ABSTRACT

Dairy processing can alter the digestion stability and bioavailability of cow milk proteins in the gastrointestinal tract. However, analysis of stable linear epitopes on cow milk allergens that could enter into intestinal mucosa is limited. Thus, this study aimed to investigate the digestion and transportation properties and residual allergen epitopes entering into gastrointestinal mucosa of 3 commercial dairy products, including pasteurized milk (PM), ultra-heat-treated milk (UHTM), and dried skim milk (DSM). In this work, the digestive stability of the 3 kinds of dairy products has been performed in a standard multistep static digestion model in vitro and characterized by Tricine-SDS-polyacrylamide gel electrophoresis and reversed-phase HPLC. With respect to gastrointestinal digestion in vitro, the main allergens including β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and caseins were degraded gradually, and the resistance peptides remained in the PM with a molecular weight of range from 3.4 to 5.0 kDa. Simultaneously, the potential allergenicity of the cow milk proteins was diminished gradually and is basically consistent after 60 min of gastrointestinal digestion. After gastrointestinal digestion, the remaining peptides were transported via an Ussing chamber and identified by liquid chromatography-MS/MS. By alignment, 10 epitopes peptides were identified from 16 stable peptides, including 5 peptides (AA 92–100, 125–135, 125–138, and 149–162) in β-LG, 2 peptides in α-LA (AA 80–93 and 63–79), 2 peptides in αS1-casein (AA 84–90 and 125–132), and 1 peptide (AA 25–32) in αS2-casein were identified by dot-blotting mainly exist in UHTM and PM. This study demonstrates dairy processing can affect the digestion and transport characteristics of milk proteins and in turn alter epitope peptides release.

Key words: cow milk allergy, in vitro digestion, intestinal transportation, epitope peptides

INTRODUCTION

Cow milk allergy (CMA) is a common problem encountered in infants with prevalence estimated as 2 to 7.5% at 1 yr of age (Mousan and Kamat, 2016). Epidemiological studies estimated CMA affect 2.2% of infants in the United States and 2.69% of infants in China, based on a birth cohort of 1,050,061 and 9,910 infants, respectively (Hill et al., 2016; Yang et al., 2019). It is reported that 53% of infants allergic to cow milk outgrow their CMA at a median age of 5.3 yr, but the allergy persists into adulthood in 21% of the children (Savage et al., 2016). Bovine milk contains at least 25 potentially allergenic proteins, among them α-LA, β-LG, and caseins were recognized as major allergens because more than half of sera from patients with CMA reacted with them (Hochwallner et al., 2014). Importantly, dairy processing increases the allergenicity of cow milk (Abbring et al., 2019). In addition, consumption of strongly heat-treated and homogenized milk in contrast to raw cow milk could increase the asthma risk in children (Høst and Samuelsson, 1988; Koivusaari et al., 2021)

Industrial dairy processing includes pasteurization, ultra-high temperature sterilization, vacuum evaporation, spray drying, and homogenization. Those heat treatments could prolong the shelf life of dairy products and may also alter the structure of food proteins, including the aggregation of proteins and the exposure of the hidden antigenic epitopes (Mills et al., 2009; Rahaman et al., 2016). Homogenization makes milk emulsion more stable through disruption of milk fat globules and casein micelles, which in turn increases the surface area of milk fat droplets to proteins absorb thus augment-
ing the allergenicity of processed milk (Jaiswal and Worku, 2021). Regarding digestibility, several studies have focused on peptides generation from heat treatment of milk during gastrointestinal digestion (Wada et al., 2017; Torcello-Gómez et al., 2020). In addition, dairy product absorption from the gastrointestinal tract result in delivery to the gut immune system and may trigger an immune response (Do et al., 2016). In general, food proteins that are resistant to acid denaturation and protease digestion have an increased probability of epitopes absorption within the intestinal mucosa immune system where sensitization could occur (Sampson, 2004). Thus, both digestibility and intestinal barrier function are important factors that determined whether allergens could invoke an allergic reaction in individuals. The comprehensive evaluation, which includes the continuous gastrointestinal digestion and absorption of commercially available heat-treated cow milk, is meaningful for dairy products for allergic individuals. However, studies on the allergenicity and residual epitopes of dairy products from digestion and absorption in the gastrointestinal tract are still insufficient.

