



In vitro digestibility and immunoreactivity of bovine milk proteins



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ABSTRACT

Current models of digestibility solely utilize pepsin stability to assess the safety of allergenic food proteins. However, *in vivo* complete protein digestion requires acid denaturation and pepsin, trypsin, and/or chymotrypsin cleavage. This study aimed to identify the immunoreactivity and allergenicity of stable bovine milk proteins, using an improved digestibility model to simulate physiological gastric and intestinal conditions *in vitro*. Gel electrophoresis and immunoblot analysis were used to determine protein stability and immunoreactivity, respectively. Immunoreactivity of bovine milk proteins, β -lactoglobulin (β -LG) and casein (CN) was greatly diminished with gastric simulation (0–60 min), but some proteins were stable and immunoreactive with simulated intestinal digestive conditions (0–60 min). This study demonstrates the need for improved digestibility models for more accurate assessment of the behavior of food allergens *in vivo*.

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1. Introduction

Bovine milk is enriched with vitamins and minerals, such as calcium, vitamin A and B₆, which are beneficial for human bones, hair, skin, and teeth (Macdonald et al., 2011), needed for the growth and development of young children and infants. Bovine milk is often consumed from infancy to adulthood, providing high quality protein and nutrients (Pereira, 2014). Food allergies among children have increased by roughly 50% between 1997 and 2011, affecting one in every thirteen children (Jackson, Howie, & Akinbami, 2013), with bovine milk protein allergy affecting 2–4% of young children and infants (Fritsché, 2003). The incidence of cow's milk protein allergy is greatly increased in infants' at risk for the development of allergy, with approximately 20% of infants developing bovine milk protein allergy within the first year of life if fed bovine milk proteins (Fritsché, 2003). Some children outgrow cow's milk allergy, while 15% of these children will continue to have cow's milk sensitivity until the approximate age of 9 years old (Saarinen, Pelkonen, Makela, & Savilahti, 2005). Bovine milk protein allergy is an IgE-mediated hypersensitivity reaction with multiple sensitizations to differing bovine milk proteins in about 75% of milk-allergic patients (Wal, 1998).

Bovine milk contains at least 20 proteins that may potentially be allergenic, such as α -lactalbumin, serum albumin and trace

amounts of lactoferrin (Fritsché, 2003), with β -lactoglobulin and caseins widely considered as the predominant milk protein allergens (Wal, 2002). Bovine milk consists of three major components: whey, casein, and milk fat globule membrane (MFGM). Whey consists of two major proteins: β -lactoglobulin (Ig) and α -lactalbumin (α -LA), while casein (CN) has four major proteins: α -s1, α -s2, β , and κ casein (Dupont et al., 2010; Gallier, Cui et al., 2013; Gallier, Zhu et al., 2013; Suju, Jing, Dongdong, Jiankang, & Weibo, 2010). Both β -LG and CN are major milk allergens recognized by human immunoglobulin E (Wal, 2002; Shek, Bardina, Castro, Sampson, & Beyer, 2005). Specifically, β -CN is widely considered to be the most immunogenic, with 75% of milk-allergic patients having IgE-mediated reactivity with β -CN (Shek et al., 2005). Milk fat globule membrane represents 1–4% of total milk protein and forms the membranous structure surrounding the lipid droplets during their secretion in the lactating mammary gland (Cavaletto, Giuffrida, & Conti, 2008). Many of the bovine milk allergenic proteins have been identified and characterized, while few studies have examined the digestive stability and immunoreactivity of total bovine milk proteins.

Breastfeeding is the only preventative measure against the development of allergy to bovine milk proteins in infants at risk for the development of allergy. However, under circumstances barring breastfeeding, infants at risk for milk allergy are provided with hypoallergenic bovine milk-based formulas in which potentially allergenic proteins are extensively hydrolyzed (Moneret-Vautrin, Hatahet, & Kanny, 2001; Rosendal & Barkholt, 2000). Nevertheless, most often hypoallergenic bovine milk-based formulas are given after the discovery of bovine milk allergy or sensitivity, in which

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the infant or child has demonstrated hypersensitivity responses (Ragno, Giampietro, Bruno, & Businco, 1993). Therefore, more studies need to be conducted for the identification and improved knowledge regarding the allergenicity; stability and behavior of bovine milk proteins *in vivo*, for the improvement of existent hypoallergenic bovine milk-based hypoallergenic formulas and for the development of more sensitive and effective antibodies needed for the detection of hidden bovine milk proteins within processed foods.

A common feature of many food allergens is their resistance to gastric and intestinal luminal digestion (Taylor, Lemanske, Bush, & Busse, 1987). Food proteins that are resistant to pepsin digestion and acid denaturation within the gastric lumen have an increased probability of reaching the intestinal mucosa where absorption can occur. The longer these allergenic food proteins remain intact within the mammalian gastrointestinal tract, the more likely they are to trigger an immune response (Taylor et al., 1987). Thus, the ability of food allergens to reach the jejunal mucosa is considered a prerequisite for allergenicity. While other studies have demonstrated the digestive stability of purified milk proteins, β -lactoglobulin and casein (Benedé et al., 2014; Peram, Loveday, Ye, & Singh, 2013; Petrat-Melin et al., 2015; Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006; Wal, 2001), few studies have examined the pepsin and/or pancreatin digestive stability of total bovine whole milk proteins. Moreover, limited digestibility studies have examined the immunoreactivity and potential allergenicity of digestive stable total bovine whole milk proteins, using allergen specific rabbit IgG antibodies and IgE

antibodies from the sera of milk-allergic patients. Therefore, the unique aim of this study was to determine the immunoreactivity and potential allergenicity of digestive stable total bovine whole milk proteins, using a validated *in vitro* digestibility model.

