Microbial transglutaminase alters the immunogenic potential and cross-reactivity of horse and cow milk proteins

J. Fotschki,^{1*} B. Wróblewska,¹ B. Fotschki,² B. Kalicki,³ N. Rigby,^{4,5} and A. Mackie^{4,5}

¹Department of Immunology and Food Microbiology, Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-748 Olsztyn, Poland

²Department of Biological Function of Food, Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-748 Olsztyn, Poland

³Department of Pediatrics, Nephrology and Allergology, Military Institute of Medicine, 04-141 Warsaw, Poland

⁴Institute of Food Research, Norwich NR4 7UA, United Kingdom

⁵School of Food Science and Nutrition, University of Leeds, LS2 9JT, United Kingdom

ABSTRACT

Horse milk is a valuable raw material and a very attractive alternative for scientific research to address the issue of cow milk (CM) allergy due to its protein profile. A decrease in immunoreactive properties can be achieved by thermal, enzymatic, and hydrolytic processing. Therefore, the aim of this study was to explore the possibility of reducing the immunoreactivity of horse milk proteins by microbial transglutaminase (TG) polymerization. To determine how TG linking alters immunoreactivity under simulated digestion of the examined milk, analyses were performed before, during, and after digestion. The dose-dependent (1, 10, and 100 U) effects of microbial TG on horse and cow milk were analyzed. A consecutive 3-stage digestion was simulated with salivary, gastric, and intestinal fluids. The effects of digestion were analyzed by SDS-PAGE, particle size analysis, and size-exclusion chromatography. Immunoreactivity was assessed using competitive ELISA (β -lactoglobulin and α -casein) and immunodot (sera from 7 patients aged 3 to 13 years who are allergic to CM proteins). Horse milk contained almost half of the amount of total proteins in CM. The dose 1 U/g of total milk protein changed the immunoreactivity of both cow and horse milk. With increasing TG doses, α -casein immunoreactivity increased, and β -lactoglobulin decreased. After total digestion, horse milk was characterized by 2.4-fold lower average IgE and 4.8-fold lower IgG reactivity than CM. We found that TG alters the IgE and IgG reactivity of CM after in vitro digestion. Horse milk was less reactive to IgE and IgG than was CM, with animal and patient sera.

The effect of TG on immunoreactivity depends on enzyme quantity and milk protein type. The diet based on modified horse milk proteins could be an alternative for some patients with CM protein allergy; however, confirmation through clinical trials is needed.

Key words: simulated gastrointestinal digestion, cow milk protein allergy, microbial transglutaminase, horse milk

INTRODUCTION

Considering the increasing prevalence and nuisance of cow milk allergy (CMA), it is important to examine an alternative source of milk proteins and nutrients with similar immunological effects and nutritional value to cow milk (CM). Horse milk (HM) could be considered a substitute for CM, especially for people who suffer from CMA. Our previous study showed that HM proteins and CM proteins have similar immunoreactive epitopes (Fotschki et al., 2015a). The use of technological processing for modification of HM may lead to the development of a product with decreased immunogenic potential. Therefore, the aim of the present study was to explore the possibility of reducing the immunoreactivity of HM proteins through microbial transglutaminase (TG) cross-linking.

Cow milk allergy is characterized by an abnormal reaction of the immune system following contact with food containing CM proteins. Other than avoidance, no safe diet without the risk of side effects is available for people with CMA (Hochwallner et al., 2014). Among all foods, CM is often the first food introduced into the diet of infants, and CMA affects approximately 2.5% of children (Høst, 2002). Horse milk is currently of scientific interest, as the properties, components, and fractions of HM are highly similar to those of breast milk (Miranda et al., 2004; Potočník et al., 2011). The administration of HM decreased serum IgE levels in mice

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*Corresponding author: j.fotschki@pan.olsztyn.pl

sensitized to CM proteins (Fotschki et al., 2016). Fewer proteolytic-resistant peptides were absorbed from HM than from CM (Fotschki et al., 2015b). High digestibility and an optimum whey protein-to-caseins ratio make a compelling argument for selecting horse milk over the milk from other animals as a dietary substitute for humans (Uniacke-Lowe et al., 2010). The homology of the main proteins in HM and CM is approximately 62%, significantly lower than the homology of the same proteins provided by the milk of other animals (buffalo, sheep, goat, pig, or donkey; Fiocchi et al., 2010). Due to its low protein homology with CM, HM may be characterized by different epitopes responsible for various immune reactions. Moreover, the concentration of caseins in HM is significantly lower than that in bovine milk (approx. 55 vs. 80% of total protein, respectively; Uniacke-Lowe et al., 2010). Due to cross-reactivity, the antibodies present in the serum of people with food allergies can interact with particular proteins that have structures similar to that of the allergen. Consistent with the data from Businco et al. (2000), HM is tolerated by 96% of studied children with CMA, and with appropriate modifications, HM can be regarded as a good substitute for CM for most children with severe IgE-mediated CMA.