Food allergen epitopes, including B cell and T cell epitopes, are key to understanding the basic mechanism of food allergy. Furthermore, their identification may provide useful information for food desensitization and immunotherapy (Matsuo et al., 2015). Compared with conformational epitopes, linear epitopes are considered more important in CMA, they are more stable in retaining their structural integrity during thermal processing and gastrointestinal tract digestion, thus easily interacting with the immune system (Morisawa et al., 2009). Extensive epitope mapping studies have been performed to identify sequential epitopes for native cow milk allergens (Cong et al., 2013; Li et al., 2015, 2016; Ueno et al., 2016). Dairy processing of cow milk proteins causes extensive protein aggregation and denaturation which results in structure alteration and allergen epitopes exposure (Abbring et al., 2019). However, analysis of stable linear epitopes on cow milk allergens that could enter into intestinal mucosa is limited.

The aim of this study was to assess the gastrointestinal digestibility, transport and allergenicity of cow milk allergens in 3 dairy products, including pasteurized milk (PM), ultra-heat-treated milk (UHTM), and dried skim milk (DSM) by simulating gastrointestinal tract digestion in vitro, using an Ussing chamber system and indirect ELISA. Further, stable epitopes of cow milk allergens derived from gastrointestinal digestion and intestinal epithelium transport were identified by liquid chromatography-MS/MS (LC-MS/MS). The IgE binding capacity of stable cow milk epitope peptides was assessed using sera from cow milk sensitized patients by dot-blot.

MATERIALS AND METHODS

Four-week-old Brown Norway rats were purchased from Charles River [Beijing, China; Permission number “SYXK (Jing) 2016-0006”]. Mice were housed at 22 ± 2°C and 50% humidity. All mice were housed for at least 1 wk before conducting the experiments. All experiments were performed in strict accordance with the NCH guidelines from China for the care and use of laboratory animals (NHC Publication No.11, Rev. 2016) and approved by the Animal Care Committee of Nanchang University (Nanchang, China).

In Vitro Digestion Model

To reproduce the gastrointestinal digestion phase, the dairy products including PM, UHTM, and DSM were performed with the standard in vitro digestion protocol (Minekus et al., 2014). Simulated gastric fluid (SGF) was prepared as follows: 1.6 mg/mL of a solution consisting of 4,000 U/mL pepsin (P7012, Sigma) in the pH 2.0 gastric stock electrolyte solution (Supplemental Table S1, https://figshare.com/articles/figure/Appendices_docx/20335143; Yang, 2022). Resolving 30 mg/mL of PM (Bright Dairy & Food Co.), UHTM (Mengniu Dairy Co.), and DSM (Fonterra Co-operative Group) were prepared with the standard in vitro digestion protocol consisting of 4,000 U/mL pepsin in water with pH 2.0, respectively, before mixing with SGF (1:1 vol/vol) incubated in a 37°C shaker. A 500-μL aliquot was collected to a 1.5-mL tube at 0, 5, 10, 30, 60, 90, and 120 min, then 1 M NaHCO$_3$ was added to each tube for stopping the reaction, respectively, and placed on ice for further use.

Simulated intestinal fluid (SIF) was prepared as follows. The SGF products were collected at 60 min and further incubated with isopycnic solution which consisted of 1 mg/mL pancreatin (P7545, Sigma), 0.2 mol/mL sodium glyceroxocholate (G9910, Sigma), and 0.2 mol/mL taurocholic acid sodium salt hydrate (T4000, Sigma) in intestinal stock electrolyte solution (Supplemental Table S1) in a 37°C shaker. The 500-μL aliquot was collected into a 1.5-mL tube at 0, 5, 10, 30, 60, 90, and 120 min, and 5 μL of 4 nmol/L 4-(2-Aminoethyl) benzenesulfonyl fluoride (A109762, Aladdin) was added to each tube and placed on ice to stop the reaction. The concentration of digested peptides was tested by the Pierce Quantitative Colorimetric Peptide Assay kit (23275, Thermo Scientific).
Electrophoresis

Digested products (15 μg) were loaded (per lane) on a Tricine-SDS-PAGE by using a discontinuous acrylamide containing 4, 10, and 16.5%. Then electrophoretic gel was separated under constant voltage of 30 V for 60 min, then 100 V for 120 min with Tris/Tricine buffer. The gels were immersed in a fixative solution containing ethanol, acetic acid, and water (4:1:5 vol/vol/vol) for 20 min and then stained with Coomassie Blue R-250. The picture of gels detected by Gel Documents System (G:Box F3; Syngene).

