4. Immunoglobulin E epitope mapping by microarray immunoassay reveals differences in immune response to genetic variants of the caseins from different ruminant species

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ABSTRACT

The allergenicity of the caseins (CN), one of the major allergens in cow milk, is well characterized and their immunoglobulin E (IgE)-binding epitopes have been identified. However, investigations about the allergenic potential of the genetic variants occurring in the caseins are lacking. Therefore, this study determined the influence of the genetic polymorphism on IgE binding to epitopes of bovine casein variants. Furthermore, differences in IgE binding between epitopes of goats and water buffaloes were analyzed. A set of 187 peptides, covering the previously identified sequential IgE-binding epitopes of α_{S1}-, α_{S2}-, β-, and κ-CN variants from cows and the corresponding homologous peptides of water buffaloes and goats, were synthesized and tested by means of peptide microarray for IgE binding, using sera from 16 cow milk-sensitized individuals. Seven of the 16 sera samples showed positive signals on microarrays and were included in this study. In 5 α_{S1}-CN variants (A, B, C, E, and I), the AA substitution or deletion affected the immunoreactivity of epitopes AA 4 to 23, AA 17 to 36, AA 83 to 102, AA 173 to 192, and AA 175 to 194, as well as of the variant-specific peptides AA 184 to 196, AA 187 to 199, AA 174 to 193, and AA 179 to 198, which were found to resist gastrointestinal digestion. Variation in IgE binding was further detected for peptides AA 103 to 123 and AA 108 to 129 of 3 β-CN variants (A^1, A^2, and B). The majority of sera showed IgE binding to α_{S1}-CN peptides of cows and the homologous counterpart of goats and water buffaloes. However, α_{S1}- and β-CN epitopes from goats and water buffaloes had lower immunoreactivity than those of cows, but, in some cases, higher or exclusive IgE binding was observed. The results of this study indicate that genetic variants of the caseins differ in their allergenicity. This might be useful in the search for a suitable protein source for cow milk-allergic patients. In addition, milk from water buffaloes and goats harbor an allergenic potential due to cross-reactivity of IgE antibodies with cow milk caseins and are, therefore, not an acceptable alternative in the nutrition of cow milk-allergic patients.

Key words: casein variant, immunoglobulin E epitope, milk allergy, peptide microarray

INTRODUCTION

From a nutritional point of view, cow milk gains considerable attention, as it provides a wide range of essential nutrients, in particular proteins, fat, vitamins, and minerals. In contrast, cow milk belongs to the most common nutritive source of allergens during infancy and early childhood. Cow milk allergy (**CMA**) affects 2 to 3% of infants (Skripak et al., 2007) and

approximately 0.1% of adults (Osterballe et al., 2005) who are typically sensitized to several cow milk proteins (**CMP**). The majority of children with CMA achieve tolerance toward cow milk, although the age of clinical recovery has differed among various studies (Savilahti and Savilahti, 2013). Reports suggest that in most children, CMA resolves by 3 to 5 yr of age, but outgrowing CMA can develop even later (Skripak et al., 2007; Savilahti and Savilahti, 2013). Cow milk allergy is mostly mediated by milk protein-specific IgE antibodies that react with the allergenic epitopes on milk proteins. The 4 caseins α_{S1}-, α_{S2}-, β-, and κ-CN were confirmed to be the major allergens in cow milk, with α_{S1}-CN found to be more allergenic than α_{S2}-, β-, and κ-CN (Shek et al., 2005; Gaudin et al., 2008). By using SPOT-membrane-based or microarray immunoassays and sera from CMA patients, a large number of IgE-binding epitopes 8 to 24 AA in length have been identified in the 4 caseins (Chatchatee et al., 2001a,b; Busse et al., 2002, Cerecedo et al., 2008; Lin et al., 2009). Recent studies demonstrated that peptides resulting from in vitro gastrointestinal digestion of milk proteins still contain some of these IgE-binding epitopes (Dupont et al., 2010a,b; Lisson et al., 2013, 2014). This might provide an explanation for the high sensitization rate to the caseins, even though they are very rapidly digestible proteins.

Within the caseins, noticeable genetic variation has been identified, which is caused by substitutions or deletions of AA. So far, a total of 39 casein variants have been determined on the protein and DNA level, with important differences among species and breeds within the *Bos* genus (Caroli et al., 2009).

To date, the only treatment for CMA is strict avoidance of CMP. This is very difficult, as milk proteins are used as additives or processing aids and, therefore, a large number of food products contain remaining amounts of CMP. Furthermore, infant formulas based on CMP still may provoke allergic reactions in selected patients, as residual allergenic activity has been reported in partially and extensively hydrolyzed formulas (Ragno et al., 1993; de Boissieu et al., 1997; Hoffman and Sampson, 1997). Moreover, CMP hydrolysates are disadvantaged by their bitter taste and the requirement of additional emulsifiers. Thus, the identification of a suitable protein source as well as the search for options to eliminate the allergenicity in milk products or infant formulas is of great importance. Genetic variants of milk proteins are not considered when discussing the allergenic potential of bovine milk. However, detection of CMP variants with reduced allergenicity may contribute to find adequate alternatives for allergic patients. Genetic polymorphisms of milk proteins are of

increasing interest in animal breeding because of their significant effect on milk production traits and on cheese-making properties (Martin et al., 2002; Heck et al., 2009). In addition, milk protein variants are also important in human nutrition, as they represent a reservoir of bioactive peptides (angiotensin I-converting enzyme inhibitory peptides, casomorphins, and caseinophosphopeptides), which affect the nutritional value of milk (Jinsmaa and Yoshikawa, 1999; Weimann et al., 2009; Tulipano et al., 2010).

On the basis of the allergenic epitopes previously identified, the present study investigated the influence of the genetic polymorphisms on the IgE-binding properties of epitopes from α_{S1}-, α_{S2}-, β-, and κ-CN variants. This study also aimed to determine if variant-specific casein peptides resisting in vitro gastrointestinal digestion still retain IgE-binding properties and differ in their allergenic potential. In addition, differences in IgE binding between epitopes of 2 mammalian species (goats and water buffaloes) were analyzed because there has been an increasing focus on the use of proteins from non-bovine milk as a possible alternative to cow milk.

MATERIALS AND METHODS

Sera from Patients with CMA

The screening was performed with sera from 16 patients (6 females, 8 males, and 2 unknown; 8 mo to 52 yr old) who had a positive skin prick test reaction to cow milk or specific IgE antibodies to cow milk, or both. The sera were obtained from the University Hospitals Bonn and Marburg (Germany). The patients had milk-specific IgE antibodies ranging from 4.5 to >100 kUA/L (where **UA** = allergen-specific units), as measured with the ImmunoCAP System (Thermo Scientific, Freiburg, Germany). Only 7 (1 female, 5 males, and 1 unknown; 8 mo to 35 yr old; IgE >100 kUA/L) of the 16 sera, which were characterized by an IgE titer ≥95.5 kUA/L, showed positive signals on microarray and were included in the current study. This study was approved by the local ethical review committees of the Justus-Liebig-University Giesen and the University of Bonn, Germany.

Synthesis and Printing of Casein Peptides

To investigate the role of genetic polymorphism as well as interspecies differences on the epitope structure, a set of 187 peptides, 11 to 20 AA in length, covering the previously

determined sequential IgE-binding epitopes of α_{S1}-, α_{S2}-, β-, and κ-CN variants from cows and the corresponding homologous peptides of water buffaloes and goats as well as casein peptide variants, which were shown to resist gastrointestinal digestion (Lisson et al. 2013, 2014), were synthesized as described below (Table 1). Moreover, 36 peptides were synthesized with a phosphorylated serine residue and 1 peptide with a phosphorylated threonine residue. A total of 9 of these 37 peptides were spotted on the microarray without the phosphorylation to study the influence of posttranslational modifications on the immunoreactivity of the epitopes (Table 1). All peptides were synthesized in a stepwise manner on a cellulose membrane by using the SPOT synthesis technology (Frank and Overwin, 1996; Wenschuh et al., 2000). The peptide library and 3 control spots (human IgG, mouse IgE, and human IgE) were spotted in triplicate on each glass slide using a microarray printing system (JPT Peptide Technologies GmbH, Berlin, Germany). This gave a general microarray layout with 3 identical subarrays.

IgE-Binding Assays

The determination of peptide antibody binding was performed by RepliTope analysis (JPT Peptide Technologies GmbH). The array slides were first incubated with blocking buffer (SmartBlock; Candor Bioscience GmbH, Wangen, Germany) for 60 min. Then, they were placed into a microarray processing station (Tecan HS4800; Tecan Group Ltd., Männedorf, Switzerland) and incubated with 200 µL of the patient's serum, diluted 1:100 in diluent buffer [SuperBlock Tris-buffered saline (**TBS**); Pierce International, Rockford, IL], and with diluent buffer only as control for 2 h. Subsequently, the peptide microarray was washed 3 times with 50 m*M* TBS buffer, including 0.1% Tween 20 (pH 7.2). Specific human IgE binding was detected by incubation with SureLight allophycocyanin-labeled secondary antihuman IgE (Abcam, Cambridge, UK) at a final concentration of 1 µg/mL in diluent buffer. Control incubation with secondary antibody only was performed in parallel. Afterward, 3 washing steps with 50 m*M* TBS buffer, including 0.1% Tween 20 (pH 7.2) were carried out, which were followed by a washing step with 3 m*M* saline-sodium citrate buffer (pH 7.0). Finally, peptide microarrays were dried using a nitrogen stream.

Table 1. The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence[1]	Source of selected peptides[2]
1	α_{s1}-CN B cow	1-15	RPKHPIKHQGLPQEV	1
2	α_{s1}-CN goat	1-15	RPKHPINHRGLSPEV	1
3	α_{s1}-CN water buffalo	1-15	RPKQPIKHQGLPQGV	1
4	α_{s1}-CN B cow, water buffalo	17-36	NENLLRFFVAPFPEVFGKEK[3]	1
5	α_{s1}-CN A cow	4-23	HPIKHQGLPQPFPEVFGKEK	2
6	α_{s1}-CN goat	17-36	NENLLRFVVAPFPEVFRKEN	1
7	α_{s1}-CN B cow	34-48	KEKVNELSKDIGSES[3]	1
8	α_{s1}-CN goat	34-48	KENINELSKDIGSES	1
9	α_{s1}-CN water buffalo	34-48	KEKVNELSTDIGSES	1
10	α_{s1}-CN B cow, water buffalo	49-63	TEDQAMEDIKQMEAE[4]	1, 4
11	α_{s1}-CN D cow	49-63	TEDQ-pT-MEDIKQMEAE	2, 4
12	α_{s1}-CN E cow	49-63	TEDQAMEDIKKMEAE	2, 4
13	α_{s1}-CN H cow	45-59	GSESTEQMEAESISS	2
14	α_{s1}-CN goat	49-63	TEDQAMEDAKQMKAG	1, 4
15	α_{s1}-CN B cow	64-78	SISSSEEIVPNSVEQ[3]	1
16	α_{s1}-CN F cow	64-78	SILSSEEIVPNSVEQ	2
17	α_{s1}-CN goat	64-78	SSSSSEEIVPNSAEQ	1
18	α_{s1}-CN water buffalo	64-78	SISSSEEIVPISVEQ	1
19	α_{s1}-CN B cow, water buffalo, goat	83-102	KEDVPSERYLGYLEQLLRLK[3]	1
20	α_{s1}-CN I cow	83-102	KDDVPSERYLGYLEQLLRLK	2
21	α_{s1}-CN B cow	106-120	VPQLEIVPN-pS-AEERL[3]	1, 4
22	α_{s1}-CN goat	106-120	VPQLEIVPK-pS-AEEQL	1, 4
23	α_{s1}-CN water buffalo	106-120	VPQLEIVPNLAEEQL	1, 4
24	α_{s1}-CN B cow, water buffalo	121-135	HSMKEGIHAQQKEPM[3]	1
25	α_{s1}-CN goat	121-135	HSMKEGNPAHQKQPM	1
26	α_{s1}-CN B cow	139-154	NQELAYFYPELFRQFY[3]	1
27	α_{s1}-CN water buffalo, goat	139-154	NQELAYFYPQLFRQFY	1
28	α_{s1}-CN B cow	159-174	YPSGAWYYVPLGTQYT[3]	1
29	α_{s1}-CN goat	159-174	YPSGAWYYLPLGTQYT	1
30	α_{s1}-CN water buffalo	159-174	YPSGAWYYVPLGTQYP	1
31	α_{s1}-CN B cow	173-192	YTDAPSFSDIPNPIGSENSE[3]	1
32	α_{s1}-CN C, E cow, goat	173-192	YTDAPSFSDIPNPIGSENSG	1, 2
33	α_{s1}-CN water buffalo	173-192	YPDAPSFSDIPNPIGSENSG	1
34	α_{s1}-CN B cow	175-194	DAPSFSDIPNPIGSENSEKT[3]	1
35	α_{s1}-CN C, E cow, goat	175-194	DAPSFSDIPNPIGSENSGKT	1, 2
36	α_{s1}-CN water buffalo	175-194	DAPSFSDIPNPIGSENSGKT	1
37	α_{s1}-CN B cow	184-196	NPIGSENSEKTTM[5]	3
38	α_{s1}-CN B cow	187-199	GSENSEKTTMPLW[5]	3
39	α_{s1}-CN C cow	187-199	GSENSGKTTMPLW[5]	3
40	α_{s1}-CN B cow	180-193	SDIPNPIGSENSEK[5]	3
41	α_{s1}-CN C cow	180-193	SDIPNPIGSENSGK[5]	3
42	α_{s1}-CN B cow	174-193	TDAPSFSDIPNPIGSENSEK[5]	3
43	α_{s1}-CN C cow	179-198	FSDIPNPIGSENSGKTTMPL[5]	3
44	α_{s2}-CN A cow	1-20	KNTMEHVSSSEESIISQETY[6]	1