A considerable number of scientific studies have attempted to reduce the antigenicity of food proteins by implementing different technologies such as polymerization, heat treatment, hydrolysis, fermentation, high-pressure processing, and microwave radiation (Sabadin et al., 2012; Wróblewska et al., 2016; Golkar et al., 2019). Transglutaminase cross-linking is a safe modification with no identified safety concerns with regard to allergenicity, according to the requirements of the 2001 FAO/WHO Decision Tree (Pedersen et al., 2004). Currently, TG is commonly used in the food industry as a protein cross-linking factor and to change some properties, such as texture, solubility, viscosity, gelation, and water retention capacity (Kieliszek and Misiewicz, 2014). According to the literature, microbial TG may significantly affect the digestibility and immunoreactivity of proteins and protein-based products (Cabrera-Chávez et al., 2009; Giosafatto et al., 2012; Wróblewska et al., 2013). Moreover, TG polymerization has been reported to reduce the immunoreactivity of some proteins known as severe milk allergens (Wróblewska et al., 2009). In contrast to cow, sheep, goat, and human breast milk, studies have reported the absence of a TG inhibitor in HM (de Jong et al., 2003); therefore, the effect of TG on the immunoreactivity of HM proteins seems to be worth studying. The effect of TG on CM has been previously studied (Olivier et al., 2012; Villas-Boas et al., 2012). To the knowledge of the present authors, no studies investigating the effect of

TG on the immunogenic potential of HM proteins have been conducted to date.

The enzymatic reactions that occur in the gastrointestinal tract cause hydrolysis of food components, exposing the collection of antigens that affect the immune system. Stability in intestinal and gastric fluids is associated with the intrinsic allergenicity of many proteins (Huby, 2000). The resistance of an allergenic food protein to *in vivo* digestion increases its potential to cause an allergic reaction in susceptible individuals (Schnell and Herman, 2009). Assessment of food allergens requires the use of both digestion and immunological assays to ensure the safety of milk proteins for consumers, taking into account the effects of the food matrix. Therefore, the determination of the influence of HM protein digestion previously modified by enzymatic reactions is a key issue to understanding the effects of new types of diets containing HM for allergic individuals in the future.

MATERIALS AND METHODS

Samples

Milk was collected from mares (Wielkopolski breed, $n = 6$, Genactiv Co., Poznań, Poland) and cows (Aberdeen breed, $n = 6$, Norwich, UK). After the animals were milked, aliquots of fresh milk were immediately chilled to 4°C and kept frozen at -20°C. The samples were heated for 10 min at 125°C to increase the availability of the enzyme to the substrate, incubated with microbial Ajinomoto Activa (Ajinomoto, Paris, France) TG for dairy use (activity 100 U/g; 37°C, 18 h) at different doses (1 U, 10 U, and 100 U per 1 g of protein), and chilled to 4°C. Protein determination was carried out using the Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA) using BSA as a standard (Bradford, 1976). Samples were stored at -20°C. The chemical composition of HM was analyzed using a MilkoScan FT2 Infrared Milk Analyzer (Foss, Hillerød, Denmark).

In Vitro Gastrointestinal Digestion

Sample digestion was carried out simultaneously, using the consensus static *in vitro* digestion protocol as previously described (Minekus et al., 2014) but without addition of salivary amylase. The following 3 consecutive stages of digestion were simulated: oral digestion, gastric digestion (**GD**), and intestinal digestion (**ID**). The digestion process was performed on 5-mL samples. Briefly, in the oral phase, the suspensions were mixed with simulated salivary fluids (50:50 vol/vol) to a final volume of 10 mL. Porcine pepsin (P700, Sigma-Aldrich,

Steinheim, Germany) and simulated gastric fluid were added to achieve a concentration of 2,000 U/mL in the final GD mixture. The samples were placed in a water bath with a magnetic stirrer for 2 h at 37°C (gastric digestion for 2 h, 2GD). To simulate the intestinal phase of digestion, 2.5 mL of gastric chyme was mixed 1:1 with simulated intestinal fluid to a final volume of 5 mL, and pancreatin (P7545, Sigma-Aldrich) was added to the digestion mixture to obtain the final mixture of 100 U/mL based on trypsin activity. Then bile (B8631, Sigma-Aldrich) was added to a final concentration of 10 mM in the final mixture, and the samples were incubated again for 2 h (2GD + intestinal digestion for 2 h, 2ID). The samples were immediately frozen to stop the reaction. The obtained digested samples were used for the subsequent analysis.

SDS-PAGE Analysis

We performed SDS-PAGE analyses according to the methodology of Laemmli (1970), using NuPAGE 4 to 12% Bis-Tris gels (Invitrogen, Paisley, UK) with 2-(*N*-morpholino)ethanesulfonic acid running buffer (Invitrogen, NP0002-02) according to the manufacturer's instructions. Mark12 Molecular Weight Standard (Invitrogen, LC5677) was used as the molecular mass standard, and gels were stained using a Coomassie-based stain (Invitrogen, SimplyBlue SafeStain). After destaining, the gels were scanned using a Pharos FX Plus Imager and analyzed using Quantity One software v. 4.6.1 (Bio-Rad, Hercules, CA).