2. Materials and methods

2.1. Materials

Bovine whole milk proteins (catalog# XPF395D3A2.5) were purchased from Greer Source Materials (Lenoir, NC), as pasteurized nonfat dry milk, and were reconstituted in ultra-pure distilled water according to the manufacturer's instructions. Purified total bovine casein proteins (catalog# ab91092) were purchased from Abcam (Cambridge, MA) in liquid form. Purified total bovine β -lactoglobulin (BLG) proteins (catalog# L3908-5G) were purchased Sigma–Aldrich Corp. (St. Louis, MO) and reconstituted in ultra-pure distilled water according to the manufacturer's instructions. Pepsin, 2546 U/mg activity (premium quality and essentially free of all impurities, catalog# P7012), and pancreatin, 26296 U/mg activity (catalog# P1750), were purchased from Sigma Chemical Company (St. Louis, MO). The gels and running buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) Criterion TGX Precast Gels (catalog# 567-1113) were purchased from Bio-Rad (Hercules, CA) and reducing sample buffer (catalog# 39000) was purchased from ThermoScientific (Waltham, MA). Western blots were performed, using the Trans-Blot Turbo Mini System (catalog#

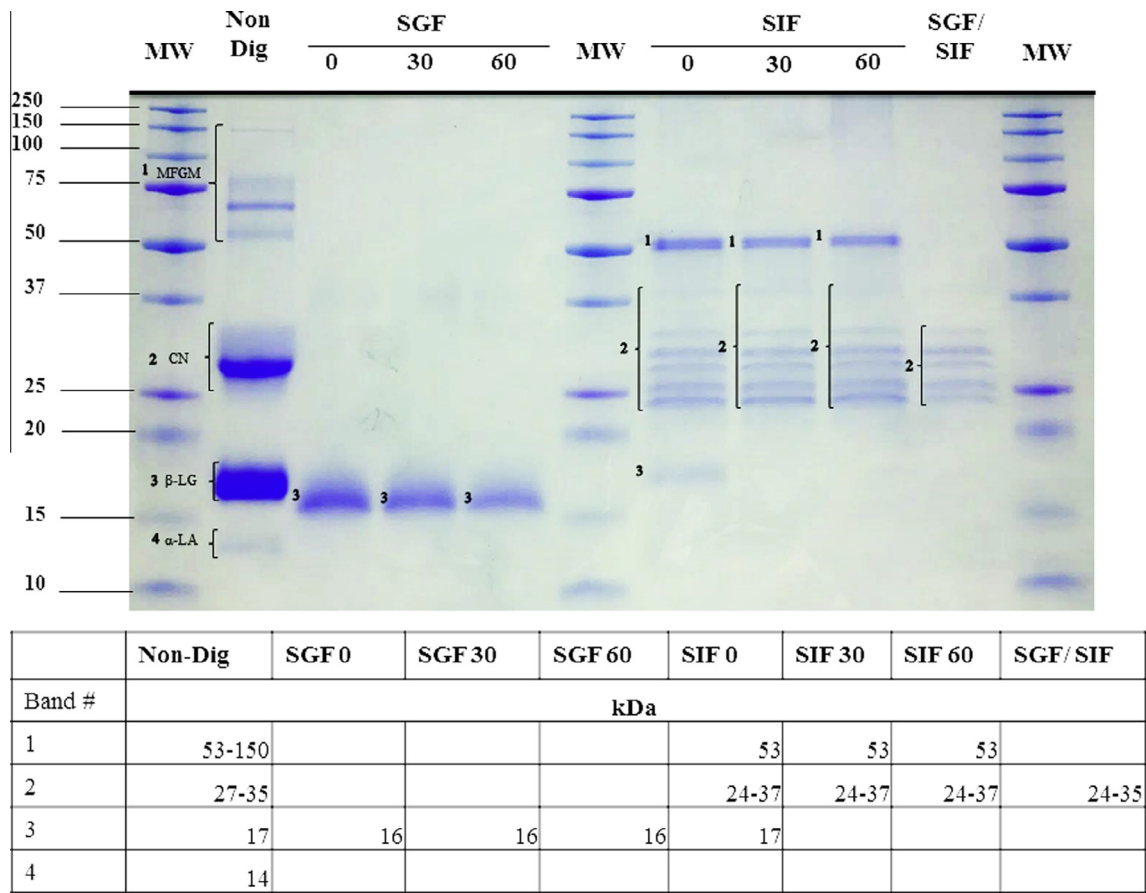


Fig. 1. Electrophoretic analysis of temporally pepsin and pancreatin *in vitro*-digested bovine whole milk proteins. Under reducing conditions, bovine milk proteins (20 μ g/ lane) were separated by SDS–PAGE and stained with Coomassie brilliant blue. Lanes: MW (molecular weight marker); non-dig (non-digested control bovine milk proteins); time (minutes) of pepsin digestion; SGF 0, SGF 30, SGF 60; Time of pancreatin digestion; SIF 0, SIF 30, SIF 60; Dual protease digestion of pepsin (60 min), followed by 30 min of pancreatin digestion = SGF/SIF. Numbered protein bands correspond to values of molecular mass (kDa) found within the table. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

170-4155), and Nitrocellulose Transfer Packs (catalog# 170-4159) were purchased from Bio-Rad. Immunoreactive digestive protein fragments and non-digested control bovine milk proteins were detected, using either rabbit anti-bovine milk IgG primary antibodies raised in-house, or rabbit anti- β -lactoglobulin IgG primary antibodies (catalog# GTX37640) purchased from GeneTex (Irvine, CA) or rabbit anti-casein IgG primary antibodies (catalog# orb10185), purchased from Biorbyt (San Francisco, California), diluted in Pierce Protein Free T20 Blocking Buffer (catalog# 37573), purchased from ThermoScientific. Donkey anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibodies (catalog# sc-2313) from Santa Cruz Biotechnology (Santa Cruz, CA) and chromogenic substrates chloronaphthol and diaminobenzidine (CN/DAB, catalog# 34000) from ThermoScientific, were used for detection. Sera from non-sensitized rabbits was used as the primary antibody as a negative control immunoblot. Rabbit bovine milk primary antibodies were produced, using a protocol that was approved by the Institutional Animal Care and Use Committee.