Continued

Table 1 (Continued). The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence[1]	Source of selected peptides[2]
45	α_{s2}-CN B cow	1-20	KNTMEHVFSSEESIISQETY	2
46	α_{s2}-CN goat	1-20	KHKMEHVSSSEEPINIFQEI	1
47	α_{s2}-CN goat	2-21	HKMEHVSSSEEPINIFQEIY	1
48	α_{s2}-CN water buffalo	1-20	KHTMEHVSSSEESIISQETY	1
49	α_{s2}-CN A cow	13-32	SIISQETYKQEKNMAINPSK[6]	1
50	α_{s2}-CN A cow	14-33	IISQETYKQEKNMAINPSKE[6]	1
51	α_{s2}-CN C cow	13-32	SIISQETYKQEKNMAINPSK	2
52	α_{s2}-CN C cow	14-33	IISQETYKQEKNMAINPSKG	2
53	α_{s2}-CN A cow	31-45	-pS-KENLCSTFCKEVVR[7]	1
54	α_{s2}-CN C cow	31-45	-pS-KGNLCSTFCKEVVR	2
55	α_{s2}-CN A cow	42-56	EVVRNANEEEYSIG-pS-[7]	1,4
56	α_{s2}-CN C cow	42-56	EVVRNTNEEEYSIG-pS-	2, 4
57	α_{s2}-CN D cow	42-56	EVVRNANEEESAEVA	2, 4
58	α_{s2}-CN A cow	57-71	SSEESAEVATEEVKI	1
59	α_{s2}-CN D cow	48-62	NEEESAEVATEEVKI	2
60	α_{s2}-CN A cow	67-86	EEVKITVDDKHYQKALNEIN[4,6]	1
61	α_{s2}-CN A cow	83-100	NEINQFYQKFPQYLQYLY[7]	1
62	α_{s2}-CN A cow	93-108	PQYLQYLYQGPIVLNP[7]	1
63	α_{s2}-CN A cow	105-119	VLNPWDQVKRNAVPI[7]	1
64	α_{s2}-CN A cow	117-131	VPITPTLNREQLSTS[7]	1
65	α_{s2}-CN C cow	117-131	VPITPTLNREQLSIS	2
66	α_{s2}-CN A cow	130-144	TSEENSKKTVDMEST[6]	1
67	α_{s2}-CN C cow	130-144	ISEENSKKTVDMEST	2
68	α_{s2}-CN A cow	143-158	-pS-TEVFTKKTKLTEEEK[7]	1
69	α_{s2}-CN A cow	157-172	EKNRLNFLKKISQRYQ[7]	1
70	α_{s2}-CN A cow	165-184	KKISQRYQKFALPQYLKTVY[7]	1
71	α_{s2}-CN A cow	169-188	QRYQKFALPQYLKTVYQHQK[7]	1
72	α_{s2}-CN A cow	191-205	KPWIQPKTKVIPYVR[7]	1
73	α_{s2}-CN A cow	7-26	VSSSEESIISQETYKQEKNM[5]	3
74	α_{s2}-CN A cow	10-29	SEESIISQETYKQEKNMAIN[5]	3
75	α_{s2}-CN B cow	1-20	KNTMEHVFSSEESIISQETY[5]	3
76	α_{s2}-CN B cow	3-22	TMEHVFSSEESIISQETYKQ[5]	3
77	α_{s2}-CN B cow	9-21	SSEESIISQETYK[5]	3
78	β-CN A^2 cow, water buffalo	1-18	RELEELNVPGEIVESLSS[8]	1
79	β-CN D cow	1-18	RELEELNVPGEIVESLSK	2
80	β-CN goat	1-18	REQEELNVVGETVESLSS	1
81	β-CN A^2 cow	16-35	LSSSEESITRINKKIEKFQ-pS-[4]	1, 4
52	β-CN A^2 cow	18-37	SSEESITRINKKIEKFQ-pS-EE[4]	1, 4
83	β-CN D cow	16-35	LSKSEESITRINKKIEKFQ-pS-	2, 4
84	β-CN D cow	18-37	KSEESITRINKKIEKFQ-pS-EE	2, 4
85	β-CN H^1 cow	16-35	LSSSEESITCINKKIEKFQ-pS-	2, 4
86	β-CN H^1 cow	18-37	SSEESITCINKKIEKFQ-pS-EE	2, 4
87	β-CN C cow	16-35	LSSSEESITRINKKIEKFQS	2, 4
88	β-CN C cow	18-37	SSEESITRINKKIEKFQSEK	2, 4
89	β-CN E cow	16-35	LSSSEESITRINKKIEKFQ-pS-	2, 4

Continued

Table 1 (Continued). The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence[1]	Source of selected peptides[2]
90	β-CN E cow	18-37	SSEESITRINKKIEKFQ-pS-KE	2, 4
91	β-CN goat, water buffalo	16-35	LSSSEESITHINKKIEKFQ-pS-	1, 4
92	β-CN goat, water buffalo	18-37	SSEESITHINKKIEKFQ-pS-EE	1, 4
93	β-CN A^2 cow, goat	34-50	Q-pS-EEQQQTEDELQDKIH[6]	1, 4
94	β-CN C cow	34-50	QSEKQQQTEDELQDKIH	2, 4
95	β-CN E cow	34-50	Q-pS-KEQQQTEDELQDKIH	2, 4
96	β-CN water buffalo	34-50	Q-pS-EEQQQMEDELQDKIH	1, 4
97	β-CN A^2 cow	42-56	EDELQDKIHPFAQTQ[8]	1
98	β-CN A^2 cow	55-72	TQSLVYPFPGPIPNSLPQ[8]	1
99	β-CN A^1, B, C, F, G cow	55-72	TQSLVYPFPGPIHNSLPQ	2
100	β-CN H^2 cow	55-72	TQSLVYPFPGPIPNSLPE	2
101	β-CN goat	55-72	AQSLVYPFTGPIPNSLPQ	1
102	β-CN water buffalo	55-72	TQSLVYPFPGPIPKSLPQ	1
103	β-CN A^2, water buffalo	70-84	LPQNIPPLTQTPVVV	1
104	β-CN H^2 cow	70-84	LPENIPPLTQTPVVV	2
105	β-CN goat	70-84	LPQNILPLTQTPVVV	1
106	β-CN A^2 cow	83-97	VVPPFLQPEVMGVSK[8]	1
107	β-CN H^1 cow	83-97	VVPPFIQPEVMGVSK	2
108	β-CN H^2, I cow	83-97	VVPPFLQPEVLGVSK	2
109	β-CN goat	83-97	VVPPFLQPEIMGVPK	1
110	β-CN water buffalo	83-97	VVPPFLQPEIMGVSK	1
111	β-CN A^2 cow, water buffalo	95-109	VSKVKEAMAPKHKEM	1
112	β-CN A^3 cow	95-109	VSKVKEAMAPKQKEM	2
113	β-CN goat	95-109	VPKVKETMVPKHKEM	1
114	β-CN A^2 cow, goat, water buffalo	106-122	HKEMPFPKYPVEPFTES[8]	1
115	β-CN A^3 cow	106-122	QKEMPFPKYPVEPFTES	2
116	β-CN B cow	106-122	HKEMPFPKYPVEPFTER	2
117	β-CN A^2 cow, water buffalo	121-135	ESQSLTLTDVENLHL[6]	1
118	β-CN B cow	121-135	ERQSLTLTDVENLHL	2
119	β-CN goat	121-135	ESQSLTLTDVEKLHL	1
120	β-CN A^2 cow	135-149	LPLPLLQSWMHQPHQ[8]	1
121	β-CN G cow	135-149	LPLLLLQSWMHQPHQ	2
122	β-CN water buffalo	135-149	LPLPLLQSWMHQPPQ	1
123	β-CN goat	135-149	LPLPLVQSWMHQPPQ	1
124	β-CN A^2 cow, water buffalo	149-164	QPLPPTVMFPPQSVLS[8]	1
125	β-CN B cow	149-164	QPLLPTVMFPPQSVLS	2
126	β-CN goat	149-164	QPLSPTVMFPPQSVLS	1
127	β-CN A^2 cow, water buffalo	167-184	QSKVLPVPQKAVPYPQRD[8]	1
128	β-CN goat	167-182	QPKVLPVPQKAVPQRD	1
129	β-CN A^2 cow, goat, water buffalo	185-204	MPIQAFLLYQEPVLGPVRGP[8]	1
130	β-CN A^2, A^1 cow	114-124	YPVEPFTESQS[9]	3
131	β-CN A^2 cow	59-72	VYPFPGPIPNSLPQ[9]	3
132	β-CN B, A^1 cow	59-72	VYPFPGPIHNSLPQ[9]	3
133	β-CN A^2 cow	59-78	VYPFPGPIPNSLPQNIPPLT[9]	3

Continued

Table 1 (Continued). The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence[1]	Source of selected peptides[2]
134	β-CN A2 cow	61-80	PFPGPIPNSLPQNIPPLTQT[9]	3
135	β-CN B , A^1 cow	59-78	VYPFPGPIHNSLPQNIPPLT[9]	3
136	β-CN B , A^1 cow	61-80	PFPGPIHNSLPQNIPPLTQT[9]	3
137	β-CN A^2 , A^1 cow	110-125	PFPKYPVEPFTESQSL[9]	3
138	β-CN B cow	109-123	MPFPKYPVEPFTERQ[9]	3
139	β-CN A^2 , A^1 cow	108-127	EMPFPKYPVEPFTESQSLTL[9]	3
140	β-CN A^2 , A^1 cow	110-129	PFPKYPVEPFTESQSLTLTD[9]	3
141	β-CN B cow	103-122	APKHKEMPFPKYPVEPFTER[9]	3
142	β-CN B cow	104-123	PKHKEMPFPKYPVEPFTERQ[9]	3
143	κ-CN A cow	1-15	QEQNQEQPIRCEKDE	1
144	κ-CN F^2 cow	1-15	QEQNQEQPIHCEKDE	2
145	κ-CN A cow	9-26	IRCEKDERFFSDKIAKYI[8]	1
146	κ-CN F^2 cow	9-26	IHCEKDERFFSDKIAKYI	2
147	κ-CN A cow	24-43	KYIPIQYVLSRYPSYGLNYY[8]	1
148	κ-CN A cow	25-44	YIPIQYVLSRYPSYGLNYYQ[8]	1
149	κ-CN A cow	34-53	RYPSYGLNYYQQKPVALINN[6]	1
150	κ-CN A cow	49-68	ALINNQFLPYPYYAKPAAVR[8]	1
151	κ-CN A cow	66-80	AVRSPAQILQWQVLS[8]	1
152	κ-CN A cow	79-94	LSNTVPAKSCQAQPTT	1
153	κ-CN A cow	95-114	MARHPHPHLSFMAIPPKKNQ[8]	1
154	κ-CN A cow	97-116	RHPHPHLSFMAIPPKKNQDK[8]	1
155	κ-CN G^1 cow	95-114	MACHPHPHLSFMAIPPKKNQ	2
156	κ-CN G^1 cow	97-116	CHPHPHLSFMAIPPKKNQDK	2
157	κ-CN C, D cow	95-114	MAHHPHPHLSFMAIPPKKNQ	2
158	κ-CN C, D cow	97-116	HHPHPHLSFMAIPPKKNQDK	2
159	κ-CN I cow	95-114	MARHPHPHLAFMAIPPKKNQ	2
160	κ-CN I cow	97-116	RHPHPHLAFMAIPPKKNQDK	2
161	κ-CN A cow	111-126	KKNQDKTEIPTINTIA[8]	1
162	κ-CN A cow	125-139	IA-pS-GEPTSTPTTEAV	1
163	κ-CN H cow	125-139	IA-pS-GEPTSTPITEAV	2
164	κ-CN B, B^2, C, F^2, G^1, J cow	125-139	IA-pS-GEPTSTPTIEAV	2
165	κ-CN A cow	134-148	PTTEAVESTVATLED[8]	1
166	κ-CN H cow	134-148	PITEAVESTVATLED	2
167	κ-CN B, B^2, C, F^2, G^1, J cow	134-148	PTIEAVESTVATLEA	2
168	κ-CN G^2 cow	134-148	PTTEAVESTVATLEA	2
169	κ-CN F^1 cow	134-148	PTTEAVESTVATLEV	2
170	κ-CN A cow	146-160	LED-pS-PEVIESPPEIN	1
171	κ-CN B, C, F^2, G^1, G^2, J cow	146-160	LEA-pS-PEVIESPPEIN	2
172	κ-CN F^1 cow	146-160	LEV-pS-PEVIESPPEIN	2
173	κ-CN B^2 cow	146-160	LEA-pS-PEVTESPPEIN	2
174	κ-CN E cow	146-160	LED-pS-PEVIEGPPEIN	2
175	κ-CN J cow	146-160	LED-pS-PEVIERPPEIN	2
176	κ-CN A cow	149-168	-pS-PEVIESPPEINTVQVTSTA[8]	1
177	κ-CN B^2 cow	149-168	-pS-PEVTESPPEINTVQVTSTA	2
178	κ-CN E cow	149-168	-pS-PEVIEGPPEINTVQVTSTA	2

Continued

Table 1 (Continued). The AA sequence of peptides spotted on the microarray

No.	Protein	Position (AA no.)	Sequence[1]	Source of selected peptides[2]
179	κ-CN J cow	149-168	-pS-PEVIERPPEINTVQVTSTA	2
180	κ-CN A, E cow	125-137	IASGEPTSTPTTE[9]	3
181	κ-CN B cow	125-137	IASGEPTSTPTIE[9]	3
182	κ-CN A cow	146-157	LED-pS-PEVIESPP[9]	3
183	κ-CN E cow	147-160	EDSPEVIEGPPEIN[9]	3
184	κ-CN A cow	148-159	D-pS-PEVIESPPEI[9]	3
185	κ-CN A, E cow	136-149	TEAVESTVATLED-pS-[9]	3, 4
186	κ-CN A, E cow	136-149	TEAVESTVATLEDS[9]	3, 4
187	κ-CN B cow	134-150	PTIEAVESTVATLEASP[9]	3

[1]Peptides previously reported as IgE-binding epitopes are underlined. Phosphorylated peptides are indicated as -pS- or -pT-.

[2]1 = peptides described in cattle; 2 = bovine genetic variant; 3 = peptides resisting gastrointestinal digestion; 4 = peptides with different phosphorylation levels.

[3]Chatchatee et al. (2001a).

[4]Lin et al. (2009).

[5]Lisson et al. (2014).

[6]Cerecedo et al. (2008).

[7]Busse et al. (2002).

[8]Chatchatee et al. (2001b).

[9]Lisson et al. (2013).

Signal Detection and Data Analysis

The peptide microarrays were scanned with the GenePix Scanner 4300SL50 (Axon Instruments, Concord, ON, Canada). Images were saved electronically in tagged image file format (*.tif). Image analysis was performed using the spot-recognition software GenePix Pro 6.1 (Axon Instruments), which showed the signal intensity (light units) as single measurements for each peptide. Each spot feature was analyzed for total intensity and background intensity. For data tables and diagrams, the mean value of all 3 instances on the microarray was calculated. In case the standard deviation divided by the mean value was larger than 0.5, the mean of the 2 closest values was used instead. Negative controls were used to define background noise. The fluorescent intensity of each peptide was determined by subtracting the mean intensity of the corresponding peptide spots from control incubation without serum. Immunoglobulin E binding was considered positive if signal intensities were above the lower limit of detection, meaning that ratios of signal-to-background noise intensities were greater than a minimum threshold of 1.5.