HPLC Analysis of Gastrointestinal Digests

To identify the change of major allergens β-LG, α-LA, and caseins after gastrointestinal digestion, reversed-phase HPLC was performed by the reported methods (Picariello et al., 2010). Briefly, samples were separated on a 4.6 mm i.d. × 250 mm, C18, 5-μm reverse-phase column (GL Sciences) with a flow rate of 1 mL/min on an LC-20AT system (Shimadzu). Solvent A was 0.1% trifluoroacetic acid (302031, Sigma; vol/vol) in water; solvent B was 0.1% trifluoroacetic acid (vol/vol) in acetonitrile (Xilong Scientific). The column was equilibrated at 5% solvent B. Digests (40 μg for each analysis) were carried out with a gradient of 5 to 60% solvent B for 70 min. The column effluent was monitored by UV detection (214 nm).

IgE Binding Ability of Digested Products by Indirect ELISA

The sera were pooled from 10 patients allergic to cow milk (Supplemental Table S2, Yang et al., 2022), which was purchased from PlasmaLab International. Ethical approval from the Second Affiliated Hospital of Nanchang University was obtained for the study. The IgE binding capacity of digests was analyzed by indirect ELISA. Briefly, cow milk protein or digests diluted in 0.05 M carbonate buffer (pH 9.6) was coated to a 96-well microtiter plate at 4°C overnight (0.2 μg/well). The next day, the plate was washed 3 times with PBS containing 0.05% Tween 20 (PBST). Each well was blocked for 1 h at 37°C with 3% gelatin (250 μL/well) in PBS. Antisera with a dilution of 1:20 were added and incubated for 1 h at 37°C. Then, the plate was washed, and 100 μL of HRP-labeled anti-human IgE diluted to 1:5,000 was added to the wells and incubated at 37°C for 1 h. After washing again, the color was developed with 3,3′,5,5′-tetramethylbenzidine (NeoBioscience). The reaction was terminated by adding 50 μL of 2 M sulfuric acid per well. Absorbance was determined at 450 nm with an ELISA plate reader (Varioscan LUX; Thermo Scientific).

Transepithelial Transport Studies by Ussing Chamber

The Ussing chamber (EM-CSY5–8, Physiology Instruments) was used for transepithelial transport studies. First, a section of jejunum was collected from 2 rats both in the sensitized group and the control group for each sample transportation (Supplemental Figure S1, https://figshare.com/articles/figure/Appendices_docx/20335143; Yang, 2022). These tissues were carefully incubated in Kreb’s solution (Supplemental Table S3, https://figshare.com/articles/figure/Appendices_docx/20335143; Yang, 2022) and were then carefully separated from underlying tissue using forceps and surgical scissors. Separated intestinal epithelial tissues were immersed in Kreb’s solution and bubbled with carbogen gas (95% O₂/5% CO₂) before being fixed in Ussing chamber inserts (P2313, Hugo Sachs Elektronik, Harvard Apparatus GmbH), exposing 0.71 cm² of the surface to 5 mL of Kreb’s solution and was kept at 37°C under constant carbogen gassing. After a 10 min equilibration period, the digests (1 mg/mL) from the stomach digestion for 60 min at pH 2.0 and followed with duodenum digestion for 60 min, were added to the mucous lateral chamber. After 3 h, all the transport products in the chamber at the side of the basolateral membrane were collected. The collected transport products were stored at −20°C for further detection.

LC-MS/MS

Transported samples were loaded on a Thermo Scientific EASY column (2 cm × 100 μm, 3 μm, 100 Å nanoViper C18), and then separated on a Thermo Scientific EASY column (10 cm, i.d. 75 μm, 3 μm, C18-A2). The mobile phases were prepared as follows: A (0.1% formic acid in water) and B (0.1% formic acid and 84% acetonitrile in water). Peptide mixtures (25 μg) were separated at a flow rate of 0.3 μL/min with a linear gradient (0–35% solution B, 0–50 min; 35–100% solution B, 50–55 min; 100% solution B, 55–60 min) in the columns. The eluted peptides were detected using an MS (Q exactive, Thermo Scientific). Based on automatic gain control at a resolution of 70,000 at m/z 200 and a dynamic exclusion duration of 60 s, the MS/MS spectra were full scan mass spectrum (m/z 300–1,800). The resolution of HCD spectra was adjusted to 17,500 m/z 200. The normalized collision energy was 30 eV and underfill radio was defined as 0.1%. The overall runtime of the analysis was 120 min.
Raw data files were converted to MGF files using version 1.1 of the protein discoverer software (Thermo Scientific) and then processed using version 2.2 of Mascot (Matrix Science) to match peptides by searching the NCBI and UniProt database.