Size-Exclusion Chromatography (SEC)

The sample solutions were separated by size-exclusion chromatography (SEC) on a BioSep-SEC-S 2000 column (Phenomenex, Torrance, CA) attached to an HPLC system (Shimadzu Prominence; Shimadzu Corp., Kyoto, Japan). A solution containing 50 mM monosodium dihydrogen orthophosphate, 150 mM NaCl, and 0.01% sodium azide at a pH of 7.0 was used as an eluant buffer. All the samples were filtered through a 0.22- μ m filter. The sample injection volume was 10 μ L, and the nominal flow rate was 0.4 mL/min. The retention time was interpolated to molecular weight (kDa) according to molecular standards of the proteins. Undigested samples were centrifuged (15 min at 135 $\times$ *g*, 10°C, Centrifuge 5424 R, Eppendorf, Hamburg Germany) and filtered (0.22 μ m).

Particle Size Analysis

Samples were analyzed with an LS 13 320 laser diffraction particle size analyzer with the Universal Liquid

Module (Beckman Coulter, Brea, CA). For determination of the size distribution, a refractive index of 1.462 (that of milk fat) was used.

Immunoreactivity of Milk Proteins: Competitive ELISA

In this study, the term “immunoreactivity” refers to the ability of an antibody to recognize a native protein or a hydrolyzed protein in CM or HM. The ELISA method was carried out according to a previously described method (Wróblewska et al., 2009; Fotschki et al., 2015a). The immunoreactivity levels of samples were compared using the antibodies targeting the CM proteins β -LG and α -CN. Microtiter plates were coated with antigen (α -CN C6780, β -LG L3908, Sigma-Aldrich) diluted in 0.05 *M* carbonate buffer (pH 9.6) and incubated overnight at 4°C. Then the samples or standard antigen and polyclonal rabbit antibodies were added and incubated (37°C for 2 h). Animal sera were obtained every 2 wk from rabbits immunized 4 times. The single allergen concentration was 2.5 mg/mL per animal, emulsified with an equivalent amount of Freund's Adjuvant (Complete, Sigma-Aldrich F5881, at first immunization; Incomplete, F5506, during the next immunizations) to obtain a final concentration of 1 mg/mL. The wells were washed and filled with horseradish peroxidase-conjugated goat anti-rabbit IgG (A6154, Sigma-Aldrich). The reagent 3,3',5,5'-tetramethylenbenzidine (T5525, Sigma-Aldrich) was used as a substrate solution. The plates were incubated (37°C for 15 to 30 min), and the reaction was stopped by adding 2 *M* H₂SO₄. Absorption was measured at the wavelength of 450 nm using a Sunrise plate reader (Tecan, Männedorf, Switzerland). Immunoreactivity was calculated from the standard curves of protein antigens in CM. All analyses were carried out in triplicate, and average values were converted to concentration equivalents (in μ g per mL). A nonlinear adjustment was applied to the data obtained for each dilution from each rabbit serum. The adjusted model was a sigmoidal curve and was obtained with the GraphPad Prism program (version 5.0, GraphPad Software Inc., San Diego, CA).

Immunodot

Human serum samples were collected from 7 patients aged 3 to 13 years with history of CMA [approved by the Local Ethics Commission (2/2010)]. The bacterial proteins that reacted with total IgG and IgE were detected according to a previously described method (Markiewicz et al., 2016), with modifications. Briefly, the membranes with applied samples were incubated (120 min, room temperature) with Odyssey Blocking

Buffer (pH 7.2 to 7.6, 927-40003, LI-COR Biosciences, Lincoln, NE). Samples were diluted with HM at a 1:4 (vol/vol) ratio and with CM at a 1:8 (vol/vol) ratio. Human sera were diluted 2× with Odyssey Blocking Buffer containing 0.1% Tween. Membranes were incubated overnight at 4°C and then incubated for 90 min at room temperature, with 2 types of secondary antibodies labeled with different fluorescent markers: a 700-nm dye (red channel) and an 800-nm dye (green channel). Commercially available goat anti-human IgG antibodies already conjugated with IRDye 800CW (926-32232, LI-COR Biosciences) and mouse monoclonal anti-human IgE antibodies (I6510, Sigma-Aldrich) labeled using the IRDye 680RD Protein Labeling Kit (928-38072, LI-COR Biosciences) were used. The anti-human IgG and anti-human IgE secondary antibodies were diluted 1:5,000 and 1:500, respectively, with Odyssey Blocking Buffer (pH 7.2 to 7.6) containing 0.1% Tween 20 and 0.01% SDS. Signal detection was carried out with the Odyssey Infrared Imaging System (LI-COR Biosciences) using autoscan settings and LI-COR Image Studio software (version 2.0). ImageJ 1.52a (Wayne Rasband, National Institutes of Health, Bethesda, MD) was used for image analysis. The reaction between samples and human IgE and IgG was expressed by the dot intensity.

Statistical Analysis

We used Statistica software, version 10.0 (StatSoft Corp., Krakow, Poland), to determine whether variables differed among treatment groups. The results were statistically analyzed using one-way ANOVA and Duncan's multiple range test. Differences with $P \leq 0.05$ were considered significant. Data are expressed as the mean and standard error of the mean (SEM), except for the chemical composition and physical parameters, and the residual IgE and IgG reactivity of HM and CM are expressed as the mean and the standard deviation (SD) of the mean. The results are based on 3 replicates.