RESULTS

Identification of IgE-Binding Epitopes in Patient Sera Samples

Among the sera samples tested in this study, IgE binding to at least 1 of the casein peptides occurred in all samples. A total of 46 casein peptides (27 α_{S1}-CN peptides, 13 β-CN peptides, and 6 κ-CN peptides) were recognized by IgE antibodies from the different sera samples (Figure 1a, b, and c). All sera showed IgE binding to α_{S1}-CN peptides. It was the most frequently detected milk allergen, whereas no significant IgE binding was found for α_{S2}-CN peptides. The α_{S1}-CN peptides AA 17 to 36, AA 139 to 154, AA 173 to 192, and AA 175 to 194 of cows, goats, and water buffaloes reacted with IgE antibodies from at least 50% of the patient sera samples and, thus, represented the major IgE-binding epitopes (Figure 1a). The α_{S1}-CN peptides AA 83 to 102, AA 159 to 174, AA 174 to 193, and AA 175 to 194 from cows were recognized by 3 of the 7 positive sera. The α_{S1}-CN peptides AA 4 to 23 (cow), AA 83 to 102 (goat and water buffalo), AA 179 to 198 (cow), and the β-CN peptide AA 16 to 35 (goat and water buffalo), as well as the κ-CN peptides AA 24 to 43 (cow) and AA 25 to 44 (cow) were identified in 2 sera samples (Figure 1a, b, and c). The other peptides of α_{S1}-, β-, and κ-CN, giving positive signals on the microarray, displayed only binding to individual sera, particularly to serum 4.

Only in 1 serum was an influence observed of the posttranslational modification on the immunoreactivity of the epitopes for the α_{S1}-CN peptide AA 106 to 120 of the water buffalo (Figure 3a). The IgE response to the peptide lacking the phosphorylated serine residue was 86% higher than to the phosphorylated peptides of cows and goats. The other sera showed no variation in the immunoreactivity between the peptides carrying phosphorylated serine residues or not.

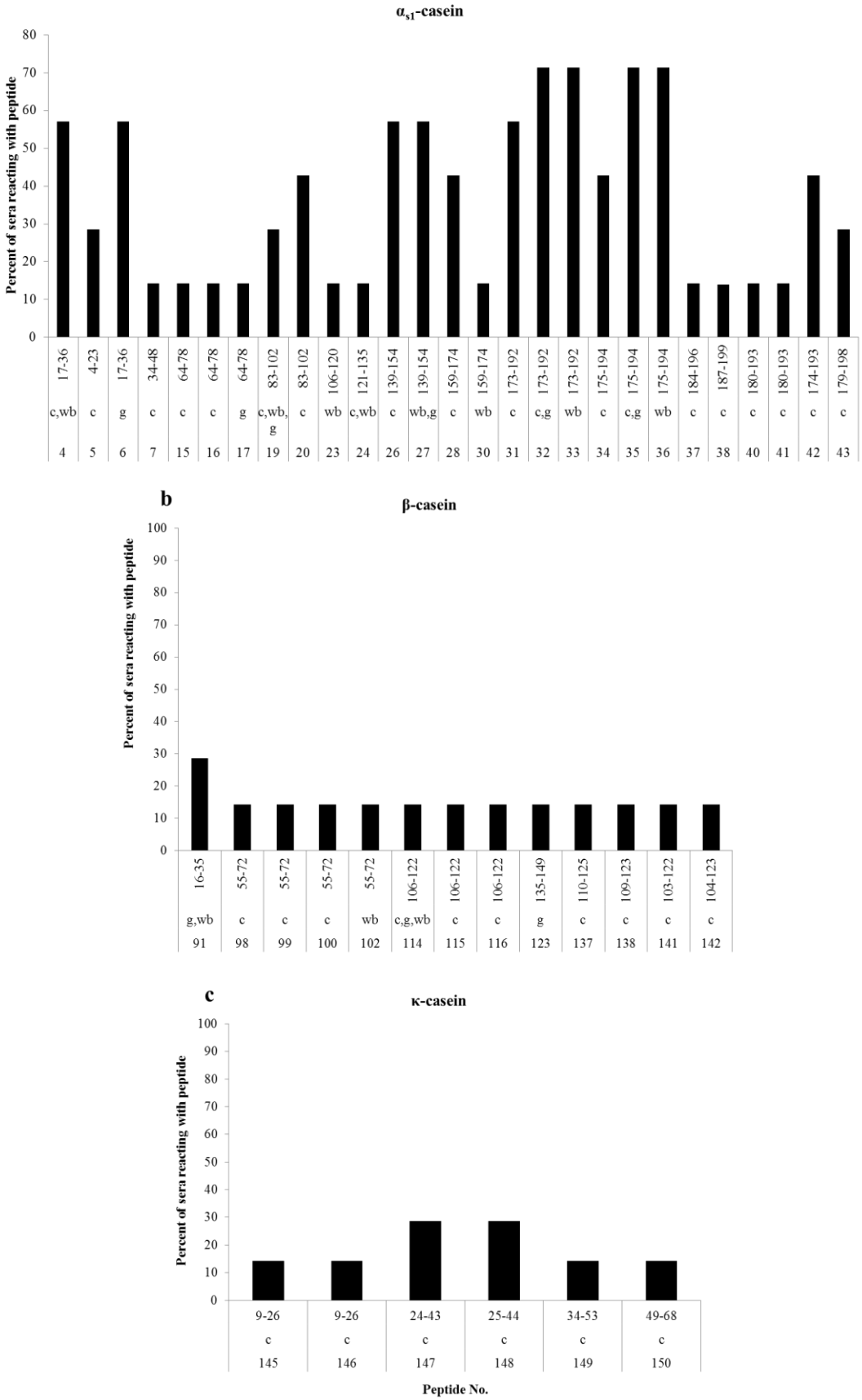


Figure 1. Immunoglobulin E binding pattern of cow milk-allergic patients to peptides of α_{S1}-CN (a), β-CN (b), and κ-CN (c) from cows, goats, and water buffaloes. The bars indicate the percentage of sera showing IgE binding to peptides (represented by AA residues, number, and species). c = cow, g = goat, wb = water buffalo.

Identification of Variant-Specific Differences on the Epitope Structure

The genetic variation affects the IgE-binding epitope structure of the caseins without inducing changes in IgE binding by the majority of sera (Table 2; Figure 2). Nevertheless, as indicated in Figure 2, some of the mutated epitopes led to abrogation or decreased or increased IgE binding in individual sera (≥15% of IgE binding remaining compared with the reference peptide).

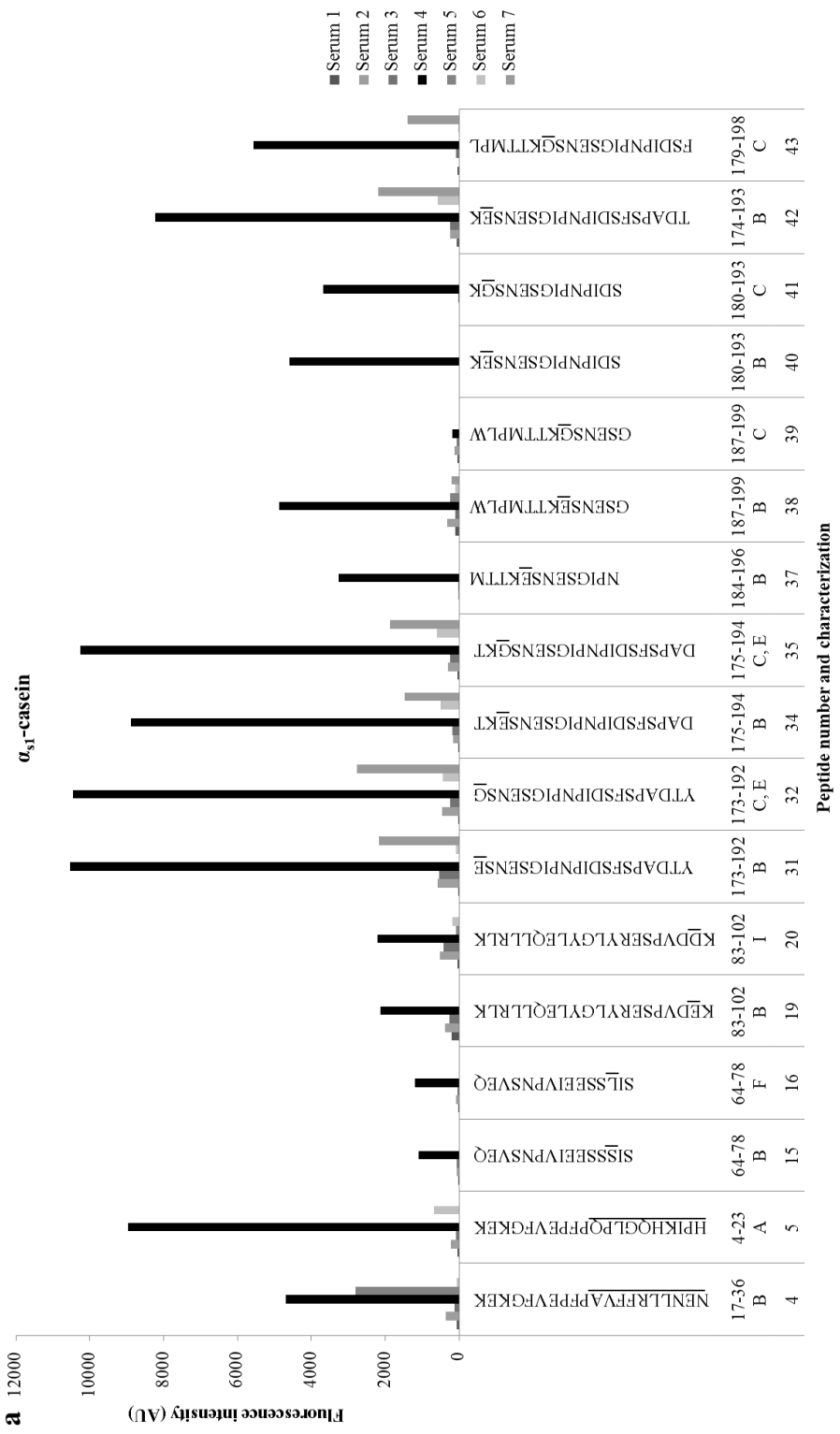
In 4 α_{S1}-CN variants (A, C, E, and I), 1 deletion and 3 AA exchanges influenced the immunoreactivity of 5 epitopes (Figure 2a). Due to the deletion of AA 14 to 26 in α_{S1}-CN A, epitope AA 4 to 23 differs in 10 AA from the reference epitope AA 17 to 36 in α_{S1}-CN B. Thus, for peptide AA 4 to 23 of α_{S1}-CN A, a loss of IgE binding could be observed in serum 5, a decrease in IgE binding in serum 2, and an increase in IgE binding in serum 4 compared with the reference peptide. Serum 6 reacted only with the peptide of α_{S1}-CN A, but showed no IgE binding to the reference peptide.

The AA substitutions characterizing epitopes AA 83 to 102 of α_{S1}-CN I as well as AA 173 to 192 and AA 175 to 194 of α_{S1}-CN C and E resulted in a loss of IgE binding in serum 3 and increased IgE binding in sera 2, 3, 4, and 7. In contrast to peptides AA 173 to 192 and AA 175 to 194 of α_{S1}-CN C and E, IgE binding to the corresponding peptides of α_{S1}-CN B was eliminated by the AA substitution occurring at position 192 (sera 2 and 6). In 3 sera, the AA exchanges had no significant effect on the immunoreactivity of epitopes AA 64 to 78 (serum 4), AA 173 to 192 (sera 2 and 4), and AA 175 to 194 (sera 4 and 6). Both the mutated peptide as well as the reference peptide displayed similar IgE-binding intensities on the peptide microarray (Figure 2a).

Five of the 7 peptides resulting from gastrointestinal digestion of α_{S1}-CN B and C showed positive IgE binding on the microarray in 3 sera (Figure 2a). The AA substitution did not cause a change in the immunoreactivity of peptides AA 180 to 193 of α_{S1}-CN B and C in serum 4. However, important differences in IgE binding were detected for peptides AA 187 to 199 and 179 to 198 of α_{S1}-CN C and the corresponding peptides of α_{S1}-CN B because sera 4, 6, and 7 revealed significantly higher or exclusive IgE binding to peptides of α_{S1}-CN B.

As indicated in Figure 2b, the highest signal intensity of the responses against the different β-CN peptides were almost 1500% lower (700 versus 11,000 arbitrary units) compared with the highest signals obtained for peptides of α_{S1}-CN. Four AA exchanges occurring in epitopes AA 55 to 72 and AA 106 to 122 of 7 β-CN variants (A^1, A^3, B, C, F, G, and H^2) only induced

positive IgE binding in serum 4 but without significant differences in the immunoreactivity between the mutated and the reference peptides.