**Peptides Synthetic**

The 16 candidate epitopes peptides in Supplemental Table S4 (https://figshare.com/articles/figure/Appendices_docx/20335143; Yang, 2022), which were both located in reported linear epitopes and the collected transported stable peptides by LC-MS/MS, were synthesized. Synthetic peptides were provided by Sangon Biotech Co. Ltd. (Shanghai, China) and stored at −20°C until use. The purity (>95%) was assayed by both reversed-phase HPLC and MALDI-TOF MS.

**Dot-Blot Assays**

For the dot-blot assay, the sera were pooled from 10 patients allergic to cow milk (Supplemental Table S2), which were purchased from PlasmaLab International (Everett, WA). An aliquot of the peptide fractions (0.5 μg) was spotted on a 0.22 μm Transblot nitrocellulose membrane (Bio-Rad) and dried at 37°C. The negative control was 0.5 μg of BSA (Sigma) incubated with sera from the infant with the allergy. The membranes were blocked with 3% gelatin (Sigma) in Tris-buffered saline (TBS) for 1 h at 37°C. The membranes were then incubated overnight at 4°C with the sera from allergic individuals diluted 1/20 in TBS with 0.05% Tween 20 (TBS-T). After washing with TBS-T, the monoclonal biotin-conjugated anti-human IgE antibody (Sigma) in TBS (1/2,000) was incubated with the membrane for 1 h at 37°C. After washing with TBS-T, monoclonal peroxidase-conjugated avidin (NeoBioscience) in TBS (1/60) was incubated with the membrane for 1 h at 37°C. The membrane was extensively rinsed with TBS-T (5 × 3 min). Enhanced chemiluminescence reagents (ECL Plus reagent, Bioscience) were used for visualizing the immunoreactive peptide spots at various exposure times.

**Mapping of Epitopes on Allergens**

The 3-dimensional structure of the β-LG and α-LA was based on the crystallographic structure with the Protein Data Bank identity of 1BSO and 6IP9, respectively. The structure of αS1-CN (UniProt ID: P02662) and αS2-CN (UniProt ID: P02663) were predicted by the online software I-TASSER (https://zhanggroup.org/I-TASSER/). Those allergens’ structures with gray color were generated with the PyMol visualization software (PyMOL Molecular Graphics System, version 4.6, Schrödinger). And other color belongs to the epitope peptides mapping within the allergen structure.

**Statistical Analyses.**

All data were analyzed for statistical significance by unpaired t-test (GraphPad Prism, version 8.0, GraphPad Software Inc.). Values are presented as mean ± standard error of the mean. Probability values <0.05 were considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

**RESULTS**

**Changes of Gastrointestinal Digested Products of Milk Proteins**

Results showed that gastric and gastrointestinal digests of milk proteins from PM, UHTM, and DSM displayed a change in molecular weight profiles (Figure 1). The band intensities of intact proteins in the 3 kinds of processed cow milk gradually decreased at each time point, and almost disappeared at intestinal digestion for 30 min. The main allergens showed different resistance to gastrointestinal digestion in the 3 processed cow milks. Herein, degradation of caseins (~25 kDa) started very early and was rapidly completed in the gastric digest phase, and caseins almost completely degraded after 5 min during the gastric digestion in PM and UHTM, whereas a few intact caseins could present until the 10 min gastrointestinal digestion in DSM (Figure 1A).