RESULTS

Characterization of the Samples

Table 1 shows the selected chemical components and physical parameters of HM and CM. Protein content in HM was 1.8-fold lower than in CM. The HM contained 1.5-fold lower concentration of lactose than did CM. The fat content in HM was 4.3-fold lower than that of CM. The SDS-PAGE electropherogram in Figure 1 shows the pattern of undigested samples of CM and HM with and without the addition of different doses of TG. As shown in lanes 1 and 2, CM and HM showed different protein compositions. Results showed that

increasing doses of TG led to changes in the structure of the milk matrix and could increase disulphide bond formation. Based on the electrophoresis data, the addition of TG enhanced protein polymerization and agglomerate formation, especially in CM, in the range of 120 to 200 kDa. The cross-linking of proteins with 1 U of TG was more clearly observed in CM samples than in HM samples. Under the polymerization with 100 U of TG, new bands were detected that did not exist in the samples with the addition of 1 and 10 U of TG, and the bands corresponded with the molecular weight of TG. Enzyme residues were observed due to the surplus of the enzyme relative to the protein substrate. We used SDS-PAGE to monitor enzymatic polymerization with TG; however, this method has some limitations in maximum separation. Therefore, polymers with high molecular weights (>200 kDa) were not visible. Samples with 100 U of TG were characterized by a streak in the upper part of the gel, due to the presence of high-molecular-mass conglomerates, which possibly could not get into the gel.

Simulation of In Vitro Digestion

To evaluate the effect of in vitro digestion on samples cross-linked with TG or control samples, we performed salivary, gastric, and duodenal digestion under simulated physiological conditions. The effects of digestion were analyzed using SDS-PAGE (Figure 2), particle size analysis (Figure 3), and SEC (Figure 4).

The results show that, upon gastric digestion of the studied samples, some proteins were partially intact. The use of the lowest enzyme dose did not result in the formation of fully cross-linked polymers, and we found no visible differences on the SDS-PAGE electropherograms of samples after gastric and total digestion (data not shown). A similar pattern was observed for CM and for CM with 10 U of TG added (+10 U TG); however, the patterns of HM and HM+10 U TG samples were different. In this analysis, the addition of 100 U of TG to CM and HM changed the protein profiles in both matrixes. After total digestion, the analyzed samples

Table 1. Chemical composition and physical parameters of horse and cow milk (mean $\pm$ SD)

Parameter	Horse milk	Cow milk
Total proteins (%)	1.75 $\pm$ 0.16	3.23 $\pm$ 0.23
Fat (%)	0.64 $\pm$ 0.20	2.73 $\pm$ 0.32
Lactose (%)	7.12 $\pm$ 0.39	4.74 $\pm$ 0.24
Urea (mg/L)	375 $\pm$ 0.12	112 $\pm$ 0.20
Freezing point (°C)	-0.56 $\pm$ 1	-0.55 $\pm$ 1
Fat-free DM (g/L)	9.61 $\pm$ 0.19	9.35 $\pm$ 0.11

Figure 1. Electropherogram from SDS-PAGE of nondigested samples of horse milk (HM) and cow milk (CM). M = molecular marker; 1 = raw CM; 2 = raw HM; 3 = CM + TG1; 4 = HM + TG1; 5 = CM + TG10; 6 = HM + TG10; 7 = CM + TG100; 8 = HM + TG100; TG = Ajinomoto Activa (Ajinomoto, Paris, France) transglutaminase (37.8 kDa); TG1, TG10, and TG100 = doses of TG (U) per 1 g of total protein. A = lactoferrin; B = BSA; C = TG residues; D = α -, β -, and κ -caseins; E = β -LG; F = α -LA. Samples were diluted to 1 mg/mL of total protein content. Samples 3 through 8 were pretreated (10 min at 125°C).

were degraded, and we found no observed differences between samples.

The particle size distribution of CM and HM with different doses of microbial TG before and during *in vitro* gastrointestinal digestion are shown in Figure 3. The results showed that the introduction of covalent bonds between or within proteins or peptides affects the particle size distribution of CM and HM in a dose-dependent manner. Undigested HM contained 2 main fractions of particles with a size range of 0 to 1 μ m and 1 to 10 μ m. Cow milk was mostly characterized by particles with a size from 1 to 20 μ m. In undigested samples, the particle size clearly increased with increasing TG dose. Based on these results, it becomes apparent that the protein network formed after extensive cross-linking of proteins by TG results in tightly packed protein nanoparticles, also indicating intramolecular cross-linking. Compared

with undigested samples, the samples digested with simulated salivary fluid and simulated gastric fluid showed decreases in the content of particles with a size 0.1 to 20 μ m. After gastric digestion, the addition of TG to CM and HM samples increased the proportion of particles with a size range of 10 to 40 μ m. This finding suggests that TG influenced the gastric digestibility of the studied samples, slowing digestion of the proteins. After gastric digestion, the aforementioned increase in the size of particles in samples with addition of 100 U of TG could have been because of the poor breakdown of aggregates by pepsin.

The interpolated kinetic profile of the molecular weight fractions of undigested and digested CM and HM with different doses of TG is shown in Figure 4. The profiles of CM and HM samples were different, taking into account the undigested samples (I) and dur-

ing (A, E) and after gastric digestion (B, F). A similar pattern of molecular weights was observed in these samples during (C, G) and after intestinal digestion (D, H). The undigested samples of CM were characterized mostly by fractions ranging from 10 to 100 kDa and 500 to 1,000 kDa, whereas HM samples ranged from 10 to 30 kDa. After both 1 and 2 h of digestion, fewer fractions above molecular weight of 10 kDa were found in HM than in CM. The addition of TG to both CM and HM caused a decrease in intensity due to the polymerization that occurred in samples. The results showed that each subsequent step of *in vitro* digestion caused an increase in the level of fractions corresponding to low molecular masses. During and after intestinal digestion, most CM and HM proteins were transformed into peptides of less than 10 kDa.