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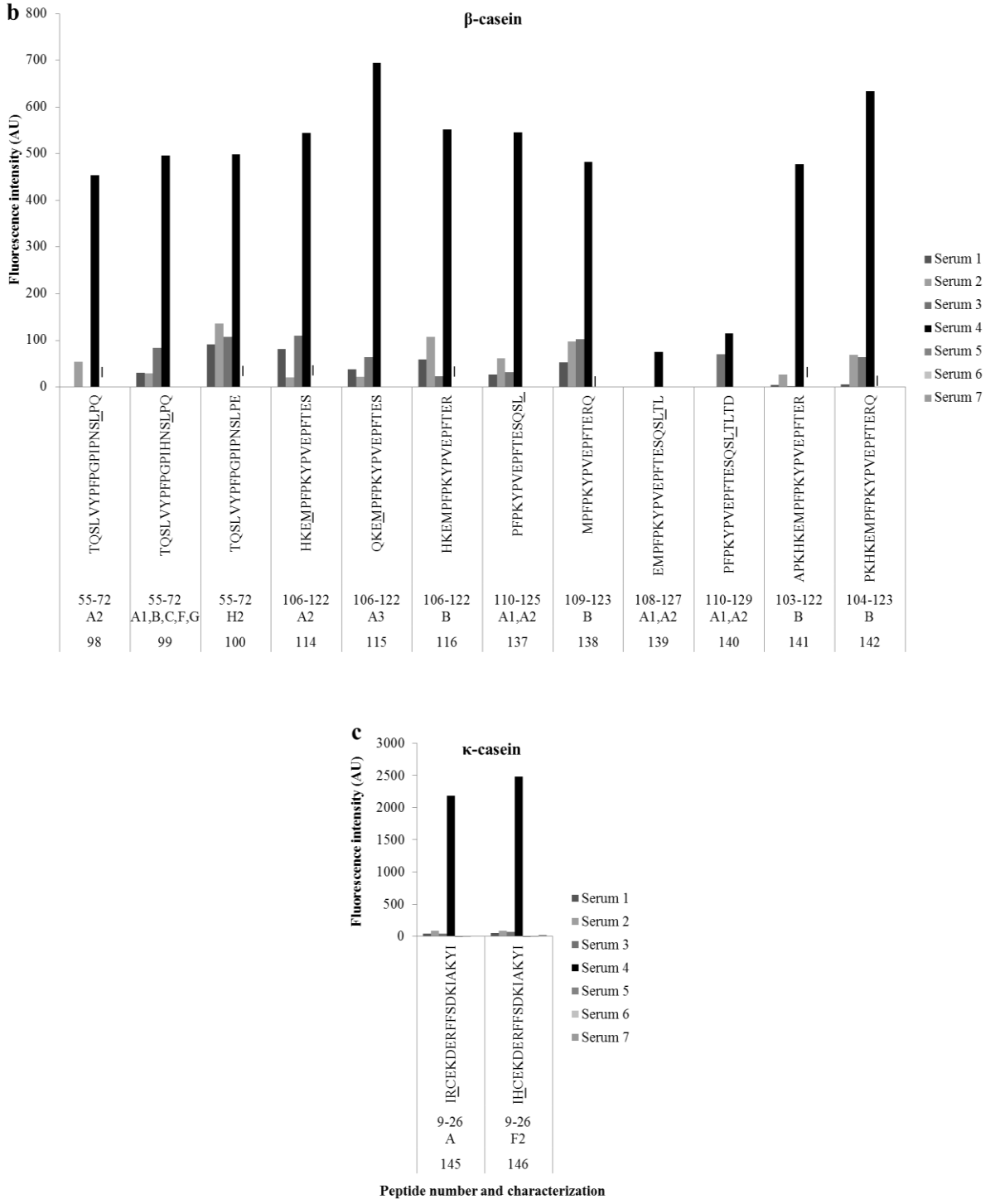


Figure 2. Differences in IgE binding between epitopes (represented by AA residues, number, and casein variant) of α_{s1}-CN (a), β-CN (b), and κ-CN (c) variants from cows, using 7 sera with cow milk-specific IgE values >100 kU_A/L, where UA = allergen-specific units. Differences in AA sequence between the reference peptides and the mutated peptides are underlined. The mean fluorescence intensity (arbitrary units, AU) of the 3 subarrays on the microarray for each peptide is presented.

Table 2. Sera of cow milk-allergic patients reacting with peptides of α_{s1}-, β-, and κ-CN variants and differences in IgE binding between variant-specific peptides and corresponding reference peptides[1]

Patient serum[2]	Age	Milk IgE [kU_A/L]	Peptides (AA no.)	Casein variant	IgE binding compared with reference peptides α_{s1}-CN B, β-CN A[2], and κ-CN A
2	35 y	>100	4-23	α_{s1}-CN A	↓[3]
			17-36	**α_{s1}-CN B**	
			83-102	**α_{s1}-CN B**, I	↑[4]
			173-192	**α_{s1}-CN B**, C, E	No differences
			175-194	α_{s1}-CN C, E	No reaction to reference peptide
3	12 y	>100	83-102	**α_{s1}-CN B**, I	↑
			173-192	**α_{s1}-CN B**	No reaction to peptide of α_{s1}-CN C, E
4	24 y	>100	4-23	α_{s1}-CN A	↑
			17-36	**α_{s1}-CN B**	
			64-78	**α_{s1}-CN B**, F	No differences
			83-102	**α_{s1}-CN B**, I	↑
			173-192	**α_{s1}-CN B**, C, E	No differences
			175-194	**α_{s1}-CN B**, C, E	No differences
			180-193	**α_{s1}-CN B**, C, E	No differences
			184-196	**α_{s1}-CN B**	No corresponding peptide in α_{s1}-CN C, E
			187-199	**α_{s1}-CN B**	No reaction to peptide of α_{s1}-CN C, E
			179-198	α_{s1}-CN C, E	Lower reaction compared with peptide 174-193 of **α_{s1}-CN B**
			174-193	**α_{s1}-CN B**	
			55-72	**β-CN A[2]**, A[1], B, C, F, G, H[2]	No differences
			106-122	**β-CN A[2]**, A[1], B, C, F, G, H[2]	No differences
			110-125	**β-CN A[2]**, A[1]	No differences between 110-125 of β-CN A[2], A[1] and 109-123 of β-CN B
			109-123	β-CN B	
			103-123	β-CN B	No reaction to corresponding peptide 108-129 of **α_{s1}-CN A[2]**, A[1]
			9-26	**κ-CN A**, F[2]	No differences
5	8 mo	95.5	17-36	**α_{s1}-CN B**	No reaction to peptide 4-23 of α_{s1}-CN A
6	10 y	>100	4-23	α_{s1}-CN A	No reaction to reference peptide 17-36 of **α_{s1}-CN B**
			173-192	α_{s1}-CN C, E	No reaction to reference peptide 173-192 of **α_{s1}-CN B**
			175-194	**α_{s1}-CN B**, C, E	No differences
			174-193	**α_{s1}-CN B**	No reaction to corresponding peptide 179-198 of α_{s1}-CN C
7	16 y	>100	173-192	**α_{s1}-CN B**, C, E	↑
			175-194	**α_{s1}-CN B**, C, E	↑
			179-198	α_{s1}-CN C, E	Lower reaction compared with peptide 174-193 of **α_{s1}-CN B**
			174-193	**α_{s1}-CN B**	

[1]Reference proteins are shown in bold.

[2]Serum 1 (age unknown; >100 kU_A/L, where U_A = allergen-specific units) showed no reaction with any of the casein variant peptides.

[3]↓ = decreased binding.

[4]↑ = increased binding.

The peptides AA 110 to 125 and AA 108 to 129 of β-CN A^1 and A^2, as well as AA 109 to 123 and AA 103 to 123 of β-CN B, which were found to resist gastrointestinal digestion of β-CN, reacted with IgE antibodies of serum 4 (Figure 2b). Significant variations in IgE binding could be demonstrated between peptides AA 103 to 122 and AA 104 to 123 of β-CN B, comprising the digested peptide AA 103 to 123, and peptides AA 108 to 127 and AA 110 to 129 of β-CN A^1 and A^2, representing the digested peptide AA 108 to 129, where peptides of variant A^1 and A^2 were not recognized by the IgE antibodies of serum 4.

Among the variant-specific κ-CN peptides, marked IgE binding was solely found for the κ-CN peptide AA 9 to 26 in serum 4. Nevertheless, the substitution of an arginine (R) by histidine (H) in position 10 of κ-CN F^2 resulted in no significant change in immunoreactivity, as the remaining IgE binding was less than 15% compared with the reference peptide of κ-CN A (Figure 2c).

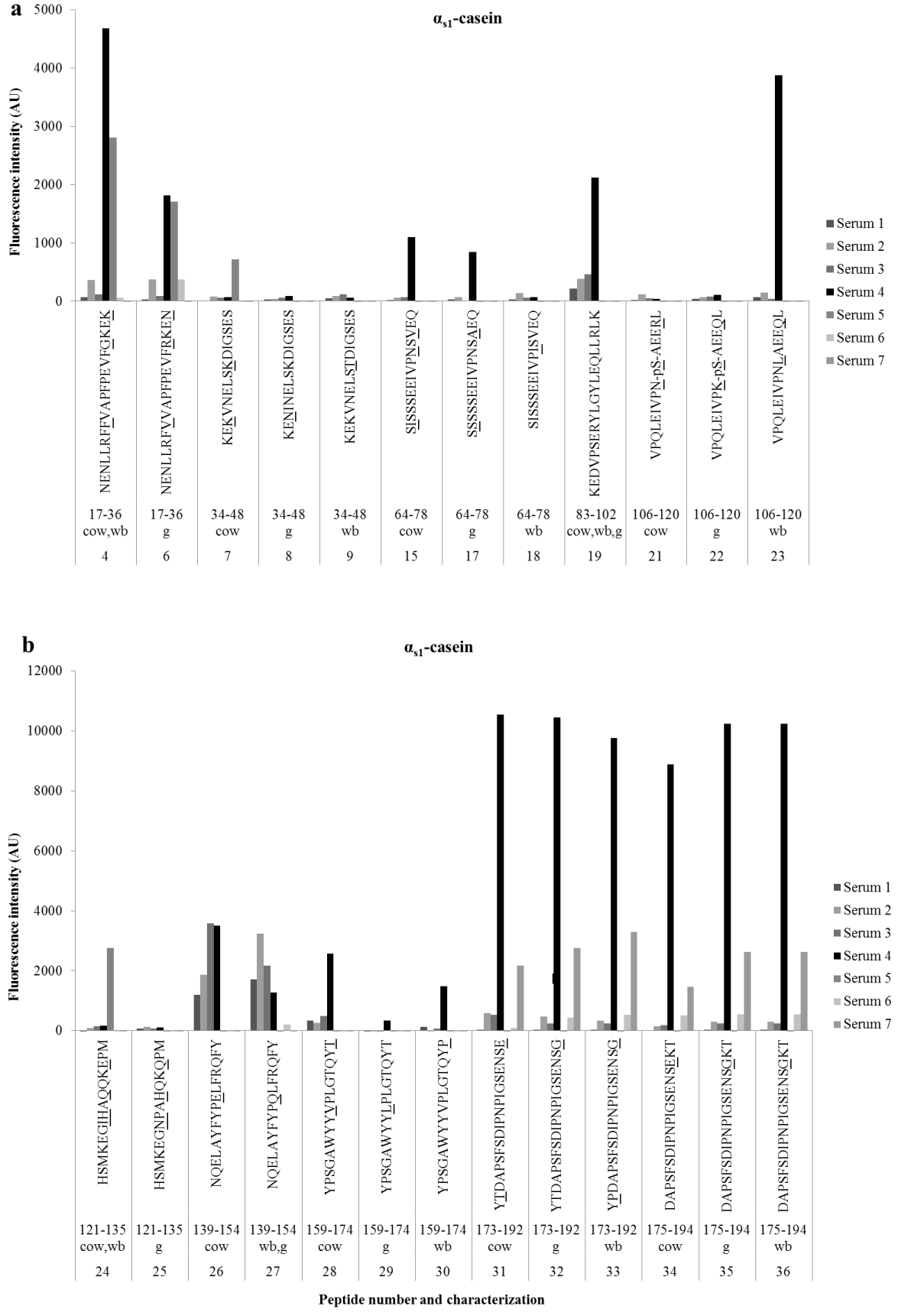
Identification of Interspecies Differences on the Epitope Structure

Due to the high degree of similarity in AA sequence between the known IgE-binding epitopes of caseins from cows and caseins from goats and water buffaloes, differences in their IgE-binding potential were investigated. The majority of sera showed IgE binding to α_{S1}-CN peptides of cows and the homologous counterpart of goats as well as water buffaloes. Some sera bound exclusively to peptides of cows, water buffaloes or goats. As illustrated in Figure 3a, b, and c, only the immunoreactivities of α_{S1}- and β-CN peptides were affected by the variation in the epitope sequences occurring in the different species. In most sera, a significant modification in IgE binding between epitopes of cows and the other species occurred if the peptides differed from each other in at least 2 AA. Nevertheless, in some epitopes, 1 AA was sufficient to cause differences in the immunoreactivities between the epitopes of the species.

For several sera, marked diminished IgE binding (≥15% of IgE binding remaining compared with the reference peptides of cows) to α_{S1}-CN peptides AA 17 to 36 (sera 4 and 5), AA 64 to 78 (serum 4), AA 139 to 154 (sera 3 and 4) from goats as well as to α_{S1}-CN peptides AA 139 to 154 (sera 3 and 4) and AA 173 to 192 (serum 2) from water buffaloes was found (Figure 3a and b). In contrast, the α_{S1}-CN peptides AA 139 to 154 (sera 1 and 2), AA 173 to 192 (serum 7), and AA 175 to 194 (serum 7) of goats and water buffaloes were characterized by marked increased IgE binding compared with the peptides of cows.

Individual sera analyses recognized the cow α_{S1}-CN peptides AA 34 to 48 (sera 2 and 3), AA 121 to 135 (serum 5), AA 159 to 174 (sera 1, 3, and 4), and AA 173 to 192 (sera 2 and 3). However, they had no or less IgE binding to the homologous peptides of goats and water buffaloes. In contrast, 3 sera showed IgE binding solely to the α_{S1}-CN peptides AA 17 to 36 (serum 6), AA 173 to 192 (serum 6), AA 175 to 194 (serum 2), and AA 106 to 120 (serum 4) of goats or water buffaloes, or both, but not to the reference peptides of cows. In 3 sera, no significant differences in immunoreactivity were found between the α_{S1}-CN peptides AA 17 to 36 (serum 2), AA 173 to 192 (sera 2 and 4), and AA 175 to 194 (sera 4 and 6) of the 3 species.

