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Peptidomics Analysis of Milk Protein-Derived Peptides Released over Time in the Preterm Infant Stomach

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Abstract

Over the course of milk digestion, native milk proteases and infant digestive proteases fragment intact proteins into peptides with potential bioactivity. This study investigated the release of peptides over 3 h of gastric digestion in 14 preterm infant sample sets. The peptide content was extracted and analyzed from milk and gastric samples via Orbitrap tandem mass spectrometry. The relative ion intensity (abundance) and count of peptides in each sample were compared over time and between infants fed milk fortified with bovine milk fortifier and infants fed unfortified milk. Bioactivity of the identified peptides was predicted by sequence homology to known bioactive milk peptides. Both total and bioactive peptide abundance and count continuously increased over 3 h of gastric digestion. After accounting for infant weight, length, and postconceptual age, fortification of milk limited the release of peptides from human milk proteins. Peptides that survived further gastric digestion after their initial release were structurally more similar to bioactive peptides than nonsurviving peptides. This work is the first to provide a comprehensive profile of milk peptides released during gastric digestion over time, which is an essential step in determining which peptides are most likely to be biologically relevant in the infant. Data are available via ProteomeXchange with identifier PXD012192.

Graphical Abstract

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The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.jproteome.8b00604](https://doi.org/10.1021/acs.jproteome.8b00604).

Figure S1: Workflow of the infant samples and the analyses performed on them (PDF)

Table S1: FASTA sequences of the human and bovine milk protein library used to identify peptides in MS/MS; Table S2: List of all milk peptides found in the preterm infant milk and gastric samples (XLSX)

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE²¹ partner repository with the data set identifier PXD012192.

Keywords

bioactive; digestion; gastric; human milk; peptide; preterm

1. INTRODUCTION

Milk has evolved as the sole source of nutrition for the human infant. Milk contains the ideal balance of macronutrients, micronutrients, and other bioactive factors to fuel infant growth and aid in development.¹ In addition to providing amino acids for protein synthesis, intact milk proteins have multiple functions within the infant, including supporting the infant immune system,^{2,3} preventing pathogen growth,⁴ serving as growth factors,⁵ and regulating sequestration and delivery of iron.⁶ Digestion of milk proteins occurs by the action of a combination of proteases native to the milk and digestive proteases secreted by the infant gastrointestinal tract.⁷ Our previous work has determined that even before expression of milk from the mammary gland, native milk proteases initiate the hydrolysis of milk proteins.⁸ However, beyond milk proteins' role as a source of amino acids and as bioactive molecules, digestion of the proteins also releases protein fragments (peptides) that have specific biological activities, including antimicrobial,⁹ antihypertensive,¹⁰ immunomodulatory,¹¹ anti-inflammatory,¹² and opioid agonistic and antagonistic effects.¹³ The evolutionary role of milk proteins in the development of the human infant thus might not be limited to intact function and amino acid release but may also encompass the release and function of bioactive peptides.

The release of these bioactive milk peptides varies by stage of digestion. As different proteases with different cleavage specificities are secreted throughout the gastrointestinal tract, more of the parent protein sequence is broken apart to release peptides with diverse sequences. As the amino acid sequence of the released peptides determines the type of activity they can exert,¹⁴ peptides released in the infant stomach may have different functions and sites of action than those already present in human milk, and peptides that survive gastric digestion intact might be released only to act further downstream in the intestine. As such, identifying the release of peptides from milk proteins across time will

provide a more complete picture of gastric protein digestion that can aid in the identification of potential bioactive peptides that are most biologically relevant.

Previous work has measured the proteolysis of intact milk proteins in real or simulated infant stomach conditions without examining the resulting peptides.^{15,16} Other studies have examined in vitro and in vivo gastric milk peptide release, but samples were only collected at a single time point.^{17–19} These types of profiles of human milk peptides are vital for efforts to identify promising peptide sequences for determination of further bioactivities that are most relevant to the human infant. However, in vitro studies do not adequately represent how digestion occurs in the infant stomach, and the previous in vivo peptidomic analysis study¹⁹ only analyzed gastric samples collected 2 h after feeding. As such, there is limited knowledge on which peptides are released and at what time during gastric digestion, and the types of functions these peptides may have in the body.