IgE immunoreactivity of digested bovine milk peptides and control non-digested proteins were detected using the sera from two bovine milk-allergic patients (ImmunoCAP >40.0 kU/l) purchased commercially from Plasma Lab International (Everett, WA), and goat anti-human IgE-HRP secondary antibodies (catalog# 074-1004) from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD), and CN/DAB substrate from ThermoScientific. Serum from a non-allergic patient, purchased from Plasma Lab International (Everett, WA), was used for the negative control immunoblot.

2.2. Simulated gastric fluid digestion assay

The protocols of [Astwood, Leach, and Fuchs \(1996\)](#) were followed with some modifications ([Toomer, Do, Pereira, & Williams, 2013](#)). Simulated gastric fluid (SGF) was prepared as described in the [United States Pharmacopeia \(2006\)](#): 1 mg/ml of a solution consisting of 2.546 U pepsin/ μ l in 0.03 M NaCl, pH 1.2 ([USP Pharmacopeia, 2006](#)). SGF was aliquoted (500 μ l) to a 5.0 ml glass tube for each time interval (0, 30, 60 min) and incubated in a 37 °C shaking water bath (Boekel™ Scientific Orbital and Reciprocating Shaking Water Bath, catalog# 05-450-101 from Fisher Scientific, Pittsburgh PA) for 10 min. Test protein (400 μ g) was added to each tube to begin the temporal SGF digestion assay at a 3:1 ratio (U pepsin: test protein), and 100 μ l of 1 N NaOH were added to each vial and placed on ice to stop the reaction. The 0 min time point was obtained by the addition of 500 μ l of SGF and 100 μ l NaOH (stop solution) simultaneously to the aliquot of test protein. Subsequently, 100 μ l of NaOH were added to the other aliquots of test proteins after 30 and 60 min of incubation at 37 °C with shaking, to obtain those respective time points of SGF digestion. SGF digestive products were subsequently stored at –20 °C until analyzed.

2.3. Simulated intestinal fluid digestion assay

Simulated intestinal fluid (SIF) was prepared as described in the [United States Pharmacopeia \(USP, 2006\)](#): 10 mg/ml of pancreatin (chymotrypsin, trypsin, amylase, and lipase) in 0.05 M KH_2PO_4 (pH 7.5) containing 26,296 U pancreatin/ml ([USP Pharmacopeia,](#)