For β-CN, significant IgE binding was seen for peptide AA 16 to 35 of goats and water buffaloes (sera 4 and 7), as indicated in Figure 3c. Additionally, serum 4 reacted with the β-CN peptide AA 55 to 72 of cows and water buffaloes. However, no reaction against the homologous peptide of goats could be observed. On the other hand, significant IgE binding was only detected for peptide AA 135 to 149 (serum 4) of goats.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α_{s1}-casein
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Serum 2
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Serum 4
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Serum 6
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Fluorescence intensity (AU)
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Serum 2
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Serum 4
Serum 5
Serum 6
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Peptide number and characterization

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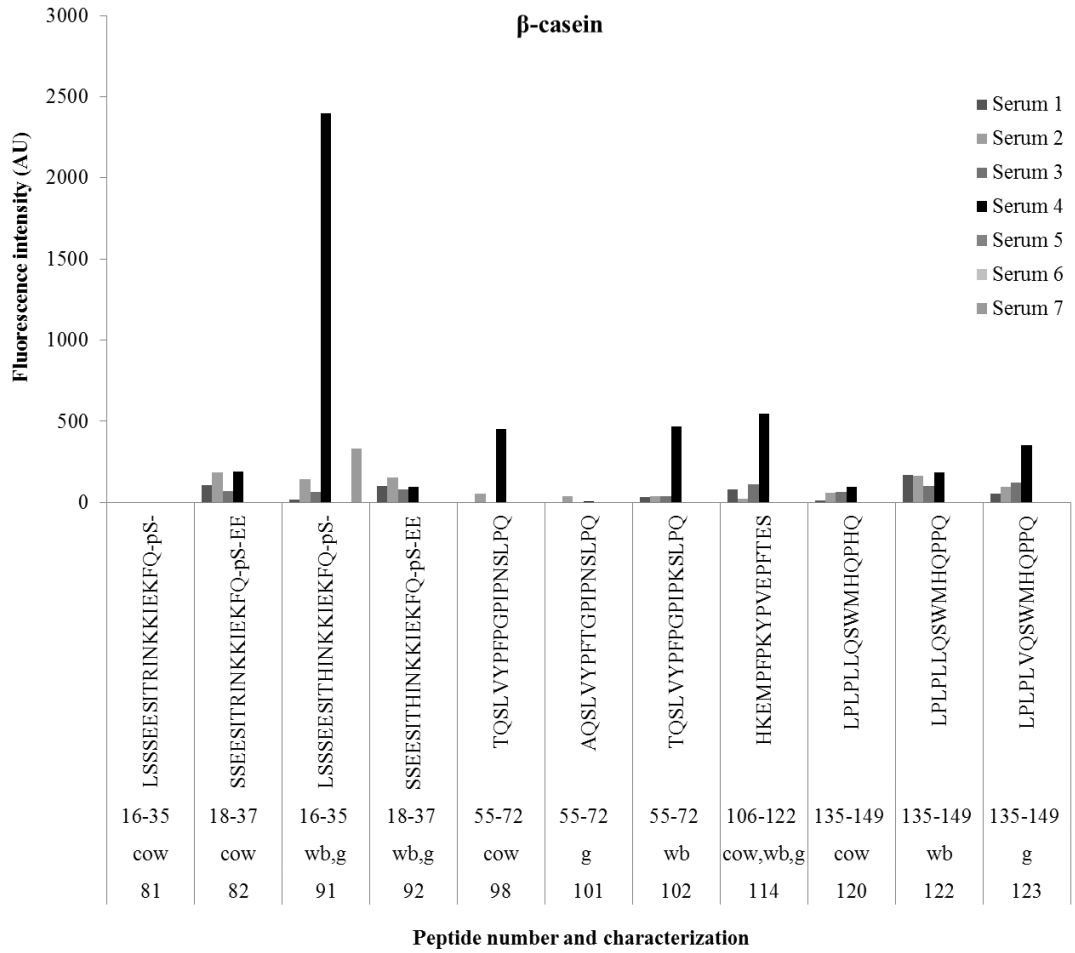

Figure 3. Differences in IgE binding between epitopes (represented by AA residues, number, and species) of α_{s1}-CN (a and b) and β-CN (c) from cows, goats, and water buffaloes, using 7 sera with cow milk-specific IgE values >100 kU_A/L, where UA = allergen-specific units. Modifications in AA sequence between peptides of the 3 species are underlined. The mean fluorescence intensity (arbitrary units, AU) of the 3 subarrays on the microarray for each peptide is presented. g = goat; wb = water buffalo.

DISCUSSION

Identification of IgE-Binding Epitopes in Patient Sera Samples

In this study, each individual serum had a heterogeneous IgE recognition pattern in terms of diversity and intensity of epitope binding. This agrees with previous observations and might be associated with clinical features of CMA, such as the course or the severity of allergic reactions (Savilahti et al., 2010; Wang et al., 2010). Most sera were monosensitized toward α_{S1}-CN, as IgE binding was mainly seen for α_{S1}-CN peptides, which is consistent with the fact that α_{S1}-CN has been described as the main allergen among the 4 caseins (Docena et al., 1996; Gaudin et al., 2008, Schulmeister et al., 2009).

The strongest fluorescence intensities and binding to most of casein peptides were obtained with serum 4. Nevertheless, for most samples, weak signal intensities on the peptide microarray were observed, but showed significant higher signals compared with noise. Conceivably, this is caused by IgG antibodies, which compete with the binding of serum IgE to the specific epitope peptides. It has been demonstrated that IgE and IgG antibodies recognize the same or similar epitopes on the various CMP (Chatchatee et al., 2001a,b; Savilahti et al., 2010). Due to this fact, it also might be that additionally to serum 4, further sera would react with other α_{S2}-, β-, or κ-CN peptides. Therefore, the possible competing interactions between IgE and IgG antibodies had to be considered in further analyses.

Identification of Variant-Specific Differences on the Epitope Structure

To date, the only treatment of CMA is strict avoidance of all CMP, including CMP-derived infant formulas and other dairy products. However, this carries the risk of inadequate nutrient intake, such as calcium or a negative calorie:protein ratio and AA composition (Fiocchi et al., 2010). Thus, the identification of a suitable protein source or safe cow milk substitutes is an important aim for pediatricians and nutritionists to ensure a balanced diet for CMA patients. Depending on the nature of the allergy and as a consequence of the specific sensitization pattern of an individual, the best alternative diet has to be defined for each patient (Restani et al., 2009; du Toit et al., 2010). In the present study, it was demonstrated that the genetic polymorphism has influence on the immunoreactivity of some casein epitopes and peptides resisting gastrointestinal digestion, although with significant differences between individual sera (Table 2). A marked heterogeneity in IgE binding was seen for the variant-specific epitopes AA 4 to 23 of α_{S1}-CN A as well as AA 173 to 192 and AA 175 to 194 of α_{S1}-CN C and E because the deletions and substitutions resulted in abrogation or decreased or increased IgE binding in particular sera. This is of importance, as the corresponding epitopes AA 17 to 36 and AA 173 to 194 of the reference protein α_{S1}-CN B were identified as immunodominant IgE-binding regions, not only in this study but also by Chatchatee et al. (2001a), because they were recognized with the highest IgE-binding intensities in the majority of patients. Therefore, abrogated or increased IgE binding to both epitopes in α_{S1}-CN A, C, and E may change the allergenicity of the whole proteins in most patients. In contrast, epitope AA 83 to 102 of α_{S1}-CN I showed almost identical IgE binding between individual sera. The AA substitution led to a significant increase in IgE binding in 3

of the 7 sera. This is quite surprising, as the substituted AA, glutamic acid (E), and aspartic acid (D) are both polar, acidic, and have the same hydrophobicity and, consequently, no change in the immunoreactivity between epitope AA 83 to 102 of α_{S1}-CN I and B may be expected. However, in this epitope, the substitution is located at position 84 at the N terminus of the epitope, which confirms the observation of Cocco et al. (2003). They reported that substitutions of hydrophobic AA in the central portion of epitopes in α_{S1}-CN were most likely to induce a loss of antibody binding, whereas a small increase in IgE binding was observed for some substitutions at the periphery of the epitope.

For the majority of variant-specific peptides, no significant differences in IgE binding could be found compared with the corresponding reference peptides. This can be explained by the fact that the occurrence of only 1 AA substitution in these epitopes is not sufficient to alter their IgE-binding properties. In general, all sera showed variations in the IgE-binding intensities when peptides differed from each other by 2 AA. For example, with serum 4, more IgE binding was determined for peptides AA 173 to 192 > AA 175 to 194 > AA 174 to 193 of α_{S1}-CN B and for peptides AA 173 to 192 > AA 175 to 194 > AA 179 to 198 of α_{S1}-CN C. This indicates that for α_{S1}-CN, a minimum of 2 AA have to be changed to alter the immunoreactivity in the majority of epitopes, which is in agreement with the results of the mutational analysis of α_{S1}-CN by Cocco et al. (2003). However, for the bulk of epitopes in β- and κ-CN, as well as in the peanut allergens Ara h1, Ara h2, and Ara h3, 1 AA substitution has been detected to be sufficient for inducing a significant modification in IgE binding (Burks et al., 1997; Stanley et al., 1997; Cocco et al., 2007; Han et al., 2008).

Some of the α_{S1}- and β-CN peptides, which were found to resist in vitro gastrointestinal digestion (Lisson et al., 2013, 2014), showed positive IgE binding on microarray immunoassay, suggesting that these digested low-molecular weight peptides retain an allergenic potential, as was also described for other food allergens (Bogh et al., 2009, 2012; Martos et al., 2012). In the current study, peptides 13 AA in length, such as AA 184 to 196 of α_{S1}-CN B as well as AA 180 to 193 of α_{S1}-CN B and C, displayed significant IgE binding on the peptide microarray, even if immunoreactivity was lower compared with the longer peptides (e.g., AA 173 to 192 or AA 175 to 194). It is generally thought that peptides have to be approximately 3,500 Da (30 AA residues) in length and must contain at least 2 IgE-binding epitopes to induce cross-linking of the IgE receptors for stimulating an immune response (Huby et al., 2000). Nevertheless, it has been reported that immunization with free peptides

as small as 6 to 14 AA long induced an acceptable antibody response (Atassi and Young, 1985; Muller, 1999). This was also demonstrated for the peanut allergen Ara h1, where peptides of less than 2,000 Da were able to sensitize and elicit allergic reactions (Bogh et al., 2009, 2012). Results of the present study suggest that this could also apply to casein peptides. Thus, they probably do not need to survive the digestion process as large fragments to react with the immune system, which might contribute to the high sensitization rate to the caseins and explain why caseins are such potent allergens despite their excellent digestibility.

One limitation of the current study was the small number of tested sera, most of which reacted solely with α_{S1}-CN. Among the 16 sera used in this study, 7 displayed positive IgE binding on the microarray and only 2 sera exhibited positive IgE binding to β- and κ-CN peptides. Thus, it is entirely possible that some epitopes of β- and κ-CN variants differ in their allergenic properties when tested with other sera. All sera had milk-specific IgE ≥95.5 kUA/L, meaning that low or lacking signals on the microarray were due to confounding factors in the methods rather than the quality of the sera. To evaluate differences in immunoreactivity between the variant-specific epitopes, further investigations with a larger number of patient sera known to have IgE against both proteins are needed.

In this study, decreased, abrogated, or even higher IgE binding to the immunodominant epitopes AA 17 to 36, AA 173 to 192, and AA 175 to 194 in α_{S1}-CN A, C, or E was identified (Table 2). For example, serum 2 showed significant lower IgE binding to peptide AA 4 to 23 of α_{S1}-CN A than to peptide AA 17 to 36 of α_{S1}-CN B, C, and E. Furthermore, peptide AA 175 to 194 of variant A, sharing the same sequence with peptide of α_{S1}-CN B, was no longer recognized from IgE antibodies of serum 2, whereas significant IgE binding was found to the corresponding peptide of α_{S1}-CN C and E. As a consequence, it is likely that the decreased and abrogated IgE binding to these 2 immunodominant epitopes in α_{S1}-CN A changed the allergenicity of the whole protein and, thus, milk from cows with genotypes α_{S1}-CN AA might be characterized by a lower or, as was detected for sera 4 and 6, higher allergenicity. This could be also relevant for cows carrying the genotype α_{S1}-CN CC and EE regarding the differences in IgE binding to epitopes AA 173 to 192 and AA 175 to 194. In the case of reduced allergenic potential, these protein variants might be suitable for the production of hypoallergenic milk or as a basis for infant formulas for a well-selected group of allergic patients monosensitized to α_{S1}-CN. This does not necessarily mean that whole milk has to be

used, but rather individual milk protein fractions, which could be used for specific processed products. To achieve this aim, selection and breeding of particular cows with genotypes α_{S1}-CN AA, CC, or EE would be necessary. However, α_{S1}-CN A is rather common, but occurs with low frequencies in Holstein-Friesian and German Red cows (Erhardt, 1993). In contrast, α_{S1}-CN C is very common in all main breeds, with higher frequencies such as 0.45 in Jerseys or 0.2 in Pinzgauers and α_{S1}-CN E has been reported only in *Bos grunniens* (Caroli et al., 2009). There is already a kind of milk with a specific genotype on the market. Since 2003, A^2 milk, containing only the A^2 variant of β-CN, has been marked in New Zealand and Australia because epidemiological evidence implies that A^2 milk is better for human health than A^1 milk (A2 Corporation Ltd., Auckland, New Zealand), although the European Food Safety Authority could not establish a relationship between A^1 milk and increased disease (EFSA, 2009). Because of the fact that IgE antibodies from individual patients also displayed increased binding to several mutated epitopes in the current study makes it difficult to consider specific genetic variants as a safe protein source for all patients with CMA.

Identification of Interspecies Differences on the Epitope Structure

In the quest to identify alternative protein sources for patients with CMA, there has been an increasing focus on the use of proteins from other species. Nevertheless, clinical cross-reactivity exists between milk from cows, sheep, goats, and water buffaloes (Järvinen and Chatchatee, 2009; Restani et al., 2009), which is due to the high degree of AA sequence homology between the proteins from cows and the other Bovidae (96.1% for buffaloes, 91.1% for sheep, and 87.6% for goats; Restani et al., 2009). Most patients who are sensitized to cow milk do not tolerate goat or sheep milk (Bellioni- Businco et al., 1999; Carroccio et al., 1999). In the present study, we found that several sera reacted with both cow peptides and the corresponding peptides of goats and water buffaloes, demonstrating cross-reactivity or co-sensitization. However, in the majority of sera, individual epitopes from goats and water buffaloes showed lower immunoreactivity compared with epitopes from cows, which suggests reduced allergenic activity of α_{S1}-CN from goat. These findings are in agreement with the results of other studies (Spuergin et al., 1997; Bevilacqua et al., 2001; Lara-Villoslada, et al., 2004; Sanz Ceballos et al., 2009; Hodgkinson et al., 2012). The lower allergenic potential of goat milk could be attributable, at least in part, to the high genetic polymorphism of α_{S1}-CN that occurs also in goat milk proteins. In goat milk, genetic variants

of α_{S1}-CN are characterized by high (3.5 g/L), medium (1.1 g/L), low (0.45 g/L), and null quantities of this protein (Ballabio et al., 2011). Several studies demonstrated that goat milk with low or null α_{S1}-CN content has reduced allergenic potential. Ballabio et al. (2011) investigated the allergenic potency of goat milk with different α_{S1}-CN genotypes. They detected lower immunoreactions to α_{S1}-CN 0_10_1 (null content of α_{S1}-CN) and 0_1F (reduced content of α_{S1}-CN) genotypes compared with goat milk samples containing α_{S1}-CN BB, BE, EE, or FF. By using guinea pig models (Bevilacqua et al., 2001; Sanz Ceballos et al., 2009) and murine models (Lara-Villoslada et al., 2004; Hodgkinson et al., 2012), it could be also shown that milk from goats producing low levels of α_{S1}-CN is potential less allergenic than milk from cows or goats containing higher levels of this protein. This may be one reason why some cow milk-allergic children are able to tolerate goat milk (Restani, 2004).