The present study aimed to identify the peptidomic profile of fortified and nonfortified human milk from preterm-delivering mothers and in gastric samples at three time points (1, 2, and 3 h postfeeding) from their preterm infants. Peptides from the milk and gastric contents were isolated and identified via Orbitrap Fusion Lumos mass spectrometry analysis to assess the change in their release over time. Bioactivity of the peptides was assessed based on sequence homology to known functional peptides in the literature.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

HPLC-grade acetonitrile (ACN) was obtained from Fisher Scientific (Waltham, MA), trifluoroacetic acid (TFA) and HPLC-grade formic acid (FA) were obtained from EMD Millipore (Billerica, MA), and trichloroacetic acid was obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Participants and Samples

This study was approved by the Institutional Review Boards of the University of California, Davis and Oregon State University. Preterm infants were enrolled if they were inpatients in the UC Davis Children's Hospital Neonatal Intensive Care Unit in Sacramento, California, had an indwelling nasogastric or orogastric feeding tube, and could tolerate full enteral feeding. Most of the enrolled infants required a feeding tube because of uncoordinated or immature capacity to suck and swallow. Infants with anatomic or functional gastrointestinal disorders were excluded from enrollment. Samples were collected from 10 preterm-delivering mother-infant pairs ranging in gestational age (GA) at birth from 23 to 32 weeks and postnatal age of 7 to 98 days. Infant characteristics are described in Table 1. Enrolled preterm infants had medical conditions typical of premature infants, including lung immaturity (respiratory distress syndrome), bronchopulmonary dysplasia, intraventricular hemorrhage, retinopathy of prematurity, patent ductus arteriosus, and sepsis/meningitis but no overt gastrointestinal tract issues. None of the infants sampled received medications known to affect gastric pH or gastric digestion capacity, including prokinetics, H2 blockers/

antagonists or proton-pump inhibitors. The enrolled infants were clinically stable at the time of sample collection.

The mothers pumped their breast milk into sterile plastic containers at home and froze it in home freezers. Mothers transported their milk to the NICU on ice where it was stored at -20°C . At time of feeding, a 1.5–2 mL sample of the breast milk was collected into a sterile Eppendorf tube after it had been thawed and fortified, or just thawed if no fortification was added. Nine preterm infants (25.2 week GA ± 0.7 , 50 ± 7 days of postnatal age) were fed their mother's milk (not pasteurized) fortified with 1 packet of Human Milk Fortifier (Similac Human Milk Fortifier Powder, Abbot Park, IL, USA) per 25 mL of milk, and five preterm infants (25 ± 3 week GA, 29 ± 9 days of postnatal age) were fed their mother's milk (not pasteurized) unfortified. The fortifier contained nonfat bovine milk, whey protein concentrate, corn syrup solids, medium-chain triglycerides, calcium phosphate, and potassium citrate. Three sets of twins were included in the study and shared milk samples (11 total milk samples).

The human milk feedings were delivered via the nasogastric tubes over 30 min. A volume of 2 mL was collected from the initial milk feed, and 0.5–2 mL of each preterm infant's gastric contents was collected in a 3 mL syringe via suction through the feeding tube at 1, 2, and 3 h after the initiation of feeding. Only infants from whom samples were successfully collected at all three time points were included in the study. Milk and gastric samples were placed into sterile plastic vials and stored immediately at -20°C to prevent further proteolysis. Human milk and gastric samples were transported to OSU on dry ice and stored at -80°C .