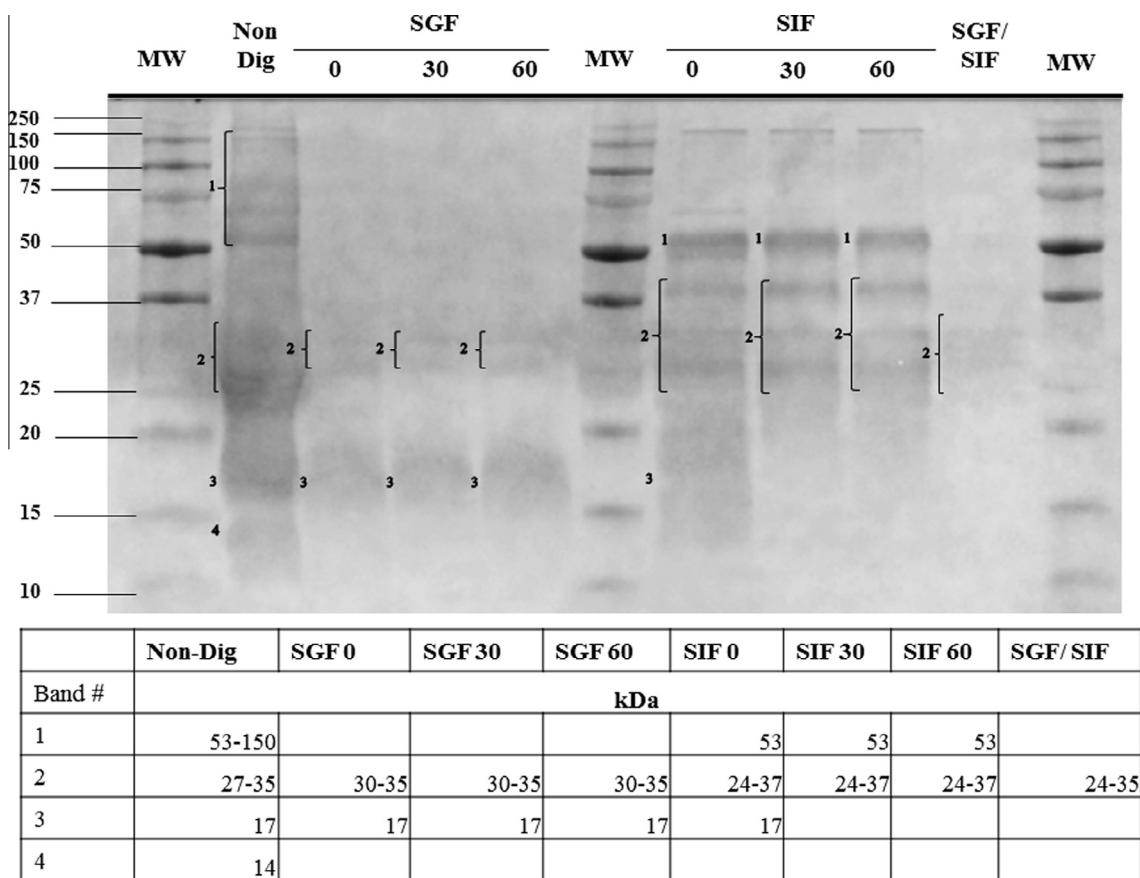


Fig. 2. IgG immunoreactivity of pepsin and pancreatin *in vitro*-digested bovine milk proteins. Under reducing conditions, bovine milk proteins (20 μ g/lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane and immunoblotted with rabbit anti-bovine whole milk IgG antibodies (1:1000) and anti-rabbit IgG-HRP secondary antibodies (1:1000) to determine immunoreactivity. Lanes: MW (molecular weight marker); non-dig (non-digested control bovine milk proteins); time (minutes) of pepsin digestion; SGF 0, SGF 30, SGF 60; time (minutes) of pancreatin digestion; SIF 0, SIF 30, SIF 60; dual protease digestion of pepsin (60 min), followed by 30 min of pancreatin digestion = SGF/SIF. Numbered protein bands correspond to values of molecular mass (kDa) found within the table.

2006). SIF was aliquoted (500 µl) to a 5.0 ml glass tube for each time interval (0, 30, 60 min) and incubated in a 37 °C water bath for 10 min with shaking. Test protein (400 µg) was added to each tube to begin the temporal SIF digestion assay at an 8:1 ratio (U pancreatin: µg test protein) at the respective time intervals (30 and 60 min), with shaking. The 0 min time point was obtained by the addition of 500 µl of SIF to the aliquot of test protein and immediately placing on ice. After the completion of the SIF digestive assay, all tubes were removed from ice and each sample was boiled for 10 min (to inactivate pancreatin enzyme) and immediately placed on ice and stored (long term) at –20 °C.

An additional tube was prepared to determine the resistance of test proteins to concurrent pepsin, trypsin, and chymotrypsin (SGF/SIF) digestion, in which 400 µg of test protein was subjected to 60 min of pepsin digestion (reaction was stopped with the addition of 100 µl of NaOH after 60 min of *in vitro* pepsin digestion at 37 °C), followed by 30 min of pancreatin digestion at 37 °C. The pancreatin digestion was stopped by placement on ice and boiled in a water bath for 10 min prior to long term storage at –20 °C.

2.4. Production of rabbit anti-bovine milk IgG primary antibodies

Rabbit antibodies were prepared by subcutaneous inoculation of adult New Zealand White rabbits (>2.0 kg). Prepared antigens were bovine whole milk proteins purchased from Greer Source Materials. One hundred microgrammes of antigen were injected into multiple sites on the shaved back of the rabbit. The initial injection was antigen suspended 1:1 in normal saline: complete

Freund's adjuvant. Rabbits were inoculated 2–3 weeks later with antigen suspended 1:1 in normal saline: incomplete Freund's adjuvant. Two subsequent injections consisted of 100 µg of protein suspended in normal saline. For blood collection, animals were anesthetized with an intramuscular injection of acepromazine (0.25–0.5 mg/kg)/Ketamine (35 mg/kg)/Xylazine (5 mg/kg). Rabbits were euthanized using intravenous injection of potassium chloride. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and rabbits were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and immunoblotting

Digested or control un-digested test proteins (20 µg for immunoblots with rabbit and human antibodies) were loaded (per lane) on a 10–20% polyacrylamide Tris–glycine gel and electrophoretically separated under constant voltage with Tris/Glycine/SDS buffer according to the manufacturer's instructions (Bio-Rad). Proteins were visualized by Coomassie Brilliant Blue staining and digitally imaged, using the Gel Doc™ EZ System (catalog# 170-8270, Bio-Rad) and band quantification and detection were determined, using the Image Lab™ Software (catalog# 170-9692, Bio-Rad). Additionally, SDS-PAGE-resolved proteins were transferred to nitrocellulose membranes electrophoretically for immunoblotting procedures. The membranes were washed three times in Tris-buffered saline with 0.1% Tween (TBST) and subsequently