Moreover, in the present study, individual sera IgE only recognized α_{S1}-CN and β-CN peptides of goats or water buffaloes but not the corresponding cow milk peptides. This indicates higher allergenicity of both proteins from the nonbovine species and might explain the observation that some patients have a selective allergy to goat or sheep milk but not to cow milk, as has been reported by others (Alvarez and Lombardero, 2002; Bidat et al., 2003; Ah-Leung et al., 2006; Mori et al., 2013).

Interestingly, in serum 4, significant IgE binding was found to the α_{S1}-CN peptide AA 106 to 120 of water buffaloes. In comparison with the corresponding peptides of cows and goats, showing no IgE binding in any serum, this peptide lacks a phosphorylated serine at position 115. Conflicting results exist about the role of phosphorylation in allergy. Cases et al. (2011) demonstrated that phosphorylation reduces the allergenicity of cow caseins in children with selective allergy to goat and sheep milk. In contrast, it has been assumed that the major phosphorylation sites act as important allergenic epitopes in the caseins, as dephosphorylation significantly diminish their allergenicity (Bernard et al., 2000), which is in concordance with the results of the present study. Twenty peptides were synthesized in phosphorylated and dephosphorylated form, but only 1 peptide (AA 106 to 120 of α_{S1}-CN) revealed differences in immunoreactivity. The other peptides displayed no significant IgE binding on the microarray immunoassay. A possible explanation is that 17 of the 20 peptides were from α_{S2}-, β-, and κ-CN and, therefore, not recognized by the IgE antibodies because most sera tested in this study were sensitized against α_{S1}-CN.

CONCLUSIONS

Genetic polymorphisms of caseins influence the allergenic potential of some immunodominant epitopes. Single AA substitutions or deletion resulted in a loss or decrease or increase in immunoreactivity in some α_{S1}- and β-CN epitopes with the tested sera. The genetic polymorphism of caseins should be taken into account in the search for a suitable protein source for CMA patients. However, a heterogeneous pattern of IgE binding to the variant-specific peptides was found between the individual sera. Thus, further clinical studies with more patient sera are needed to verify their suitability for CMA patients. The results of the present study confirm that the milk of goats and water buffaloes harbor an allergenic potential due to cross-reactivity between the proteins and are, consequently, not an appropriate replacement for patients with CMA. Nevertheless, these species are also characterized by genetic polymorphisms that affect the allergenic properties of their IgE-binding epitopes, as has been already shown for goat milk.

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5. General discussion of the results

5.1. In vitro gastrointestinal digestion analyses of casein variants

The question what makes some dairy proteins allergens still remains open but abundance in the food supply, durability during food processing, and resistance to digestion in the gastrointestinal tract are thought to be important factors (Bredehorst & David, 2001). Thus, the physical behavior of allergens during gastrointestinal digestion is of especial relevance to assess their potential allergenicity (Astwood et al., 1996). Despite their physiological importance, the implementation of in vivo assays is hardly to achieve due to ethical reasons, technical constraints, costs, impracticability of large studies, and high inter-individual variability. Therefore, in vitro digestion systems mimicking the physiological process of gastrointestinal digestion are an alternative tool to investigate the digestibility of proteins (Wickham et al., 2009; Ménard et al., 2014). This work compared the digestibility of common α_{S1}-, α_{S2}, β-, and κ-CN variants using an in vitro digestion model according to Moreno et al. (2005a) that involve an gastric and subsequent duodenal stage with digestive enzymes in physiological concentrations, a good adaption of pH and enzyme/substrate ratio, as well as surfactants like bile salts (Moreno et al., 2005a; Moreno, 2007). Recently, it has been demonstrated that this model of in vitro digestion utilizing commercial non-human enzymes provides a good estimation of the gastrointestinal digestibility and potential allergenicity of proteins as marked similarities in the results obtained with an in vitro digestion system using human oral and gastrointestinal digestive fluids were found (Benedé et al., 2014). In accordance with previous studies (Astwood et al., 1996; Fu et al., 2002; Dupont et al., 2010a,b), the caseins were rapidly digested and no intact proteins were detected at the end of gastric digestion by matrix assisted laser desorption ionisation-time of flight-mass spectrometry (MALDI-TOF-MS). After duodenal digestion, the greatest number of peptides was identified for β-CN $A^{1,}$ A^2 and B with 93 fragments and molecular weights up to 3,039 Da. A lot of these peptides were either totally or partially coincident with fragments previously determined after gastroduodenal hydrolysis of β-CN using a similar model of in vitro digestion (Dupont et al., 2010a,b; Benedé et al., 2014). There are some studies describing the digestion of β-CN with physiologically relevant in vitro models (Mandalari et al., 2009a; Defernez et al., 2010; Dupont et al., 2010a; Picariello et al., 2010; Stanic et al., 2010; Benedé et al., 2014), whereas comparable studies investigating the in vitro digestion of isolated α_{S1}-, α_{S2}, and κ-CN fractions are lacking. The particular interest in β-CN might be explained by the

fact that it is known to be an important allergen binding IgE in more than 50% of CMA patients (Docena et al., 1996; Shek et al., 2005). Moreover, it is the most important precursor of peptides including a huge number of bioactive peptides with various activities (Boutrou et al., 2013). Furthermore, the discussion about the health implications of the bioactive peptide β-casomorphin-7 in milk containing the β-CN variant A^1 may contribute to the focus on this protein (Bell et al., 2006; EFSA, 2009).

At the end of duodenal digestion, 58 fragments with molecular weights up to 2,998 Da were determined for α_{S1}-CN B and C, 67 fragments with up to 2,556 Da for α_{S2}-CN A and B, as well as 69 fragments with up to 3,123 Da for κ-CN A, B, and E covering almost the entire protein sequences. Some of these α_{S1}-, α_{S2}-, and κ-CN peptides could also be found in a study dealing with the in vitro digestion of whole raw, pasteurized, and sterilized milk, as well as yoghurt (Dupont et al., 2010b). Nevertheless, existing differences in the α_{S1}-, α_{S2}-, and κ-CN peptide patterns between both studies are due to the fact that Dupont et al. (2010b) used whole and processed milk for digestion instead of purified casein fractions as analyzed in this work. The investigation of whole and processed milk has the advantage to take into account the influence of the dairy matrix, food processing, and potential interaction with other milk ingredients on the protein digestibility (Moreno, 2007). Thus, Dupont et al. (2010b) demonstrated that milk processing, and particularly heat treatment of milk, increases the resistance of caseins to digestion. The authors made the generation of heat-induced aggregates of α_{S2}- and κ-CN with whey proteins through the formation of disulphide bridges responsible for this observation resulting in an increased protection of the caseins against digestion. Moreover, it has been shown that proteolysis of milk proteins can be affected as a result of interaction with food ingredients. Phosphatidylcholine (PC), which is both abundant in milk and secreted by the stomach itself, has a protective effect against digestion of α-La and β-LG (Moreno et al., 2005b; Mandalari et al., 2009b), whereas the presence of PC did not affect the digestibility of the caseins (Mandalari et al., 2009a; Defernez et al., 2010). The presence of polysaccharides such as gum arabic, pectins, or xylan has also been found to reduce the proteolysis of β-LG (Mouécoucou et al., 2004).

However, for a basic screening and to estimate the protein persistence of food allergens, in vitro digestion assays are commonly performed with purified proteins (Moreno et al., 2005a,b; Eiwegger et al., 2006; Smole et al., 2008; Mandalari et al., 2009a; Dupont et al., 2010a). Furthermore, the use of purified proteins allows a more detailed and easier

identification of the peptide profile by mass spectrometry due to the reduction of spectra complexity and suppression effects as well as the avoidance of overlapping isotopic distributions (Schmelzer et al., 2007). Protein digestion in the human gastrointestinal tract is a complex process and associated with large inter-individual differences in gastric and duodenal secretion and with intra-individual variations that are caused by the age and health status of a person as well as the type and amount of food consumed (Benedé et al., 2014). Nevertheless, to perfectly reflect what happens with milk proteins during digestion, there is a need to develop effective in vitro digestion models that mirror the conditions and processes occurring actually in vivo and take into account the impact of the food matrix and food processing (Dupont et al., 2010a).

Despite the significant enzymatic hydrolysis of the α_{S1}-, α_{S2}-, β-, and κ-CN variants during gastrointestinal digestion, peptides were of sufficient size to contain intact IgE-binding epitopes or at least substantial parts thereof. It could be demonstrated that 3 previously identified IgE-binding epitopes in α_{S1}-CN (AA 109-120, AA 123-132, and AA 173-194), 3 in α_{S2}-CN (AA 1-20, AA 105-114, and AA 117-128), 6 in β-CN (AA 45-54, AA 55-70, AA 83-92, AA 107-120, AA 149-164, and AA 167-184), and 5 in κ-CN (AA 21-44, AA 67-78, AA 95-116, AA 111-126, and AA 137-148) survived the in vitro gastrointestinal digestion. The exact lower size limit of peptides triggering an allergic response is not known but it has been assumed to be approximately 30 AA residues corresponding to a molecular weight of about 3,000 Da. Furthermore, these peptides must contain at least 2 IgE-binding epitopes, each of which with a minimum of 15 AA in length, to enable antibody binding and the induction of cross-linking of IgE-receptors (Huby et al., 2000). This means that the allergen or fragments thereof have to be of sufficient size to retain IgE-binding and T-cell stimulating capacity in order to sensitize the mucosal immune system and to cause an allergic reaction (Breiteneder & Mills, 2005; Wickham et al., 2009). In the present work, peptides comprising IgE-binding epitopes of α_{S1}-, α_{S2}-, β-, and κ-CN variants and resisting gastrointestinal digestion range from 12 to 25 AA in length suggesting that these fragments are in theory too small to contain more than 1 IgE-binding epitope and are therefore incapable of causing mast cell degranulation. Nevertheless, immunization with free peptides that are 6 to 14 AA in length has been described to induce acceptable antibody responses (Atassi & Young, 1985). Corresponding results were obtained in a study on sensitizing capacity of the digested peanut allergen Ara h 1 showing that this protein retained both the sensitizing and eliciting

abilities when digested into small peptide fragments of less than 2,000 Da (Bøgh et al., 2009). Aggregation of peptides to complexes of larger sizes is thought to be responsible for the sustained allergenic capacity of digestion products of allergens indicating that the size of the breakdown products is not a determining factor to mount an immune response (Bøgh et al., 2009, 2012). Similarly, this may also apply to casein peptides and would imply an allergenic activity of small fragments comprising only 1 or no intact IgE-binding epitope. Accordingly, this might be one explanation for the high allergenicity and sensitizations rate to the caseins despite their excellent digestibility.

To our knowledge, the first digestion study with genetic casein variants was made within this work. The genetic polymorphisms have an influence on the arising peptide pattern of the α_{S1}-, α_{S2}-, β-, and κ-CN variants as common or different peptides between these variants could be identified. Thereby, the AA substitutions contribute to the formation of distinct peptides in several ways. Due to the AA exchange, specific cleavage sites for the enzymes disappear as it was seen for peptides AA 193-199 of α_{S1}-CN B and AA 9-21 of α_{S2}-CN B. These peptides could not be detected in the digests of α_{S1}-CN C, respectively, α_{S2}-CN A. Furthermore, the AA substitutions may change the recognition sequence of the enzymes or contribute to steric hindrance, which impedes the access for the proteases and result in different peptides between the variants. In consequence, the IgE-binding epitopes are also affected because they correspond to these peptides or are parts thereof. Thus, for α_{S1}-CN B and C, 2 peptides (AA 174-193 of α_{S1}-CN B and AA 179-198 of α_{S1}-CN C) comprising the intact IgE-binding epitope AA 173-194 were found (Chatchatee et al., 2001a). In α_{S2}-CN, peptide AA 1-20 has been reported as a major IgE-binding epitope, which correspond to peptide AA 1-22 of α_{S2}-CN B and to a large portion of peptide AA 7-29 of α_{S2}-CN A (Cerecedo et al., 2008). Peptides AA 59-72, AA 59-80, and AA 58-80 of β-CN A^1, A^2, and B, peptides AA 103-123 and AA 109-123 of β-CN B, as well as peptides AA 108-129 and AA 110-125 of β-CN A^1 and A^2 contained the IgE-binding epitopes AA 55-70, respectively, AA 107-120 (Chatchatee et al., 2001b). For κ-CN, the IgE-binding epitope AA 137-148 survived gastrointestinal digestion within peptides AA 136-149 of κ-CN A and E and peptide AA 134-150 of κ-CN B. It is interesting to note that identical or similar peptides were observed in regions AA 172-198 of α_{s1}-CN as well as AA 57-80 and AA 106-125 of β-CN in an in vivo study on the hydrolysis of milk caseins in jejunal effluents collected from healthy human subjects (Boutrou et al., 2013). Moreover, investigations about the in vitro digestibility of β-CN with simulated and

human oral and gastrointestinal fluids also demonstrated the presence of peptides AA 59-68, AA 59-72, AA 59-80, and AA 108-125 after digestion (Benedé et al., 2014). The similarities between the α_{S1}- and β-CN peptides released by in vivo and in vitro digestion indicates that the in vitro model by Moreno et al. (2005a) used in this work provides an adequate estimation of the digestibility of the caseins as it was already assumed by Benedé et al. (2014). To assess the allergenic potential of the gastrointestinal digestion products of the α_{S1}-, α_{S2}-, β-, and κ-CN variants containing the specific AA substitutions, their IgE-binding properties were analyzed by microarray immunoassay with sera from CMA patients. The major immunoreactive fragments of α_{S1}-CN were peptides AA 174-193 of α_{S1}-CN B and AA 179-198 of α_{S1}-CN C in terms of frequency and intensity of IgE-binding, which is probably due to the fact that both peptides comprise the intact immunodominant epitope AA 173-194 (Chatchatee et al., 2001a). However, also peptides AA 184-196, AA 187-199, and AA 180-193 of α_{S1}-CN B and C showed a significant IgE-binding albeit with lower intensities. This confirms an allergenic potential of small peptide fragments containing no full epitope anymore and agrees mainly with the results found after gastrointestinal digestion of other food allergens such as peanut, egg, or yellow mustard (Eiwegger et al., 2006; Bøgh et al., 2009; Martos et al., 2012; Sirvent et al., 2012). Thus, it can be supposed that these proteins do not need to survive the digestion process as large fragments to interact with the immune system, which implies that the correlation between resistance to digestion and allergenicity is not a generally parameter for the risk assessment of the allergenic potential of a protein (Bøgh et al., 2009, 2012). For the variant-specific gastrointestinal digestion products of β-CN, a significant IgE-binding was detected for peptides AA 110-125 of β-CN A^1 and A^2 as well as AA 109-123 and AA 103-123 of β-CN B but not for peptides AA 59-68, AA 59-72, AA 59-80, and AA 58-80. The reverse was true in the study of Benedé et al. (2014), where peptide AA 57-68 released from in vitro digestion of β-CN revealed the highest immunoreactivity, whereas peptides AA 106-119 and AA 108-125 of β-CN did not react with any of the sera. The discrepancies might arise from the sera employed in both studies as is was reported that individual sera display a great variability in the IgE epitope profiles (Cocco et al., 2007; Han et al., 2008; Savilahti et al., 2010). There was no IgE-binding to the digestion products of the α_{S2}- and κ-CN variants, which can be explained by the observed monosensitization towards α_{S1}-CN in most of the sera used in this study.