Immunoreactivity with Animal Sera

The ELISA results showed that in undigested HM and CM with increasing doses of the TG enzyme,

the immunoreactivity of α -CN was increased, and β -LG was decreased (Figure 5). The addition of TG, regardless of the dose, decreased the immunoreactivity of studied proteins in HM both during and after the gastric digestion step. The polymerization of CM proteins with a TG dose of 100 U increased the immunoreactivity of α -CN and β -LG during and after gastric digestion (Figure 6). Polymerization decreased the immunoreactivity of α -CN in HM and increased the immunoreactivity of α -CN in CM (10 and 100 U of TG) after 1 h of intestinal digestion and had no effect on HM and CM after total digestion. After 1 and 2 h of intestinal digestion, the immunoreactivity of HM increased in a dose-dependent manner and decreased in CM (1ID with 10 and 100 U of TG; 2ID with all tested doses). During and after gastric digestion, and after 1 h of intestinal digestion, the immunoreactivity of α -CN from CM was significantly higher than from HM. The differences in the immunoreactivity of α -CN between samples after total digestion were negligible. The immunoreactivity of β -LG was significantly lower in all

Figure 2. Electropherogram from SDS-PAGE of samples of horse milk (HM) and cow milk (CM) after 2 h of gastric digestion (A: 1 = CM; 2 = CM + TG10; 3 = CM + TG100; 4 = HM; 5 = HM + TG10; 6 = HM + TG100; 7 = nondigested TG) and total digestion (gastric and intestinal, B: 1 = CM; 2 = CM + TG10; 3 = CM + TG100; 4 = HM; 5 = HM + TG10; 6 = HM + TG100). M = molecular marker; TG = Ajinomoto Activa (Ajinomoto, Paris, France) transglutaminase; TG10 and TG100 = dose of TG (U) per 1 g of total protein.

tested HM samples than in CM after 1 h of gastric digestion. Surprisingly, after 2 h of gastric digestion, the β -LG immunoreactivity of CM+100 U TG remained at a high level, in contrast to samples with lower doses of TG. However, after 1 h of intestinal digestion, CM+100 U TG was characterized by a lower β -LG immunoreactivity than HM+100 U TG. Finally, the post-digestion β -LG immunoreactivity of CM was significantly higher than that of all tested HM samples.

Immunodot with Human Sera

Immunodot analyses were carried out to assess the IgE and IgG reactivity of CM and HM samples with and without TG before, during, and after digestion (Figure 7 and Table 2). Figure 7 shows the results of each sample normalized to the same amount of protein from different origins. All sera from patients were analyzed by immunodot technique and showed binding to individual CM proteins (Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-17264>). Various signal inten-

sities from the IgE and IgG sera of individual patients were observed. Addition of TG did not change the average signal of undigested CM and HM. Significant differences in IgE and IgG dot intensity were observed between the CM and CM+100 U TG samples after total digestion. The average IgG signal of the undigested raw CM was higher than that of the raw HM, and the signal of digested CM was higher than that of digested HM. After gastric digestion, a lower average signal was observed for HM and HM+100 U TG than for CM and CM+100 U TG. Table 2 shows the results of each sample normalized to the same volume of milk from different origins. Considering the average signals of both IgE and IgG, the TG cross-linking did not change the parameters of the undigested and gastrically digested CM and HM. Increased average IgE and IgG signals were observed for CM samples with TG after total digestion. The lowest average IgE intensity among the undigested products was observed for raw HM and IgG HM. The highest-value average IgE and IgG intensities among the non-digested products were observed in

Figure 3. Particle size distribution of undigested cow milk (A; CM) and horse milk (B; HM), and gastrically digested CM (C) and HM (D), with different doses of transglutaminase (TG; Ajinomoto Activa, Ajinomoto, Paris, France). ND = not digested; NP = not pasteurized; 2GD = samples gastrically digested for 2 h; TG1, TG10, and TG100 = doses of TG (U) per 1 g of total protein. All samples were initially preheated (125°C for 10 min). Values in the tables for each panel are expressed as the sums of the percentages in each category of particle sizes (<1, 1 to 10, or 10 to 100 μm) ± SD. Different lowercase letters (a–d) denote statistically significant differences ($P < 0.05$).

CM+100 U TG. We found that HM was characterized by 2.4-fold lower average IgE reactivity with human sera than CM after total digestion, whereas HM+100 U TG was 2.8-fold lower than CM+100 U TG. The residual average IgG reactivity after digestion for HM was 4.7-fold lower than that for CM, and HM+100 U TG was 9.4-fold lower than CM+100 U TG.