2.3. Sample Preparation

Samples were prepared as in our previous study²⁰ with the following changes. Fifteen microliter aliquots of the skimmed samples were collected and dissolved in 85 μL of 0.1% FA solution to reduce the viscosity of the gastric samples. Milk proteins were precipitated from the samples by addition of 100 μL of 24% trichloroacetic acid. After mixing for 10 s with a vortex mixer, the samples were centrifuged at 10 000g for 20 min at 4°C , and the supernatant containing the peptides was collected. Peptides were loaded onto C18 reverse-phase preparative chromatography 96-well plates (Glygen, Columbia, MD). Trichloroacetic acid, salts, oligosaccharides, and lactose were washed out with 1% ACN, 0.1% TFA, and peptides were eluted in 80% ACN, 0.1% TFA. The peptide solutions were frozen at -80°C and lyophilized using a freeze-dry system (Labconco FreeZone 4.5 L, Kansas City, MO). After drying, the samples were rehydrated in 15 μL of 0.1% FA.

2.4. Liquid Chromatography Nanoelectrospray Ionization Mass Spectrometry

Peptides were analyzed with an Orbitrap Fusion Lumos (Thermo Scientific, Waltham, MA) mass spectrometer connected to a Waters Nano Acquity UHPLC (Water Corporation, Milford, MA). One μL of peptides were loaded onto a C18 180 $\mu\text{m} \times 20$ mm, 5- μm bead nanoAcquity UPLC Trap Column (Waters) for enrichment and desalting, and separated with a 100 $\mu\text{m} \times 100$ mm, 1.7- μm bead Acquity UPLC Peptide BEH C18 column (Waters) over 120 min. The mobile phase consisted of 0.1% FA in H_2O (Solvent A) and 0.1% FA in ACN (Solvent B). The separation gradient consisted of 3–10% solvent B over 3 min, 10–30%

solvent B over 99 min, 30–90% solvent B over 3 min, 90% solvent B for 4 min, 90–3% solvent B over 1 min then finally held at 3% solvent B for 10 min. A 30 min column wash was performed after each sample run.

Mass spectra were collected in positive ionization mode and with data dependent acquisition. Peptides were ionized with an electrospray voltage of 2400 V and ion transfer tube temperature of 300 °C. The mass spectrometer scanned masses between 400 and 1500 *m/z*. Full scan MS data were acquired in the Orbitrap at 120 K resolution at 200 *m/z*. The automatic gain control target was 4.0×10^5 over a maximum injection time of 50 ms. Precursor ions were selected for most intense peaks with an ion intensity threshold of 5.0×10^3 and charge state 2–7. Precursor ions were fragmented using collision-induced dissociation with a collision energy of 35%. Following fragmentation, precursors were excluded (10 ppm mass error) for 1 min.

Raw files were analyzed in Thermo Proteome Discoverer (v2.1.0.81), and a SequestHT search engine identified peptides using an in-house human and bovine milk protein sequence database (Table S1). Potential modifications allowed included phosphorylation of serine and threonine, and oxidation of methionine. Only peptides identified with high confidence were included ($P < 0.01$), and peptide sequences with multiple modifications were grouped into a single peptide for counts. Counts measured the number of unique peptide sequences identified in a sample. Abundance measured the area under the curve of the eluted peak (ion intensity), as determined by Proteome Discoverer. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE²¹ partner repository with the data set identifier PXD012192.

2.5. Data Analysis

A workflow of sample analysis is included as Figure S1. Identified peptides were examined for homology with literature-identified bioactive peptides using our recently created Milk Bioactive Peptide Database (MBPDB, <http://mbpdb.nws.oregonstate.edu/>).²² The MBPDB is a comprehensive source for all milk bioactive peptides. The search was performed as a sequence search that searches for bioactive peptides matching the input peptide sequence. The similarity threshold was set to 80%, with the amino acid scoring matrix set to identity. “Get extra output” was selected to obtain the specific percentage similarity between the query sequence and the database sequence.

The total abundances of peptides were summed and mapped to the parent sequence of human milk proteins using an in-house tool (PepEx), which can be accessed at <http://mbpdb.nws.oregonstate.edu/pepex/>.

