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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 B_6, 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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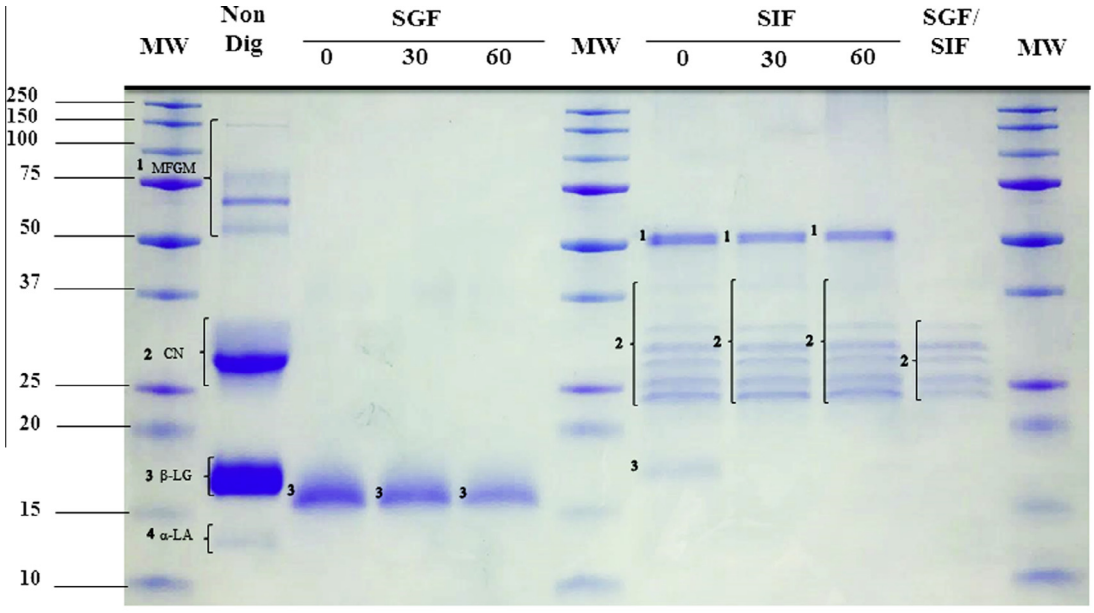
the infant or child has demonstrated hypersensitivity responses (Ragno, Giampietro, Bruno, & Businco, 1993). Therefore, more studies need to be conducted for the identification and improved knowledge regarding the allergenicity; stability and behavior of bovine milk proteins *in vivo*, for the improvement 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#



	Non-Dig	SGF 0	SGF 30	SGF 60	SIF 0	SIF 30	SIF 60	SGF/ SIF
Band #	kDa							
1	53-150				53	53	53	
2	27-35				24-37	24-37	24-37	24-35
3	17	16	16	16	17			
4	14							

Fig. 1. Electrophoretic analysis of temporally pepsin and pancreatin *in vitro*-digested bovine whole milk proteins. Under reducing conditions, bovine milk proteins (20 μg/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.