The bands of β-LG (18.3 kDa) and α-LA (14.2 kDa) in UHTM and DSM were shallower than in PM (Figure 1A), correspondent to the intense of β-LG [(tr) ∼55.90 min] and α-LA [(tr) ∼55.39 min] were decreased in UHTM and DSM, especially in UHTM almost decreased to zero (Figure 1B). In addition, the electrophoretic pattern of UHTM and DSM showed high molecular weight bands (Figure 1A). Those aggregations in UHTM persisted throughout the gastrointestinal intestinal digestion, and could slightly be dissociated and accompanied by deepening of β-LG and α-LA during gastric digestion. The monomer protein of β-LG in UHTM, PM and DSM almost disappeared completely at 10 min of intestinal digestion. The content of α-LA in UHTM, PM, and DSM almost disappeared completely at 5, 0, and 30 min of intestinal digestion, respectively. (Figure 1A). In addition, it is worth noting that resistant peptides with molecular weight ranging from 3.4 to 5.0 kDa were only formed in the digests of PM at each time point of the intestinal digestion (Figure 1A).
To determine the allergenicity of digestive bovine milk proteins, proteins and peptides were analyzed by indirect ELISA with sera from patients allergic to cow milk (Figure 2). In general, the relative IgE binding ability of PM, UHTM, and DSM decreased with prolonged exposure to gastric conditions. Interestingly, the IgE binding capacities of UHTM ($P < 0.05$) and DSM ($P < 0.01$) increased significantly at early gastric digestion stage (Figure 2B and 2C). It was indicated that the formation of those polymers could protect epitopes from disruption under UHT sterilization. After simulating gastric fluid digestion for 60 min, the IgE binding capacity of PM, UHTM, and DSM, relative to the original protein was reduced to $56.00 \pm 0.30\%$, $64.84 \pm 0.93\%$, and $55.73 \pm 1.50\%$, respectively. Until gastrointestinal digestion was conducted for 60 min, the IgE binding capacity of PM, UHTM, and DSM were gradually decreased, and relative to the original protein was reduced to $20.43 \pm 0.15\%$, $19.06 \pm 0.57\%$, and $13.92 \pm 0.24\%$, respectively. It suggested that the IgE epitopes in UHTM and DSM could be released at
the beginning of gastric digestion, and the IgE binding capability of the 3 dairy products decreased during sustained gastrointestinal digestion.

**Analysis of Cow Milk Peptides from Gastrointestinal Digestion and Transport**

Peptides were released after 60 min of gastric digestion plus 60 min of intestinal digestion and then identified by LC-MS/MS (Supplemental Table S5, https://figshare.com/articles/figure/Appendices_docx/20335143; Yang, 2022). Consistent with the number of peptides for each allergenic protein, peptides generated after gastrointestinal digestion covered more than 20% of the protein sequences of β-LG, αS1-CN, and αS2-CN (Figure 3). In contrast, the peptide sequence coverage rate of α-LA, β-CN, and κ-CN were generally lower than 20%, indicating that these allergens are sensitive to gastrointestinal digestion (Figure 3). Especially, α-LA cannot be detected in the digests of DSM after simulated gastrointestinal digestion in vitro.

To study the transepithelial transport, in vitro digests sampled after 60 min of gastric digestion followed by 60 min of intestinal digestion were applied to intestinal transportation and analyzed by LC-MS/MS (Supplemental Tables S6 and S7, https://figshare.com/articles/figure/Appendices_docx/20335143; Yang, 2022). The intestine was separated from 2 rats in each group (Supplemental Figure S1). The number of allergen peptides found in gastrointestinal digests for each sample was more than the transported products (the total peptides of PM were 31/15, UHTM were 28/16, and DSM were 32/2). Furthermore, the length distribution of peptides in the transport products appears to be shorter compared with gastrointestinal digests (Figure 4). Considering that intestinal brush border enzymes are

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**Figure 2.** The relative IgE binding ability of gastric and gastrointestinal digests (0.2 μg/well) of (A) pasteurized milk (PM), (B) ultra-heat-treated milk (UHTM), and (C) dried skim milk (DSM). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 indicate a significant difference as compared with the original protein. All data are represented as the mean ± SD (n = 3).
localized in the intestinal epithelium (Picariello et al., 2015), therefore, those allergen peptides derived from gastrointestinal digests could be further broken down as they pass through the intestinal epithelium. The number of allergen peptides in transported products of the sensitized group was more than the control group (the total peptides of PM were 15/6, UHTM were 16/2, and DSM were 2/1), suggesting the intestinal permeability was increased in cow milk sensitized rats. It is noted that peptide AA 76–83 (TKIPAVFK) of β-LG was stable exist in the gastrointestinal digestion and transport products, and its shortened form, AA 78–83 (IPAVFK) could be transported across the intestinal barrier of the control group and DSM sensitized group. According to previous studies on cow milk allergens’ epitopes, we obtained the digestion and transported stable peptides from the PM, UHTM, and DSM as the IgE binding epitope candidates (Table 1). In the sensitized groups of PM, UHTM, and DSM, the potentially allergenic peptides derived from β-LG, α-LA and caseins could stably exist in the process of digestion and transportation, including β-LG (AA 41–60, 61–69, 76–83, 84–91, 92–100, 125–135, 125–138 and 142–148), α-LA (AA 80–93, 99–108 and 115–122), αs1-CN (AA84–90) and αs2-CN (AA 25–32) for PM; β-LG (AA 61–69, 76–83, 125–135, 125–138 and 142–148), α-LA (AA 63–79, 80–93, 99–108 and 115–122), αs1-CN (AA 84–90 and 125–132) and α S2-CN (AA 25–32 and 71–76) for UHTM; β-LG (AA 78–83, 92–100) for DSM. Overall, the peptides derived from the main allergens in PM were similar to UHTM during gastrointestinal digestion and transportation. In addition, there was a small range of peptides and only one epitope presented in the transported products of DSM.