DISCUSSION

Many aspects, such as protein content and profile, indicate that HM is a valuable matrix for creating hypoallergenic products. In contrast to other ruminant milk, such as camel, goat, and sheep milk, HM proteins show low similarity to CM proteins, which is also reflected by different epitope patterns (Fiocchi et al., 2010). Horse milk is characterized by lower protein and fat

contents than are found in CM, as shown in other studies (Uniacke-Lowe et al., 2010; Danków et al., 2012). Businco et al. (2000) claimed that HM, with appropriate modifications, can be regarded as a good substitute for HM for children with severe IgE-mediated CMA. Processing can change the allergenic capacity of food due to alterations in the epitopes of food proteins (Mills et al., 2009; Paschke, 2009). The structural and conformational changes induced by food processing, such as polymerization, might change the immunoreactivity and allergenicity of milk proteins (Stanic et al., 2010; Wróblewska et al., 2011). The differences in the molecular structure of agglomerates could possibly be an outcome of the TG inhibitor in bovine milk serum (Bönisch et al., 2007), which is not present in HM. Differences in the polymerization of proteins among different milks cannot always be explained by the rheomorphic nature

Figure 4. Interpolated kinetic profile of molecular weight (MWT) fractions of undigested (I) and digested cow milk (A through D) and horse milk (E through H) with different doses of transglutaminase (TG; Ajinomoto Activa, Ajinomoto, Paris, France), analyzed by size-exclusion chromatography (BioSep-SEC-S 2000; Phenomenex, Torrance, CA). CM = cow milk; HM = horse milk; ND = not digested; GD = gastric digestion; ID = intestinal digestion following 2-h GD; TG1, TG10, and TG100 = units of TG (U) per 1 g of total protein in the sample. (I) Undigested samples were centrifuged (15 min, $135 \times g$, 10°C , Centrifuge 5424 R, Eppendorf, Hamburg, Germany) and filtrated ($0.22 \mu\text{m}$).

of proteins and the number of glutamine and lysine residues in each protein (Macierzanka et al., 2011).

The size of particles in food products is currently exploited in the food industry because size affects structure and allergenic properties (Mills and Mackie, 2008). An excessively large droplet could generate creaming due to poor stability, giving a product a greasy taste. On the other hand, an excessively small droplet may lead to flocculation. From a physiological point of view, the size of a particle considered an allergen can affect the absorbance in the intestine and exposure of the epitope to the immune system and can stimulate the synthesis of an allergen-specific reaction. Milk is an emulsion of fat globules surrounded by a membrane consisting of phospholipids and proteins within a water-based fluid. The differences detected in the particle size distribution

analysis could be explained by the differences in the size of fat globules in CM and HM. According to Ménard et al. (2010), individual globules of fat in buffalo milk and CM were 4.93 ± 0.04 and 3.56 ± 0.15 μm , respectively. Our results are in agreement with El-Zeini (2006), who reported that milk with a high fat content usually contains larger fat globules than milk with a low fat content. The results of the microscope analysis supported the particle size results. Horse milk contains smaller fat globules than does CM (Supplemental Figure S2; <https://doi.org/10.3168/jds.2019-17264>). This has also been shown to affect the digestion of milk proteins (Hussein et al., 2015). Studies assessing resistance to digestive enzymes in food samples show that cross-linking can decrease digestibility (de Souza et al., 2009; Hiller and Lorenzen, 2009). In this study on CM and HM samples,

Figure 5. Immunoreactivity of α -CN (A, C) and β -LG (B, D) from undigested horse (A, B) and cow milk (C, D) with addition of microbial transglutaminase (TG; Ajinomoto Activa, Ajinomoto, Paris, France). 1TG, 10TG, and 100TG = activity units (U) of TG per 1 g of total protein in the sample; CM = cow milk; HM = horse milk. Different lowercase letters denote statistically significant differences ($P < 0.05$). Error bars indicate SEM.

Figure 6. Kinetic profiles of the immunoreactivity of α -CN (A through D) and β -LG (E through H) in digested horse milk (HM) and cow milk (CM) with different amounts of microbial transglutaminase (TG). TG1, TG10, and TG100 = amounts of enzyme, calculated as units (U) per 1 mg of total protein in the sample; 1GD = measurement after 1 h of gastric digestion (A, E); 2GD = measurement after 2 h of gastric digestion (B, F); 1ID = measurement after 2GD and 1 h of intestinal digestion (C, G); 2ID = measurement after total digestion, 2GD and 2 h of intestinal digestion (D, H). All samples were heated for 10 min at 125°C and incubated with Ajinomoto Activa (Ajinomoto, Paris, France) TG (37°C, 18 h). Different lowercase letters denote statistically significant differences ($P < 0.05$). Error bars indicate SEM.

the large agglomerates assembled by TG were difficult to break down into smaller particles with the simulation of gastric digestive processes.