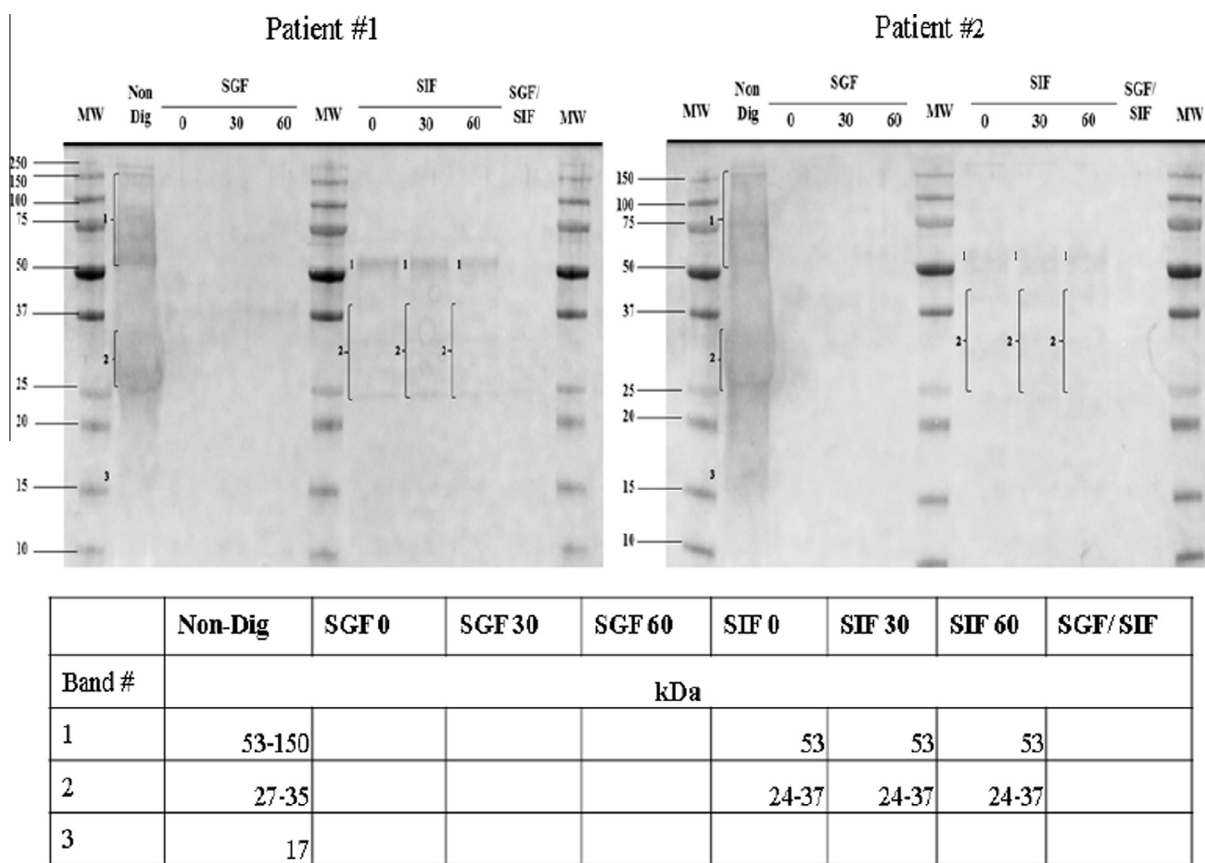


Fig. 3. IgE immunoreactivity of pepsin and pancreatin *in vitro*-digested bovine milk proteins. Under reducing conditions, bovine milk proteins (20 µg/lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane and immunoblotted with the serum from milk-allergic patients (1:4) and goat anti-human IgE-HRP secondary antibodies (1:1000) to determine IgE immunoreactivity. Lanes: MW (molecular weight marker); non-dig (non-digested control bovine milk proteins); time (minutes) of pepsin digestion; SGF 0, SGF 30, SGF 60; time (minutes) of pancreatin digestion; SIF 0, SIF 30, SIF 60; dual protease digestion of pepsin (60 min), followed by 30 min of pancreatin digestion = SGF/SIF. Numbered protein bands of both patients correspond to values of molecular mass (kDa) found within the table.

blocked in Pierce Protein Free T20 Blocking Buffer (catalog# 37573) from ThermoScientific for 2 h at room temperature. The membranes were incubated at 4 °C overnight in either (1) a 1:1000 dilution of rabbit anti-bovine milk primary antibodies, (2) a 1:500 dilution of rabbit anti- β -lactoglobulin primary antibodies, (3) a 1:500 dilution of rabbit anti-casein primary antibodies or (4) a 1:4 dilution of human sera from bovine milk-allergic and non-allergic (control) donors in Pierce Blocking Buffer. The membranes were washed three times in TBST and incubated in secondary antibodies donkey anti-rabbit IgG-horseradish peroxidase (HRP) (dilution 1:1000 in Pierce Blocking Buffer) or goat anti-human IgE-HRP (dilution 1:1000 in Pierce Blocking Buffer) at room temperature for 1 h. Bio-detection was determined utilizing chromogenic peroxidase substrate for CN/DAB-based detection of HRP activity.

3. Results and discussion

3.1. SDS–PAGE analysis

Electrophoretic analysis under reducing conditions of total bovine milk proteins revealed the existence of minor resolved MFGM protein bands ranging from 53 to 150 kDa (band 1), CN at 27–35 kDa (band 2), β -LG at 17 kDa (band 3) and faint banding of α -LA at 14 kDa (band 4) in the non-digested control sample (Fig. 1), which parallels findings by Gallier, Cui et al. (2013), Gallier, Zhu et al. (2013). Upon digestion with SGF, protein bands of 14 kDa, 27–35 kDa and 53–150 kDa were not stable at 0, 30 and 60 min of pepsin digestion (Fig. 1), while a protein/peptide

band of approximately 16 kDa was stable at all time points of pepsin digestion. At 60 min of pepsin digestion, this resolved protein/peptide band appeared to be partially hydrolyzed; however it was still visible at this time point, as in findings by Gallier, Cui et al. (2013).