**Dot-blotting Analysis of Stable Peptides**

The allergen peptides contained 5 to 15 AA that could be the IgE binding epitope. Sixteen stable peptides from digestion and transportation of cow milk protein were identified as P1 to P16 and synthesized for further analysis (Supplemental Table S7). The interaction between epitopes peptides and IgE could be displayed dot in the nitrocellulose filter membrane. As shown in Figure 5, P2 (AA 61–69), P5 (AA 92–100), P6 (AA 125–135), P7 (AA 125–138) and P8 (AA 142–148) on β-LG, P9 (AA 63–79) and P10 (AA 80–93) on α-LA, P13 (AA 84–90) and P14 (AA 125–132) on αs1-CN, and P15 (AA 25–32) on αs2-CN could actively bound to IgE. The majority of peptides were present in transported products of PM and UHTM, and only antigen peptides AA92–100 on β-LG belong to DSM. The antigen peptides of β-LG in PM were more stable than UHTM, and antigen peptides of α-LA and αs1-CN were opposite (the number of epitopes peptides in PM and UHTM on β-LG were 5/4, on α-LA were 1/2, on αs1-CN 1/2 and on αs1-CN 1/1).

**Mapping of IgE Binding Epitopes of Allergens**

These 10 antigen epitopes peptides were mapped into modeled 3-dimensional structures of corresponding allergens using PyMOL software (Figure 6). Five peptides were mapped on β-LG structure (UniProt: P02754; Figure 6A), 2 peptides were mapped on α-LA (UniProt: P00711; Figure 6B), 2 peptides mapped on αs1-CN to the modeled spatial structure predicted by I-TASSER (UniProt ID: P02662; Figure 6C), and one peptide were mapped on αs2-CN within the modeled...
3-dimensional structure predicted by I-TASSER (UniProt ID: P02663; Figure 6D). Of note, most surviving epitopes were distributed within α helices on the surface of the proteins, and 3 are distributed in β folded structure for β-LG.

**DISCUSSION**

Epitope exposure in the intestine mucosal system is one of the keys to the cause of cow milk allergy. Therefore, there has been a considerable increase in the number of studies attempting to define the behavior of dietary proteins during digestion and absorption. Such as using in vitro digestion models (dynamic and static digestion model) and in vivo animal model to mimic the phases of physiological digestion. Based on a simple and standardized digestion method that has been reported (Minekus et al., 2014), the static in vitro digestion method and some parameters were adopted in this study. An Ussing chamber could better simulate the absorption of substances under physiological conditions; it has been widely applied in the field of nutrient absorption and transportation (Luo et al., 2018; Kent-Dennis and Penner, 2021). Regardless of the pattern of gastrointestinal digestion and transport, some milk-derived peptides and allergen epitopes exhibit proteolytic resistance depending on the processing of the dairy products.