Major food allergens share several common features, and one shared feature is the relative stability in the presence of acid and proteases (Mandalari et al., 2009). Resistance to *in vivo* digestion is considered a predictive factor in assessing the allergenic potential of a protein to induce an allergic reaction in susceptible individuals. It is essential to evaluate the digestion process of proteins within the matrix in which they are usually consumed, not that of the isolated protein, especially in the field of food allergy. Havenaar et al. (2013) indicated that the *in vitro* digestion of caseinate treated with TG does not result in the formation of pepsin-resistant peptides (with a molecular mass >4 kDa), whereas digestion of unmodified caseinate resulted in the formation of these fragments. Grimshaw et al. (2003) showed that the food matrix has a critical influence on allergen availability. Therefore, *in vitro* study in the whole food matrix has been undertaken in this research, to directly compare the gastric and intestinal digestive characteristics of CM and HM protein immunoreactivity. We studied β -LG and α -CN because they are 2 of the most allergenic proteins in CM (Natale et al., 2004; Wróblewska et al., 2011). The matrix components may contribute to the ability of a protein to reach the sites of active

immune sampling in the gastrointestinal mucosa and, thus, could influence its potential allergenicity (Teuber, 2002). Certainly the lower content of casein fractions, major allergenic proteins, in HM than in CM may at least partly explain the lower immunoreactivity of HM. Cow milk contains approximately 10 g/L of α _{S1}-CN, the dominant fraction, and contains α _{S2}-CN at 3.7 g/L. Horse milk is characterized by a low concentration of these proteins, namely 2.5 g/L of α _{S1}-CN and 0.2 g/L of α _{S2}-CN (Miranda et al., 2004).

In this study, we showed that polymerization with TG decreased β -LG immunoreactivity of HM and CM. These results are in agreement with the results of a study by Villas-Boas et al. (2010), which previously showed that polymerization with TG modified or hid epitopes, reducing the potential antigenicity of β -LG in the presence of cysteine. Furthermore, the polymerization of chemically denatured β -LG with TG facilitated the action of gastrointestinal enzymes, and the digested products of chemically denatured β -LG presented lower antigenic properties than did those of the untreated protein (Villas-Boas et al., 2012). In our study, after total digestion, TG cross-linking did not change the immunoreactivity of α -CN but affected the immunoreactivity of β -LG, which increased in HM and decreased in CM. The results of ELISA showed that post-digestion immunoreactivity remained; in some

cases, immunoreactivity decreased, and in some cases, it was even enhanced. As seen from the study results, the reduction in allergenicity was still insufficient to obtain hypoallergenic products. In our study, we showed that patients with CMA can develop a specific response to different protein fractions. Patients with CMA are often sensitized to several different proteins. We showed that appropriate processing with TG can decrease the immunoreactivity of specific HM proteins, such as β -LG. This finding may be important for patients with a strong immune system reaction to a defined protein fraction.

Our results showed that HM was less reactive with human allergic patient sera than was CM. The use of high temperature during milk processing is an indispensable technological process aimed at ensuring microbiological quality. Clinical trials have reported that almost 75% of children with IgE-mediated CMA may tolerate baked foods containing milk, such as muffins, cakes, breads, and waffles (Nowak-Węgrzyn et al., 2008). Heat treatment altered serum response of individual patients to HM and CM, possibly caused by the destruction or masking of some epitopes, as has been observed in

other studies (Rahaman et al., 2016). In most cases, TG cross-linking does not change the IgE and IgG signal from the allergic sera obtained in response to undigested samples. However, the observation of slight variations in the signal cannot be ignored, particularly in the prediction of clinical symptoms. Sampson (2001) found that in 95% of patients with milk-specific IgE levels above 15 kUA/L, clinical symptoms can be expected during an oral challenge. In the present study, covalent molecular polymerization with TG increased the immune IgE and IgG reactions of sera from allergic patients to CM after total digestion, whereas almost no signal was detected from HM. The results from the assay with human sera showed that in patients with CMA, the response to analyzed milk proteins varies; therefore, a personalized diet should be designed for patients with CMA.

CONCLUSIONS

Polymerization with microbial TG, which induced conformational changes in proteins, modulates the exposure of CM and HM protein epitopes and their

Figure 7. Immunodot analysis of specific IgE (I) and IgG (II) in samples before, during, and after in vitro digestion. CM = cow milk; HM = horse milk; Raw = unprocessed sample; TG = Ajinomoto Activa (Ajinomoto, Paris, France) microbial transglutaminase; 100TG = the amount of enzyme, calculated as 100 U/1 mg of total protein in sample; 2GD = measurement after 2 h of gastric digestion; 2ID = measurement after 2GD and 2 h of intestinal digestion; Avg. int. = mean intensity of dots from all patients' sera, $\pm$ SD. All samples with TG were heat pretreated (10 min at 125°C) and incubated with TG (37°C, 18 h). Samples were normalized to similar protein contents. Samples with HM were diluted 1:4 (vol/vol), and samples with CM were diluted 1:8 (vol/vol). Values followed by the same lowercase letter (a–e) within the same step of digestion are not significantly different ($P \leq 0.05$).