Upon *in vitro* pancreatin digestion (SIF) of bovine whole milk (Fig. 1), most of the milk proteins identified in the non-digested control sample were not stable at 0 min of digestion, with the appearance of polypeptides resolved at 53 kDa (band 1), 24–37 kDa (band 2), and 17 kDa (band 3). Band 1 (53 kDa) is consistent with a polypeptide of MFGM also seen in the non-digested control sample (Fig. 1). Polypeptide bands ranging from 24 to 37 kDa (band 2) were consistent CN bands, while band 3 (17 kDa), was significantly hydrolyzed at this the 0 time point compared to the 17 kDa band seen in the non-digested control sample (Fig. 1). At 30 and 60 min of pancreatin (SIF) *in vitro* digestion, band 1 (53 kDa) and band 2 (24–37 kDa) were stable, while band 3 (17 kDa) was hydrolyzed (Fig. 1). After SGF/SIF digestion, milk proteins of band 1 (53 kDa) were completely hydrolyzed as compared to the pancreatin-digested bovine milk samples (0, 30 and 60 min), while proteins between 24 and 35 kDa were stable (Fig. 1), indicating that the protein at 53 kDa was not stable to the acidic SGF conditions *in vitro*.

3.2. Immunoblotting analysis

To determine the immunoreactivity of digestive stable bovine milk proteins, proteins were analyzed by immunoblotting analysis with rabbit anti-bovine whole milk IgG antibodies (Fig. 2). In the

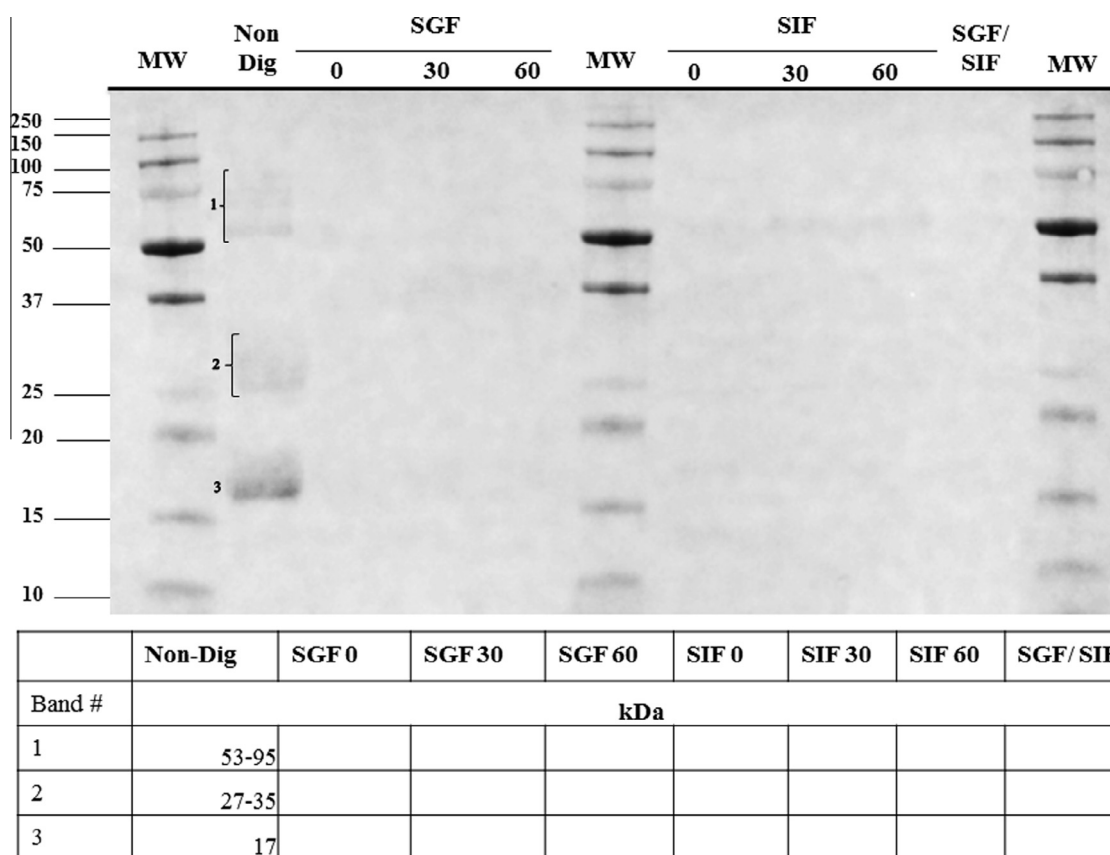


Fig. 4. Pepsin- and pancreatin-digested bovine milk proteins blotted with rabbit anti- β -lactoglobulin (BLG) IgG antibodies. Under reducing conditions, bovine milk proteins (20 μ g/lane) were separated by SDS–PAGE and transferred to a nitrocellulose membrane and immunoblotted with rabbit anti- β -LG IgG antibodies (1:1000) and anti-rabbit IgG-HRP secondary antibodies (1:1000) to determine immunoreactivity. Lanes: MW (molecular weight marker); non-dig (non-digested control bovine milk proteins); time (minutes) of pepsin digestion; SGF 0, SGF 30, SGF 60; time (minutes) of pancreatin digestion; SIF 0, SIF 30, SIF 60; Dual protease digestion of pepsin (60 min), followed by 30 min of pancreatin digestion = SGF/SIF. Numbered protein bands correspond to values of molecular mass (kDa) found within the table.

non-digested control sample, polypeptides of band 1 (53–150 kDa), band 2 (27–35 kDa), band 3 (17 kDa) and band 4 (14 kDa) were immunoreactive (Fig. 2). Band 2 (30–35 kDa) and band 3 (17 kDa) were pepsin-stable and immunoreactive after 0, 30 and 60 min of *in vitro* digestion (Fig. 2). In pancreatin digested sample at the 0 min time point, polypeptides of band 1 (53 kDa), band 2 (24–37 kDa) and band 3 (17 kDa) were immunoreactive to rabbit anti-bovine milk IgG antibodies (Fig. 2). However, only band 1 (53 kDa), and band 2 (24–37 kDa) were stable and immunoreactive to 30 and 60 min of *in vitro* pancreatin digestion (Fig. 2), while immunoreactivity of band 3 (17 kDa) was absent (Fig. 2). Digestive-stable proteins/peptides (band 2; 24–35 kDa, Fig. 1) were minimally reactive to rabbit anti-bovine milk IgG antibodies, after 60 min of digestion with pepsin, followed by 30 min of pancreatin digestion (SGF/SIF) *in vitro* (Fig. 2), suggesting the loss of epitope binding following the dual action of proteases and acid denaturation. As a negative control, immunoblot analysis was performed, utilizing non-digested and digested (pepsin, pancreatin) bovine milk proteins with the sera from normal rabbits and no reactivity of either was observed (data not shown).