Caseins are sensitive to processing-induced chemical modifications and aggregation (Pellegrino et al., 2011), in spite of less tertiary structure and few disulfide bonds of caseins. A simple digestion system has been used to simulate the digestion of cow milk proteins in vitro, demonstrating that the intact caseins entirely
degraded to peptides after 0 min incubation (Do et al., 2016). We observed caseins in PM and UHTM were almost completely degraded after 0 min of gastric digestion, whereas the intact caseins were still present until the 10 min of gastrointestinal digestion in DSM. And a similar result was obtained by Sánchez-Rivera, who noticed more stability of caseins in heated skim milk compared with the unheated sample in a dynamic in vitro gastric digestion model (Sánchez-Rivera et al., 2015). This could be due to chemical modification including glycation and oxidation, which mainly occur in the drying process (van Lieshout et al., 2020). The β-LG could be co-precipitated with casein in UHTM and remains partly unhydrolyzed by digestive proteases (Picariello et al., 2010). However, the monomer β-LG in UHTM was sensitive to gastrointestinal digestion. It agreed with the findings that heating could facilitate the gastrointestinal digestion of β-LG (Sletten et al., 2008; Rahaman et al., 2017). It is noteworthy that the electrophoretic band of β-LG and α-LA reappeared at the beginning of gastric digestion in UHTM and DSM (Figure 1A). It might result from the acid, which worked at the beginning of digestion, could interrupt the interaction between the aggregations.

Once ingested, cow milk proteins are absorbed from the gastrointestinal tract in the form of peptides and AA to provide nutrients. The peptides that could enter the gastrointestinal mucosa must be the dominant peptides that could tolerate the harsh environment of the gastrointestinal tract. In our study, the peptides sequence was aligned for 16 stable peptides that survived from the intestinal epithelial and were considered candidate epitopes (Table 1). And more peptides were transepithelial transport in allergen sensitized rats than

| Allergen | Dairy product¹ | GenBank accession no. | Stable transported peptides identified by LC-MS/MS | Reported IgE linear epitope location |
|----------|-----------------|-----------------------|---------------------------------------------------|-----------------------------------|
| β-LG     | PM              | CAA32835.1            | 41–60, 61–69, 76–83, 84–91, 92–100, 125–135, 125–138, 142–148 | 1–15, 56–70, 76–90, 136–150 (Cong and Li, 2012); 58–77, 76–95, 121–140 (Cerecedo et al., 2008); 1–16, 31–60, 67–86, 127–152 (Jarvinen et al., 2001) |
|          | UHTM            |                      | 61–69, 78–83, 125–135, 125–138, 142–148 |                                    |
|          | DSM             |                      | 78–83, 92–100 |                                    |
| α-LA     | PM              | AAA30615             | 80–93, 99–108, 115–122 | 41–46, 55–60, 62–72, 74–76, 85–90, 92–99 (Li et al., 2016); 1–19, 15–34, 45–64, 60–79, 90–109, 105–123 (Hochwallner et al., 2010) |
|          | UHTM            |                      | 63–79, 80–93, 99–108, 115–122 |                                    |
|          | DSM             |                      | 78–83, 111–122 |                                    |
| αS1-CN   | PM              | NP_851372            | 84–90 | 6–20, 11–35, 126–140, 171–185 (Cong et al., 2013); 16–35, 28–50, 73–92 (Cerecedo et al., 2008); 17–36, 39–48, 69–78, 83–102, 109–120, 122–132, 139–154, 159–174, 173–194 (Chatcatherine et al., 2001) |
|          | UHTM            |                      | 84–90, 125–132 |                                    |
|          | DSM             |                      | 84–90, 125–132 |                                    |
| αS2-CN   | PM              | NP_776953            | 25–32 | 1–20, 13–32, 67–82, 106–125, 122–141, 157–182, 181–207 (Cerecedo et al., 2008); 31–41, 43–56, 83–100, 93–108, 105–114, 117–128, 142–158, 157–172, 165–188, 191–200 (Busse et al., 2002) |
|          | UHTM            |                      | 25–32, 71–76 |                                    |
|          | DSM             |                      | 71–76 |                                    |

¹Protein supplement: PM = pasteurized milk; UHTM = ultra-heat-treated milk; DSM = dried skim milk.

Figure 5. Dot-blot analysis of IgE reactivity of synthetic β-LG (P1–P8), α-LA (P9–P12), αS1-CN (P13–14), and αS2-CN (P15–16) peptides. The signal was visualized by enhanced chemiluminescence. The control was coated with BSA as a negative control.
in unimmunized rats because sensitization induced by food allergens increased intestinal permeability (Chen et al., 2014). Among the 16 stable peptides, 4 peptides belonged to caseins, although caseins can be rapidly degraded during gastrointestinal digestion and transport. This is consistent with a report that caseins especially sensitize via the gastrointestinal tract retain their allergenicity even after a complex process of digestion in vivo (Hochwallner et al., 2014). The β-LG is known to exhibit a certain resistance to gastrointestinal hydrolysis, whereas UHT treatment facilitated its enzymatic digestion (Wada and Lonnerdal, 2014). In our study, 50% of peptides were derived from β-LG presented in PM, 5 peptides in UHTM, and only 2 peptides in DSM. With regard to α-LA, we found a small number of peptides derived from α-LA in PM and UHTM, whereas absent in DSM during gastrointestinal digestion and transportation. Considering that chemical modifications including glycation and oxidation, occur during the drying process (van Lieshout et al., 2020), some peptides cannot be detected by the LC-MS/MS used in our study.