Table 2. The IgE and IgG reactivity normalized to the dilution based on immunodot analysis of samples before, during and after in vitro digestion¹

Item	Intensity							Intensity, mean $\pm$ SD
	1	2	3	4	5	6	7	
Patient serum (IgE)								
Not digested								
Raw HM	120.15	71.58	120.32	119.51	94.37	73.80	79.30	97.01 $\pm$ 22.70 ^b
HM	89.49	103.39	111.31	223.93	103.80	172.52	95.92	128.63 $\pm$ 50.27 ^{bc}
HM + 100TG	100.27	113.94	124.16	233.29	114.65	192.70	106.90	140.85 $\pm$ 51.19 ^{bc}
Raw CM	389.68	159.70	229.05	249.72	187.28	177.74	133.85	218.15 $\pm$ 85.29 ^c
CM	553.82	237.92	397.73	511.47	249.30	463.13	208.55	374.56 $\pm$ 142.11 ^a
CM + 100TG	624.00	255.12	340.21	548.53	363.66	456.42	202.63	398.66 $\pm$ 152.89 ^a
Digested (2GD)								
HM	161.02	142.41	161.66	196.48	154.84	154.54	98.58	152.79 $\pm$ 29.18 ^b
HM + 100TG	184.22	132.77	193.24	237.81	185.40	171.44	126.31	175.89 $\pm$ 37.93 ^b
CM	476.16	330.16	400.80	480.11	422.50	381.98	374.59	409.47 $\pm$ 54.69 ^a
CM + 100TG	479.52	406.38	445.39	547.74	459.56	260.79	360.54	422.85 $\pm$ 92.27 ^a
Total digestion (2GD + 2ID)								
HM	77.93	53.79	78.27	81.83	73.46	55.39	49.58	67.18 $\pm$ 13.67 ^c
HM + 100TG	83.44	59.44	84.58	83.90	77.68	73.46	60.992	74.79 $\pm$ 10.72 ^c
CM	192.25	119.46	200.79	207.51	172.50	138.78	128.02	165.62 $\pm$ 36.55 ^b
CM + 100TG	283.54	184.69	249.31	209.94	187.22	175.38	212.87	214.71 $\pm$ 39.08 ^a
Patient serum [IgG]								
Not digested								
Raw HM	169.14	130.34	62.49	117.25	110.70	295.25	142.09	146.75 $\pm$ 73.16 ^b
HM	57.13	148.02	52.59	122.84	56.94	22.02	83.86	77.63 $\pm$ 43.98 ^b
HM + 100TG	84.33	123.20	54.94	136.97	78.06	89.32	79.24	92.29 $\pm$ 28.26 ^b
Raw CM	457.53	475.92	550.10	711.61	327.54	176.29	535.78	462.11 $\pm$ 170.99 ^c
CM	513.22	677.66	611.14	778.09	545.22	617.65	440.67	597.66 $\pm$ 111.10 ^a
CM + 100TG	635.11	778.62	535.15	657.86	691.05	580.44	400.02	611.18 $\pm$ 121.44 ^a
Digested (2GD)								
HM	57.57	55.73	51.17	46.10	52.68	51.168	44.596	51.42 $\pm$ 4.55 ^b
HM + 100TG	81.26	65.83	82.56	63.94	83.33	67.376	75.52	74.26 $\pm$ 8.43 ^b
CM	228.84	190.54	204.30	167.93	290.46	144.52	157.57	197.74 $\pm$ 49.99 ^a
CM + 100TG	272.37	216.82	271.44	184.33	294.26	121.464	146.23	215.28 $\pm$ 67.31 ^a
Total digestion (2GD + 2ID)								
HM	39.98	37.24	16.11	24.48	22.33	32.068	39.62	30.26 $\pm$ 9.41 ^c
HM + 100TG	53.12	43.80	19.35	29.33	30.94	44.092	41.27	37.42 $\pm$ 11.45 ^c
CM	134.74	132.98	209.70	125.88	118.31	128.488	164.46	144.94 $\pm$ 32.06 ^b
CM + 100TG	316.49	303.29	429.68	213.63	540.67	251.144	397.77	350.38 $\pm$ 113.04 ^a

^{a-f}Values followed by the same letter within the same step of digestion are not significantly different ($P \leq 0.05$).

¹CM = cow milk (dilution, 1:8, vol/vol); HM = horse milk (dilution, 1:4, vol/vol); Raw = unprocessed sample; TG = microbial transglutaminase (Ajinomoto Activa, Ajinomoto, Paris, France); 100TG = amount of enzyme, calculated as 100 U/mg of total protein in the sample; 2GD = measurement after 2 h of gastric digestion; 2ID = 2 h of intestinal digestion; Avg. int. = mean intensity of dots from all patient sera. All samples with TG were heat pretreated (10 min at 125°C) and incubated (37°C, 18 h).

recognition by elements of the immune system. Linking of TG modulates the level of immunoreactivity of the studied HM proteins based on the quantity of the enzyme and the type of protein. The highest amount of enzyme (100 U of TG) increased the immunoreactivity of α -CN and decreased the immunoreactivity of β -LG, in both HM and CM. After digestion, HM was characterized by 2.4-fold lower average IgE and 4.8-fold lower IgG reactivity than CM with patients' sera. In this study, patient-specific differences in serum IgE and IgG signals were observed. Future research in this area should focus on HM processing techniques that result in the destruction of additional allergenic epitopes using combined techniques, such as cross-linking and lactic acid fermentation, to develop novel protein food

materials with low immunogenic properties. Continued work on new methods of processing dairy food proteins will be of critical importance for decreasing the allergenicity of novel food proteins. The application of cross-linking with TG could reduce the allergenicity of some milk proteins and might be useful in producing "allergoids" for desensitization immunotherapy during individualized designed treatment.

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ORCIDS

- J. Fotschki <https://orcid.org/0000-0002-0116-0909>
 B. Wróblewska <https://orcid.org/0000-0002-7383-7819>
 B. Fotschki <https://orcid.org/0000-0002-9727-7481>