To determine the immunoglobulin E (IgE) reactivity and potential allergenicity of bovine whole milk proteins, pepsin and/or pancreatin digested bovine milk proteins were immunoblotted with the serum from two milk-allergic patients. In the non-digested control sample, protein band 1 (53–150 kDa), band 2 (27–35 kDa) and band 3 (17 kDa) were IgE-reactive in both patients (Fig. 3). Upon 0, 30 and 60 min of *in vitro* pepsin digestion, these proteins were no longer IgE-reactive (Fig. 3). Upon 0 and 30 min of *in vitro* pancreatin digestion, band 1 (53 kDa) and band 2 (24–

37 kDa) remained IgE-reactive, while band 2 (24–37 kDa) was IgE-non-reactive after 60 min of *in vitro* pancreatin digestion (Fig. 3). However, all SGF/SIF (60 min pepsin digestion and 30 min pancreatin digestion) stable bovine milk proteins were IgE non-reactive, suggesting the hydrolysis and/or loss of IgE-reactive epitopes (Fig. 3). In contrast, pepsin- and pancreatin-stable milk proteins/peptides of 24–35 kDa were immunoreactive with rabbit anti-bovine milk IgG antibodies (Fig. 2), while IgE was non-reactive (Fig. 3). As a negative control, immunoblot analysis was performed, using the serum of a non-milk-allergic patient, with non-digested and digested bovine milk proteins (pepsin, pancreatin) and no reactivity of either was observed (data not shown).

To confirm identification and determine the immunoreactivity of β -LG proteins within the bovine whole milk immunoblot, proteins were analyzed by immunoblot analysis with rabbit anti- β -LG IgG antibodies (Fig. 4). In the non-digested control sample, polypeptides of band 1 (53–95 kDa) and band 2 (27–35 kDa) were weakly immunoreactive, but band 3 (17 kDa), corresponding to the molecular weight of β -LG, was more prominent than were bands 1 & 2 (Fig. 4). At all-time points, in both pepsin- and pancreatin-digested samples, polypeptides of band 1 (53–95 kDa), band 2 (27–35 kDa) and band 3 (17 kDa) were non-reactive to rabbit anti- β -lactoglobulin IgG antibodies, suggesting hydrolysis of β -LG proteins and destruction of epitopes recognized by this antibody (Fig. 4). Polypeptides were also non-reactive to rabbit anti- β -LG IgG antibodies after 60 min of digestion with pepsin, followed by 30 min of pancreatin digestion (SGF/SIF) *in vitro* (Fig. 4).