2.6. Statistical Methods

For all statistical analyses, twin samples were considered as separate sample sets (14 complete milk/gastric sample sets). Repeated measures ANOVA followed by Tukey’s honest significant difference post hoc test (GraphPad Prism software, version 7.04) were applied to compare human milk and gastric samples at the three times postingestion for peptide abundance and count. Bonferroni-corrected *t*-tests were used to compare amino acid

percentage between surviving and nonsurviving peptides. Chi-squared test with Yates correction was used to compare proportion of peptides with modifications.

A multiple linear regression model (RStudio, version 3.3.2) was used to assess the impact of meal fortification to peptide content over time, starting with the initial model:

$$Y = \text{fortification} + \text{time} + \text{fortification} \times \text{time} + \text{body weight} + \text{body length} + \text{postconceptual age} \quad (1)$$

Body weight (kg) refers to the infant's weight at time of sample collection, body length (cm) refers to the infant's length at time of collection, and postconceptual age (days) is the age of the infant plus its gestational period. Model selection was performed among the covariates for body weight, body length, and postconceptual age using the function "regsubsets" in the package "leaps". *P* values were determined for the coefficients for fortification, time, and fortification $\times$ time. Differences were designated significant at *P* = 0.05.

3. RESULTS AND DISCUSSION

3.1. Peptidomic Profile of Milk Peptides during Digestion

This research is the first study in which in vivo proteolysis and peptide release were tracked over time in the preterm infant stomach. Both in vitro^{17,18} and in vivo¹⁹ studies have been performed in order to measure milk protein digestion and identify the peptides released in the infant stomach. Because these were "snapshot" studies, there is no information on whether the identified peptides represent the end point of gastric digestion, or some time in between the beginning of feeding and the completion of gastric emptying. The inclusion of time as a variable in the study parameters allowed us to gain a deeper understanding of when milk proteins are digested and peptides are released and which peptides survive further digestion. In addition, the use of a state-of-the-art Orbitrap Fusion Lumos mass spectrometer identified a larger number of identified peptides in each sample than previous studies, thus improving the accuracy and comprehensiveness of the final peptide profile.

The peptidomic data from all 56 samples included 11 592 unique peptides (13 545 when counting different post-translational modifications as unique peptides) derived from 299 different milk proteins (Table S2). Of these peptides, 8037 were human milk peptides from 202 human milk proteins, and 3304 were bovine milk peptides from 97 bovine milk proteins. Another 251 identified peptides may have derived from either human or bovine milk proteins due to shared sequences between the species. Table 2 lists the proteins with the highest abundance and count of peptides from all samples. A majority of peptides (8747) were not present in human milk, and were identified in at least one of the three gastric time points (Figure 1). Compared with a previous in vivo digestion study by Dallas et al. that identified 661 unique peptides in milk or after 2 h of digestion,¹⁹ this study identified 8916. Most (88.4%) of the peptides in the Dallas study were also identified in the present study. A likely reason for the large difference in number of peptides identified is due to our use of the Orbitrap Fusion Lumos mass spectrometer, which is able to acquire high-accuracy mass spectra much faster than the Q-TOF used in the previous study.

Both milk peptide count and abundance continuously increased over 3 h of gastric digestion. With milk samples representing the zero time point of gastric digestion for the statistical analyses, the average abundance of peptides increased after each hour of digestion (Figure 2A). Significant increases in abundance occurred between milk and 3 h gastric digestion, and between 1 h digestion and 3 h digestion. Milk to 2 h digestion trended to increase, but was not significant ($P = 0.057$). Milk peptide counts also significantly increased from 671.2 ± 52.7 unique peptides in milk to 1254.9 ± 142.8 in 1 h gastric samples, 1490.6 ± 113.2 in 2 h samples, and 1629.1 ± 102.1 in 3 h samples (Figure 2B). Interestingly, of the peptides found in both milk and 3 h samples, more peptides were significantly higher in milk than 3 h (Figure 2C). The majority of the increase in peptide abundance in 3 h samples is thus likely due to the release of additional peptides that were not present in milk.