The variant-specific peptides of α_{S1}-CN B and C, β-CN A^1, A^2, and B, as well as κ-CN A, B, and E comprise not only IgE-binding epitopes but are also precursors of peptides, which exhibit a wide range of biological functions such as the antibacterial peptides AA 180-193 of α_{S1}-CN and AA 138-158 of κ-CN, the β-casomorphins 4 to 11 that are a family of opioid peptides resulting from region AA 60-70 of β-CN with a length of 4-11 AA all starting with tyrosine residue in position 60, as well as the anticariogenic phosphopeptides AA 59-78 of α_{S1}-CN and AA 7-24 of β-CN (Malkoski et al., 2001; Monaci et al., 2006; Kamiński et al., 2007; BIOPEP database, 2013). With exception of the β-casomorphins, these bioactive peptides refer to the reference proteins and not to their genetic variants. Thus, the genetic polymorphisms occurring within the antibacterial peptides AA 180-193 of α_{S1}-CN C at position 192 and AA 138-158 of κ-CN B, C, F^1, F^2, G^1, G^2, and J at position 148, respectively, of κ-CN B^2 at position 153 and of κ-CN E at position 155, as well as within the anticariogenic phosphopeptides AA 59-78 of α_{S1}-CN E and J at postiton 59, and AA 7-24 of β-CN D at position 18 change the sequence of these fragments, which might result in the release of peptides with other or modified activities. It has already been demonstrated that the AA substitutions or deletions characterizing the genetic variants influence the manner and activity of bioactive peptides. The opioid and immune suppressant β-casomorphin-7 (AA 60-66) was shown to be yielded only by successive gastrointestinal proteolytic digestion of β-CN A^1, B, C, F, and G but not of the other β-CN variants because they possess a histidine instead of a proline at position 67, which determines the enzymatic cleavage of the peptide bond (Hartwig et al., 1997; Jinsmaa & Yoshikawa, 1999; Kamiński et al., 2007). It has been assumed that a high consumption of milk with β-CN A^1 (B, C, F, and G) carrying the histidine at position 67 increases the risk of human heart disease, atherosclerosis, type 1 diabetes, and sudden infant death syndrome (Bell et al., 2006; Kamiński et al., 2007). In New Zealand and Australia, A^2 milk containing only the A^2 variant of β-CN is already available on the market (A2 Corporation, 2013), although the European Food Safety Authority (EFSA) could not establish a relationship between the dietary intake of β-casomorphin-7 and, in consequence, A^1 milk and any of the indicated diseases (EFSA, 2009). Furthermore, the genetic polymorphisms located within the peptides AA 148-150 of κ-CN B, C, and F^1 and AA 96-99 of κ-CN C and G^1 lead to the existence of 4 distinct angiotensin I converting enhyme (ACE)-inhibitory peptides, which display significant differences in their ACE-inhibitory properties (Weimann et al., 2009). Moreover, it has been demonstrated that the phosphopeptides AA 29-41 of β-CN C and AA

126-135 of α_{s2}-CN C versus the peptides of the other β- and α_{s2}-CN variants differentially affect the calcium deposition in the extracellular matrix (Tulipano et al., 2010).

However, as described above, some of the bioactive peptides are parts of the IgE-binding epitopes and might therefore carry an allergenic potential. Their application in functional foods, nutraceutical products, and cosmetics is associated with an increased risk for allergic reactions triggered by the use of those products from CMA consumers (Monaci et al., 2006). As the appearance, the becoming, or the elimination of bioactive peptides is affected by the genetic polymorphisms, it can be suggested that the same could apply to the allergenic potential of the bioactive peptides.

5.2. Influence of genetic polymorphisms on the allergenicity of milk protein variants

Peptide microarray immunoassays have been shown to be very useful for epitope mapping of milk allergens measuring their IgE and IgG levels and classify them into major and minor allergens (Lin et al., 2009; Hochwallner et al., 2013). The identification of the immunodominant regions in milk proteins by IgE-epitope mapping is of great importance to obtain information about the relationship between allergen structure and immunoreactivity as well as the patient's immune response and may provide an important tool for diagnosis, prognosis, and treatment of CMA (Lin et al., 2009; Savilahti et al., 2010; Wang et al., 2010). By the use of microarray immunoassays with synthetic peptides, representing the previously described IgE-binding epitopes of the caseins as well as their genetic variants, the α_{S1}-CN peptides AA 17-36, AA 139-154, AA 173-192, and AA 175-194 were shown to be the most immunoreactive fragments in terms of frequency and IgE binding, which is in agreement to the results of the studies of Chatchatee et al. (2010a) and Lin et al. (2009). Five out of the 7 sera appeared to be monosensitized towards α_{S1}-CN as it was the most frequently recognized milk allergen, which was also described by others (Docena et al., 1996; Gaudin et al., 2008; Schulmeister et al., 2009). In the current study, further IgE-binding epitopes were recognized by individual sera. Data revealed IgE-binding to epitopes AA 34-48, AA 64-78, AA 83-102, AA 121-135, AA 139-154, and AA 159-174 of α_{S1}-CN, to epitopes AA 55-72 and AA 106-122 of β-CN, as well as to epitopes AA 9-26, AA 24-44, AA 34-53, and AA 49-68 of κ-CN. In contrast, none of the sera reacted with any of the α_{S2}-CN peptides. This is coincident with the results found by Matsumoto et al. (2009), where individual sera showed an IgE-binding

only to epitopes AA 67-82, AA 109-124, and AA 172-188 of α_{S1}-CN, AA 1-16, AA 55-70, AA 106-121, AA 130-145, and AA 148-163 of β-CN, as well as AA 25-40, AA 52-67, AA 67-82, AA 136-151, and AA 154-169 of κ-CN, some of which were also identified in the present study. Nevertheless, Cong et al. (2013) reported deviant findings with respect to the major IgE-binding regions on α_{S1}-CN that were located at AA 6-20, AA 11-25, AA 126-140, and AA 171-185. It is important to note that the IgE recognition patterns and binding affinities for casein-derived peptides are different in the serum for each individual patient and, thus, the results depend heavily on patient selection (Cocco et al., 2007; Matsumoto et al., 2009; Savilahti et al., 2010; Wang et al., 2010).

The present study demonstrated that the genetic polymorphisms characterizing the milk protein variants affect the IgE-binding epitope structure and, in consequence, influence the allergenicity of milk protein variants. Previous studies showed that single or multiple AA substitutions within IgE-binding epitopes of α_{S1}-, β-, and κ-CN, as well as β-LG result in a loss or reduced binding of milk-specific IgE antibodies by pooled or individual sera from CMA patients (Cocco et al. 2003, 2007; Han et al., 2008). In accordance to Cocco et al. (2003, 2007), single AA substitutions or deletions occurring within IgE-binding epitopes or gastrointestinal digestion products of α_{S1}- and β-CN variants led to a loss or reduced or increased IgE-binding in individual sera. Differences in IgE-binding were found between epitopes AA 17-36, AA 4-23, AA 83-102, AA 173-192, AA 175-194, AA 187-199, AA 174-193, and AA 178-198 of the α_{S1}-CN variants A, B, C, E, and I. Modifications in IgE-binding to epitopes of β-CN variants could only be determined for the gastrointestinal digestion products AA 103-123 of β-CN B and AA 108-129 of β-CN A^1 and A^2 that differ in 12 AA from each other. Epitopes AA 55-72 of β-CN A^1, B, C, E, G, H^2, and AA 106-122 of β-CN A^3 and B, as well as the eptiope AA 9-26 of κ-CN F^2 showed no change in IgE-binding compared to the reference proteins β-CN A^2 and κ-CN A, respectively. This agrees with the results of Cocco et al. (2007) as they reported that single AA substitutions were inadequate to eliminate IgE-binding to epitopes AA 57-70 and AA 107-120 of β-CN, as well as AA 11-24 of κ-CN by using individual sera from CMA patients. In the studies of Cocco et al. (2003, 2007) and Han et al. (2008), substitutions of AAs were most likely to eliminate IgE-binding if they were hydrophobic and located in the central portion of the epitope, a finding which could only be partially confirmed within the present study. However, although an IgE-binding to epitope AA 55-72 was observed in all β-CN variants, peptides AA 59-68, AA 59-72, AA 59-80, and AA

58-80, which correspond to products of gastrointestinal digestion of β-CN A^1, A^2, and B, were not recognized by the IgE antibodies of any sera indicating that differences of 4 AA were critical for IgE-binding. One limitation of this study was the small number of tested sera and, hence, for a better assessment of the allergenicity of milk protein variants, a larger group of patients shall be investigated in the following by the means of peptide microarray immunoassay. Nevertheless, as described by others (Cocco et al., 2003, 2007; Han et al., 2008; Savilahti et al., 2010; Wang et al., 2010), all sera displayed a great heterogeneity in IgE-binding and, thus, it strongly depended on the tested serum whether the casein variants had a higher or a lower allergenic potential than the corresponding reference proteins. This is of importance in regard to a conceivable use of milk protein variants with a lower allergenicity for patients with CMA. α_{S1}-CN A seemed to be characterized by a reduced allergenicity in 2 sera showing a lacking or decreased IgE-binding to the immunodominant epitopes AA 17-36, respectively, AA 4-23, AA 173-192, and AA 175-194, whereas α_{S1}-CN C and E tended to have a higher allergenic potential than the reference protein in most sera utilized in this study. However, the reverse case could be observed for 2 other sera and, in consequence, the determination of the individual reactivity profiles of allergic subjects to milk protein variants is necessary to assess their potential tolerability for these patients. The routine application of microarray technology will allow this purpose within a single assay that requires only tiny amounts of patients' sera (Hochwallner et al., 2013).

Allele frequencies of α_{S1}-CN A are generally low but this variant occurs in common (Holstein Friesian or German Red) and not in rare or special breeds (Erhardt, 1993). α_{S1}-CN C is very common and appears with high frequencies in all main breeds, whereas α_{S1}-CN E seems to be specific for *Bos grunniens* (Caroli et al., 2009). Thus, selection and breeding of cows with the particular genotype α_{S1}-CN AA or CC could be practicable. Milk from such cows might be suitable for the production of hypoallergenic milk or infant formulas for a well-selected group of allergic patients' monosensitized against α_{S1}-CN. In addition, the exclusive use of individual milk protein fractions for specific processed products could also be considered as milk proteins are applied as food additives or adjuvants due to their technical functions ranging from thickening, gelation, stabilization, emulsification, and film forming functionalities to tenderization (coffee, beef, bread, and sausages etc.) (Monaci et al., 2006). Before selecting and breeding of animals suitable for the generation of hypoallergenic milk and products thereof, further investigations with large patient groups followed by clinical

studies utilizing SPT and oral challenges are necessary to verify the reactivity to milk with particular genotypes in allergic individuals.

In the context of the identification of an adequate and alternative protein source for patients with CMA, milk from other species has become the focus of increasing interest. Nevertheless, cross-reactivity between milk proteins from cow, sheep, goat, and water buffalo has been described because of the high degree of AA sequence homology between the proteins from cow and the other Bovidae (96.1% for water buffalo, 91.1% for sheep, and 87.6% for goat) (Järvinen & Chatchatee, 2009; Restani et al., 2009). Thus, most of the patients with CMA cannot tolerate goat´s or sheep milk and probably buffalo milk, although, no clinical study has been published on the tolerance to buffalo milk in patients with CMA until now (Bellioni-Businco et al., 1999; Carroccio et al., 1999).

In the present study, we demonstrated that the majority of sera reacted both with α_{S1}- and β-CN peptides of cow as well as the corresponding peptides of goat and water buffalo indicating cross-reactivity. In some sera, the immunoreactivity to most α_{s1}-CN peptides of goat and water buffalo was lower than to those of cow. Accordingly, Spuergin et al. (1997) investigating the cross-reactivity of α-CN among different species showed that the IgE-response to caseins from goat and sheep was weaker than that to cow. Generally, goat milk is the subject of intense discussion regarding a less allergenic potential than cow milk, which is due to the high degree of genetic polymorphisms among goats resulting in different α_{S1}-CN variants that are characterized by high (3.5 g/L), medium (1.1 g/L), low (0.45 g/L), and null contents of this protein (Caroli et al., 2006). Several studies using guinea pig models (Bevilacqua et al., 2001) or murine models (Lara-Villoslada et al., 2004; Hodgkinson et al., 2012) have reported that goat milk with lower α_{S1}-CN level has reduced allergenic effects compared with milk from goat or cow containing higher levels of this protein. Furthermore, Ballabio et al. (2011) performed SPT and in vitro testing with sera from CMA patients to compare the reactivity of milk from goats with different α_{S1}-CN genotypes and found less immunoreaction to 2 goat milk samples characterized by null or low α_{S1}-CN content. Due to this fact, it is likely that milk from goat with α_{S1}-CN null alleles will be tolerated from patient's monosensitized against this protein and may explain why some subjects with CMA can consume goat milk and derivates (Ellis et al., 1991; Restani et al., 2002, 2009). However, Ballabio et al. (2011) also observed an increased immunoreactivity against milk homozygous for the null allele. In the current study, 2 sera showed an exclusive IgE-binding to the

immunodominant α_{S1}-CN epitopes AA 17-36, AA 173-192, and AA 175-194 of goat and water buffalo but not to the corresponding peptides of cow suggesting a higher allergenic potential of this protein in the non bovine species. In this context, several cases of a selective allergy to goat and sheep milk but not to cow milk have been described in children and adults (Alvarez & Lombardero, 2002; La Muñoz Martín et al., 2004; Ah-Leung et al., 2006; Cases et al., 2011; Mori et al., 2013). Combined, these data propose that in general goat milk is not an adequate replacement for patients with CMA but it might be suitable in some cases and, thus, the individual tolerability of goat milk with a particular genotype has to be ascertained individually. Interestingly, Bernard et al. (2012) examined the structural determinants that account for the absence of cross-reactivity between the highly homologous β-CN of cow and goat in patients allergic to goat milk but tolerant to cow milk and found domain AA 49-79 of β-CN from goat of decisive importance. The authors assumed that the 3 AA substitutions, which differentiate peptide AA 49-79 of goat from that of cow, are the reason for the lack of cross-reactivity between these highly homologous allergens. In addition to the results of the present study, the findings of Bernard et al. (2012) confirm that the AA substitutions and deletions have an influence on the allergenicity of milk proteins not only in cows but also in the other Bovidae such as goat and sheep. Moreover, a genetic variability could also be identified within α_{S1}-CN of camel milk (Shuiep et al., 2013) and, recently, for the first time within κ-CN of camel milk (Pauciullo et al., 2013). The constantly growing number of genetic variants in cow and in the other species necessitates their consideration in the field of human nutrition. On basis of in vitro (Restani et al., 1999, 2002) and in vivo studies (Shabo et al., 2005; Ehlayel et al., 2011a), there is some evidence that camel milk might be a promising protein source for patients with CMA as the homology with proteins from cow is only 60%, which corresponds to the lowest level of similarity among the species. Additionally, β-LG, one of the most dominant milk allergen, is not present in camel milk (Restani et al., 2009; Hinz et al., 2012). Further studies on the allergenicity of camel milk are needed as cross-reactivity with cow milk could be observed in 25% of CMA patients, which also tested positive by SPT to camel milk (Katz et al., 2008).