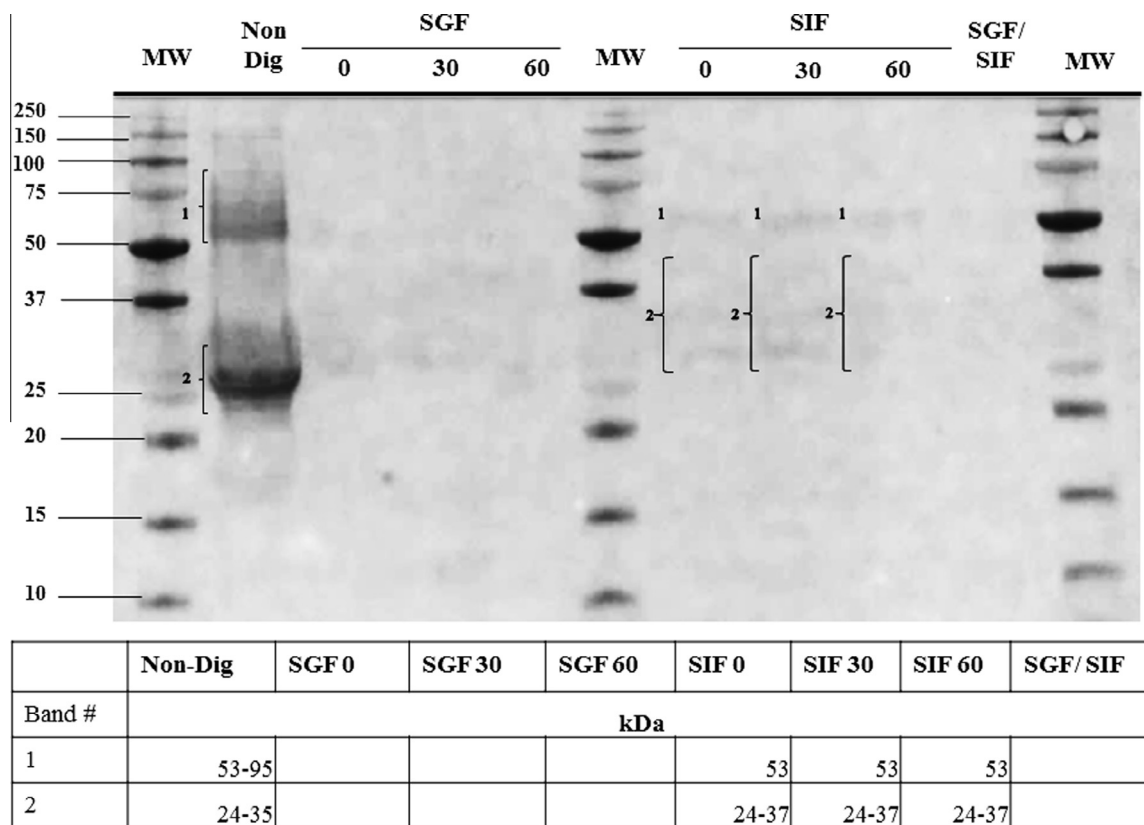


Fig. 5. Pepsin- and pancreatin-digested bovine milk proteins blotted with rabbit anti-casein (CN) IgG antibodies. Under reducing conditions, proteins (20 μ g/lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane and immunoblotted with rabbit IgG CN antibodies (1:1000) and anti-rabbit IgG-HRP secondary antibodies (1:1000) to determine immunoreactivity. Lanes: MW (molecular weight marker); non-dig (non-digested control bovine milk proteins); time (minutes) of pepsin digestion; SGF 0, SGF 30, SGF 60; time (minutes) of pancreatin digestion; SIF 0, SIF 30, SIF 60; dual protease digestion of pepsin (60 min), followed by 30 min of pancreatin digestion = SGF/SIF. Numbered protein bands correspond to values of molecular mass (kDa) found within the table.

To confirm identification and to determine the immunoreactivity of CN proteins, proteins were analyzed by immunoblotting analysis with rabbit anti-casein IgG antibodies (Fig. 5). In the non-digested control sample, polypeptides of band 1 (53–95 kDa) and band 2 (24–35 kDa) were immunoreactive, while band 2 (24–35 kDa), corresponding to the molecular weight of CN proteins, was more prominent (Fig. 5). Upon digestion with SGF, protein bands 1 and 2 were non-reactive at the 0, 30, or 60 min time points (Fig. 5). In the pancreatin-digested samples at the 0, 30 and 60 min time points, polypeptides of band 1 (53 kDa) and band 2 (24–37 kDa) were immunoreactive to rabbit anti-casein IgG antibodies, but were less prominent than were band 1 and band 2 in the non-digested control sample (Fig. 5). All digestive-stable proteins were non-reactive with rabbit anti-CN IgG antibodies after 60 min of digestion with pepsin, followed by 30 min of pancreatin digestion (SGF/SIF) *in vitro* (Fig. 5).

In summary, these results demonstrate that non-digested control milk proteins/peptides of approximately 53–150 kDa, 27–35 kDa and 17 kDa were immunoreactive with both rabbit anti-bovine milk IgG (Fig. 2) and human IgE antibodies (Fig. 3), suggesting potential allergenicity. While β -LG milk proteins (approximately 17 kDa) visualized in Fig. 1 were pepsin-stable, these proteins were only immunoreactive with the rabbit anti-bovine milk IgG antibody (Fig. 2), and non-reactive with the rabbit anti- β -LG IgG antibody (Fig. 4). Casein milk proteins/peptides (ranging from approximately 24 to 37 kDa) were pepsin-stable, pancreatin stable and immunoreactive with rabbit anti-bovine milk IgG antibody (Fig. 2) and IgE-reactive (Fig. 3). In contrast, the pepsin-digested CN milk proteins were not reactive with the rabbit anti-CN IgG antibody (Fig. 5). The pancreatin stable band at approximately 53 kDa was prominently immunoreactive with rabbit anti-bovine milk IgG antibody (Fig. 2) and human IgE antibodies (Fig. 3), but minimally reactive with the rabbit anti-CN IgG antibody (Fig. 5), suggesting potential allergenicity. These results imply that the rabbit anti-bovine milk IgG antibody possessed different epitope binding sites in contrast to the rabbit anti- β -LG IgG antibody and rabbit anti-CN antibody, and it may better identify potentially allergenic digestive-stable milk proteins/peptides.

None of the pepsin/pancreatin (SGF/SIF) stable milk proteins/peptides (seen in Fig. 1) were immunoreactive with IgE antibodies (Fig. 3), rabbit anti- β -LG IgG antibody (Fig. 4) or rabbit anti-CN IgG antibody (Fig. 5), but these proteins/peptides were reactive with rabbit anti-bovine milk IgG antibody (Fig. 2). Therefore, these results also demonstrate IgG and IgE epitope binding differences of digestive-stable milk proteins and thus the need to analyze the reactivity of both antibodies by immunoblot analysis.

4. Conclusion

These findings imply that the allergenicity of known bovine milk allergens, β -LG and CN are greatly reduced with simulated gastric conditions of acid denaturation and pepsin cleavage, while these allergenic proteins remain stable and immunoreactive during intestinal digestion with pancreatin. However, under physiological circumstances, causing rapid transit through the gastric mucosa (dumping syndrome), non-digested milk proteins may transit intact to the small intestine and elicit an immunological response in sensitive consumers.

This study demonstrates the need for improved digestibility models for better assessment of the stability and allergenicity of food proteins *in vivo*; while these *in vitro* digestibility models are representative of human digestion (gastric and intestinal), they do not predict protein half-life or protein stability *in vivo*. An improved digestion model for food allergens, however, may enhance our understanding regarding the stability of the digestive products of food allergens in the human gut and facilitate the development of more sensitive and effective antibodies for use in food allergen detection.

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