A major cause of cow milk allergy was that epitopes survived in gastrointestinal digestion and absorption. By dot-blot, we identified casein epitopes that survived from proteolysis and intestinal epithelial transportation, including 2 regions AA 84–90 and AA 125–132 of αS1-CN and AA 25–32 of αS2-CN in PM and UHTM but not in DSM. The sequence part of the IgE epitope of caseins in our study was shorter than the reported epitopes of AA 83–102 and AA 122–132 of αS1-CN (Spuergin et al., 1996; Chatchatee et al., 2001), and AA 13–32 of αS2-CN (Cerecedo et al., 2008). These findings presented epitope peptides in caseins involved in eliciting immunoreactions in CMA individuals. Moreover, 2 epitopes of AA 63–79 and AA 80–93 derived from α-LA were also reported in Li’s study (Li et al., 2016), in which they were corresponding to 4 regions in AA 62–72, AA 74–76, AA 85–90 and AA 92–99 of α-LA. Bovine β-LG accounts for almost 80% of cow milk allergy (Rahaman et al., 2017), which is owed to its strong gastric digestion resistance (Untersmayr and Jensen-Jarolim, 2008). However, the heat treatment could improve the sensitivity of β-LG in gastric digestion. In the current study, 50% of epitopes belong to β-LG and the peptides of AA 61–69, 125–135, 125–138, and 149–162 in PM and UHTM, whereas AA 92–100 was identified in PM and DSM. The previous study reported that 3 synthesis peptides AA 72–86, AA 92–106, and AA 152–166 of β-LG exhibited major IgE binding activity by alanine scanning of immunodominant epitopes (Cong and Li, 2012). And their short fragment was also determined as the epitopes in our study, excepting the AA 76–83. The peptide of AA 78–83 with no IgE binding capacity but with angiotensin I-converting enzyme inhibitory activity (Nielsen et al., 2017) can be transported across the in-

![Figure 6](image_url)
testinal. Moreover, a resistant IgE epitopes peptide AA 125–135 of β-LG could survive in the gastrointestinal digestion and transport by a Caco-2 monolayers model (Picariello et al., 2013), which existed in UHTM and PM. Overall, there was a small range of peptides and only one epitope presented in the transported products of DSM. This was in line with the finding by Zenker et al. (2020) who observed lower numbers of peptides and a lower sequence coverage in high temperature dry heated cow milk protein. Generally, the availability of larger peptides increases the possibility of recognition by the immune system. Peptide length distribution found in the transport products (Figure 4) indicated a favored transport of peptides within a length between 6 and 20 AA. In our study, IgE binding epitopes that were stable in gastrointestinal digestion and transport were identified within the range of 7 to 14 AA. In addition to peptide size, transport in intestinal epithelium can also be determined by charge and hydrophobicity (Amigo and Hernandez-Ledesma, 2020). Further studies would be considered to determine the properties and chemical modification of peptides to explore the factors that affect the epitope transepithelial transport. In addition, in vitro cell culture studies and in vivo animal experiments are required to further explore the function of those IgE binding epitopes identified in this study.

CONCLUSIONS

In the present study, we found that the pepsin-sensitive caseins in DSM showed more stable in vitro digestion. Both α-LA and β-LG also in PM and DSM showed a stronger resistance to gastric digestion, compared with the sample in UHTM. As the major allergens, they contributed to the higher allergenicity of processed dairy products. Based on the sequence alignment, we found 16 stable peptides during digestion and transportation in vitro, and 10 of them were identified by dot-blot with allergic sera in infants. Among them, 5 peptides derived from β-LG, whereas 2 peptides were in both α-LA and αS1-CN, respectively, and one peptide belonged to αS2-CN. These 10 peptides mainly existed in PM or UHTM, and only one peptide was presented in DSM. However, future study is needed to design appropriate in vivo bioavailability assays to track the metabolic fate of those proteins and to validate the milk-derived peptides actually involved in allergic reactions.

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