5.3. Concluding remarks and future approaches

With the present study, it could be demonstrated that the genetic polymorphisms influence the allergenicity of milk protein variants. To confirm the observation of an higher or lower

allergenic potential of some α_{S1}- and β-CN variants made in this study and to clarify whether the immunoreactivity of epitopes in the α_{S2}- and κ-CN variants as well as the α-LA and β-LG variants are affected by the genetic polymorphisms, a larger group of patients with CMA should be tested in the following. Furthermore, the corresponding peptides of all milk proteins together with the genetic variants from goat, sheep, water buffalo, and camel should be included in these investigations to identify potential cross-reactivity as well as differences in the allergenicity between the peptides of cow and the other species. However, the IgE recognition pattern for casein-derived peptides varies in the serum of each patient. Therefore, the determination of the individual reactivity profile for patients with CMA is necessary to assess the possible tolerability of milk protein variants from cow as well as from other species. The characterization of the patients IgE reactivity not only to peptides of the genetic variants but also to the whole proteins inclusive the identification of cross-reactivity's with other proteins would be reasonable and able by the routine application of protein microarray technology. Currently, there is already a commercial protein microarray chip available (ImmunoCAP ISAC-CRD 103, Phadia, Uppsala, Sweden). The ImmunoCAP ISAC Allergen Chip is a miniaturized immunoassay platform that allows the measurement of specific IgE antibodies to 103 allergenic components using only 20 µL of patient sera, which is of particular importance in case of the diagnosis of allergy in infants and children. Purified natural or recombinant major and minor allergen components of the most significant food and inhalation allergens are located on the chip. They include also whole casein, α-LA, β-LG, bovine serum albumin, and lactoferrin but do not represent the genetic variants of milk proteins from cow and the other species. Thus, the development of a custom-made protein array containing all the actually known milk protein variants of cow as well as of goat, sheep, water buffalo, and camel will be required for a first assessment of the individual tolerability of milk with a particular genotype.

6. Summary

Within the screening of milk samples from different bovine breeds, the known genetic casein variants α_{S1}-CN B and C, α_{S2}-CN A, B, and D, β-CN A^1, A^2, and B, as well as κ-CN A, B, and E were confirmed and used for in vitro gastrointestinal digestion analyses. The results showed that, despite the significant enzymatic hydrolysis of the α_{S1}-, α_{S2}-, β-, and κ-CN variants during gastrointestinal digestion, peptides were of sufficient size to contain intact IgE-binding epitopes or at least substantial parts thereof. Three previously identified IgE-binding epitopes in α_{S1}-CN (AA 109-120, AA 123-132, and AA 173-194), 3 in α_{S2}-CN (AA 1-20, AA 105-114, and AA 117-128), 6 in β-CN (AA 45-54, AA 55-70, AA 83-92, AA 107-120, AA 149-164, and AA 167-184), and 5 in κ-CN (AA 21-44, AA 67-78, AA 95-116, AA 111-126, and AA 137-148) survived the in vitro gastrointestinal digestion. Furthermore, it could be demonstrated that the genetic polymorphisms have an influence on the arising peptide pattern as different peptides between the α_{S1}-, α_{S2}-, β-, and κ-CN variants were found. In α_{S1}-CN B and C, the 2 peptides AA 174-193 of α_{S1}-CN B and AA 179-198 of α_{S1}-CN C comprising the intact IgE-binding epitope AA 173-194 were determined. In α_{S2}-CN B, peptide AA 1-22 and in α_{S2}-CN A, peptide AA 7-29 could be detected both corresponding to the major IgE-binding epitope AA 1-20 or parts thereof. Peptides AA 59-72, AA 59-80, and AA 58-80 of β-CN A^1, A^2, and B, as well as peptides AA 103-123 and AA 109-123 of β-CN B, and AA 108-129 and AA 110-125 of β-CN A^1 and A^2 contained the IgE-binding epitopes AA 55-70, respectively, AA 107-120. For κ-CN, the IgE-binding epitope AA 137-148 survived gastrointestinal digestion within peptides AA 136-149 of κ-CN A and E and peptide AA 134-150 of κ-CN B. Microarray immunoassays with sera from patients (n = 7) with CMA indicated an allergenic activity of some of these digested casein fragments. This might be one explanation for the high allergenicity and sensitizations rate to the caseins despite their excellent digestibility. Major immunoreactivity was identified for peptides AA 174-193 of α_{S1}-CN B and AA 179-198 of α_{S1}-CN C but also for the smaller peptides AA 184-196, AA 187-199, and AA 180-193 occurring in both α_{S1}-CN variants. Significant IgE-binding was further detected to the gastrointestinal digestions products AA 110-125 of β-CN A^1 and A^2 as well as AA 109-123 and AA 103-123 of β-CN B. None of the sera used in the present study reacted with any of the digestion products of the α_{S2}- and κ-CN variants.

This study confirmed an influence of the genetic polymorphisms on the IgE-binding properties of epitopes from α_{S1}-, α_{S2}-, β-, and κ-CN variants of cow and revealed differences

in IgE-binding to corresponding epitopes of goat and water buffalo. Single AA substitutions or deletions existing within IgE-binding epitopes AA 17-36, AA 4-23, AA 83-102, AA 173-192, and AA 175-194 of the α_{S1}-CN variants A, B, C, E, and I or gastrointestinal digestion products AA 187-199, AA 174-193, and AA 178-198 of α_{S1}-CN B and C, as well as AA 108-129 and AA 103-123 of β-CN A^1 and A^2, respectively, B led to a loss or reduced or increased IgE-binding with a marked heterogeneity in individual sera. The modifications in the immunoreactivity mainly affected the immunodominat epitopes and, in consequence, it can be accepted that the allergenicity of the whole proteins are altered.

The majority of sera showed IgE-binding to α_{S1}-CN peptides of cow but also to the homologous counterparts of goat and water buffalo indicating cross-reactivity, which is due to the high degree of AA sequence homology between the milk proteins from cow and the other Bovidae. However, individual sera exhibited a lower, higher, or exclusive immunoreactivity to epitopes of α_{S1}- and β-CN from goat and water buffalo.

The results of our investigations support the suggestion that genetic variants differ in their allergenic properties and might therefore provide a new approach for the identification of an alternative and suitable protein source for CMA patients. Furthermore, milk of goat and water buffalo cannot be recommended in general as substitution for cow milk in the nutrition of CMA patients. Nevertheless, the milk proteins of these species are also characterized by genetic polymorphisms, whose effects on IgE-binding and allergenicity have to be defined.

7. Zusammenfassung

Im Rahmen des Screenings von Milchproben verschiedener Rinderrassen wurden die bekannten Kaseinvarianten α_{S1}-CN B und C, α_{S2}-CN A, B und D, β-CN A^1, A^2 und B, sowie κ-CN A, B und E bestätigt und für in vitro gastrointestinale Verdaustudien herangezogen. Die Ergebnisse zeigten, dass die Peptide, trotz der umfassenden enzymatischen Hydrolyse der α_{S1}-, α_{S2}-, β- und κ-CN-Varianten während des gastrointestinalen Verdaus, noch groß genug waren, um intakte IgE-bindende Epitope oder signifikante Teile davon zu enthalten. So überstanden 3 bereits identifizierte IgE-bindende Epitope des α_{S1}-CN (AA 109-120, AA 123-132 und AA 173-194), 3 des α_{S2}-CN (AA 1-20, AA 105-114 und AA 117-128), 6 des β-CN (AA 45-54, AA 55-70, AA 83-92, AA 107-120, AA 149-164 und AA 167-184) und 5 des κ-CN (AA 21-44, AA 67-78, AA 95-116, AA 111-126 und AA 137-148) den gastrointestinalen Verdau unverändert. Zudem konnte nachgewiesen werden, dass der genetische Polymorphismus einen Einfluss auf das entstehende Peptidmuster besitzt, da unterschiedliche Peptide zwischen den α_{S1}-, α_{S2}-, β- und κ-CN-Varianten detektiert wurden. In den α_{S1}-CN-Varianten B und C wurden die 2 Peptide AA 174-193 (α_{S1}-CN B) und AA 179-198 (α_{S1}-CN C) gefunden, die das intakte IgE-bindende Epitop AA 173-194 umfassen. Im α_{S2}-CN B konnte Peptid AA 1-22 und im α_{S2}-CN A Peptid AA 7-29 bestimmt werden, die beide dem IgE-bindenden Hauptepitop AA 1-20 oder Teilen davon entsprechen. Die Peptide AA 59-72, AA 59-80 und AA 58-80 der 3 β-CN-Varianten A^1, A^2 und B sowie die Peptide AA 103-123 und AA 109-123 des β-CN B und AA 108-129 und AA 110-125 des β-CN A^1 und A^2 enthalten die IgE-bindenden Epitope AA 55-70 bzw. AA 107-120. Bei dem κ-CN überstand das IgE-bindende Epitop AA 137-148 den gastrointestinalen Verdau als Bestandteil von Peptid AA 136-149 des κ-CN A und E und von Peptid AA 134-150 des κ-CN B. Mittels Microarray Immunoassay und Seren von Patienten (n= 7) mit CMA konnte bei einigen dieser verdauten Kaseinfragmente eine allergene Aktivität bestätigt werden, was eine Erklärung für die hohe Allergenität und Sensibilisierungsrate gegenüber den Kaseinen trotz ihrer exzellenten Verdaulichkeit sein könnte.

Die Peptide AA 174-193 des α_{S1}-CN B und AA 179-198 des α_{S1}-CN C wiesen die stärkste Immunreaktivität auf, aber auch für die kleineren Peptide AA 184-196, AA 187-199 und AA 180-193 beider α_{S1}-CN-Varianten konnte eine deutliche IgE-Bindung festgestellt werden. Des Weiteren wurde eine signifikante IgE-Bindung an die gastrointestinalen Verdauprodukte AA 110-125 des β-CN A^1 und A^2 sowie AA 109-123 und AA 103-123 des β-CN B identifiziert.

Keines der verwendeten Seren reagierte mit einem der Verdauprodukte der α_{S2}- und κ-CN-Varianten.

Die Ergebnisse der Microarray Immunoassays bestätigten einen Einfluss des genetischen Polymorphismus auf die IgE-bindenden Eigenschaften der Epitope der α_{S1}-, α_{S2}-, β- und κ-CN-Varianten beim Rind und wiesen zudem Unterschiede in der IgE-Bindung zu den entsprechenden Epitopen von Ziege und Wasserbüffel auf. Einzelne Aminosäuresubstitutionen oder -deletionen, die innerhalb der IgE-bindenden Epitope AA 17-36, AA 4-23, AA 83-102, AA 173-192 und AA 175-194 der α_{S1}-CN-Varianten A, B, C, E und I oder der gastrointestinalen Verdauprodukte AA 187-199, AA 174-193 und AA 178-198 des α_{s1}-CN B und C sowie AA 108-129 und AA 103-123 des β-CN A^1, A^2, bzw. B auftraten, führten zu einem Verlust oder einer Abnahme oder einer Zunahme der IgE-Bindung mit deutlichen Heterogenitäten zwischen den einzelnen Seren. Die Veränderungen in der Immunreaktivität betrafen hauptsächlich die immunodominanten Epitope und folglich sind Auswirkungen auf die Allergenität des gesamten Proteins anzunehmen.

Die Mehrheit der Seren zeigte eine IgE-Bindung an die Peptide des α_{S1}-CN vom Rind, aber auch an die homologen Gegenstücke von Ziege und Wasserbüffel, was auf eine Kreuzreaktion hinweist, die durch den hohen Grad der Homologie in der Aminosäuresequenz zwischen den Milchproteinen des Rindes und der anderen *Bovidae* bedingt ist. Allerdings wiesen einzelne Seren eine niedrigere, höhere oder ausschließliche IgE-Bindung an die Epitope des α_{S1}- und β-CN von Ziege und Wasserbüffel auf.

Aus den Ergebnissen dieser Untersuchungen wird deutlich, dass sich die genetischen Varianten in ihren allergenen Eigenschaften unterscheiden. Sie bieten daher einen neuen Ansatz für die Identifikation einer geeigneten Proteinquelle für Patienten mit Kuhmilchallergie. Zudem kann die Milch von Ziege oder Wasserbüffel generell nicht als ein Ersatz für Kuhmilch in der Ernährung von kuhmilchallergischen Patienten empfohlen werden. Allerdings sind auch die Milchproteinvarianten dieser Tierarten durch genetische Polymorphismen charakterisiert, deren Effekte auf die IgE-Bindung und Allergenität der Proteine noch bestimmt werden müssen.

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Ich erkläre:

Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.

Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze zur wissenschaftlichen Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

α_{s1}-casein epitope AA 17-36:

17 36

NENLLRFFVAPFPEVFGKEK α_{s1}-casein B

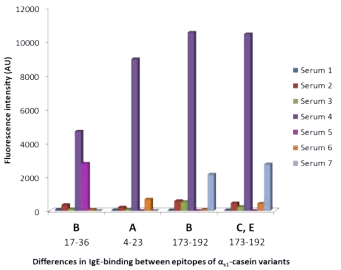
———————— PFPEVFGKEK α_{s1}-casein A

α_{s1}-casein epitope AA 173-194:

173 194

YTDAPSFSDIPNPIGSENSEKT α_{s1}-casein B

YTDAPSFSDIPNPIGSENSGKT α_{s1}-casein C, E



Differences in IgE-binding between epitopes of α_{s1}-casein variants

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