Milk peptides that were present in a majority of the infants (8 or more out of 14) for each sample type accounted for the majority of peptide abundance. One hundred and ninety-one peptides were found in a majority of milk samples, 314 in a majority of 1 h gastric samples, 454 in a majority of 2 h samples, and 613 in a majority of 3 h samples. These common peptides accounted for the majority of peptide abundance at $62.9\% \pm 23.9\%$, $63.1\% \pm 15\%$, $60.5\% \pm 10.9\%$, and $69.1\% \pm 10.7\%$ of sample type abundances, respectively. A small subset of these peptides was found in every infant for each sample type. Sixteen peptides were found in all milk samples, 9 in all 1 h gastric samples, 7 in all 2 h samples, and 11 in all 3 h samples. These shared peptides are indicated on Table S2.

These findings support our previous finding of an increase in total proteolysis in the infant stomach over time.²³ As average gastric half-emptying time ranges from 45 to 100 min for preterm infants,^{24–26} 3 h likely represents the end point of gastric digestion and captures all peptides released by pepsin and milk proteases in the stomach before transit to the duodenum. Pepsin is secreted by the infant stomach, and additional proteases, such as cathepsin D and plasmin,^{7,27} are naturally present in milk and known to be active even in somewhat acidic gastric conditions in the infant. The combined activity of pepsin and native milk proteases on intact milk proteins and any peptides already released in the mammary gland maximizes the count of unique peptides and peptide abundance prior to milk entering the small intestine. As this study concluded with the end of gastric digestion, it is not known whether 3 h gastric digest samples represent maximum peptide content, or whether the activity of pancreatic proteases continues to increase the count and abundance of milk peptides rather than reduce them through further degradation to their component amino acids.

3.2. Comparison between Fortified and Nonfortified Samples

Preterm infant milk is often fortified with additional milk protein to provide for the infant's increased protein requirements. However, fortification is typically sourced from bovine milk, thus altering the total protein composition of the milk. Human milk is the ideal source of nutrition for preterm infants as it reduces risk of sepsis, necrotizing enterocolitis, and other infections compared with formula.^{28,29} Human milk provides the infant with bioactive proteins such as immunoglobulins, cytokines, growth factors, and antimicrobial proteins that are not active in bovine milk-based fortifier.³⁰ It is therefore possible that human milk

peptides are also more beneficial to the infant than other peptide sources. To determine the effect of fortification on peptide release, fortified milk-fed infants were compared with nonfortified milk-fed infants. Three sets of infant samples were removed from these comparisons, as the milks collected from these infants' mothers was reported as nonfortified yet the infant gastric samples reflected fortified milk feeding. The final sample sets included five infants in the nonfortified group and six infants in the fortified group. Peptides that could not be distinguished as either human or bovine milk protein-derived were included in the total peptide analysis, but excluded from the human- and bovine-specific analyses.

Neither total peptide abundance nor human milk peptide abundance changed significantly over time, but nonfortified infants had significantly greater total and human milk peptide abundance than fortified infants (Figure 3A). Bovine milk peptide abundance significantly increased during digestion, but no comparisons were made between fortified and nonfortified samples, as nonfortified infants were only fed human milk. Human milk peptide count, bovine milk peptide count, and total peptide count all significantly increased across 3 h of gastric digestion (Figure 3B). Nonfortified total and human milk peptide counts were also significantly higher than fortified counts. Since the nonfortified samples generally were collected from infants at a lower corrected gestational age (Table 1), it is unlikely that maturity or development of the GI tract (capacity for efficiently releasing milk peptides) is the explanation for the observed differences. A more likely justification for the observed differences is that pepsin activity in fortified infants is divided between digesting human milk proteins and fortifier proteins during the same time of digestion, thus releasing fewer human milk peptides than infants who only receive human milk.

We identified additional significant differences between fortified and nonfortified infants concerning how peptides were released from individual milk proteins. Figure 4 compares the release of peptides from the five highest abundance and count human milk proteins. Fortified infants had significantly lower β -casein, α_{s1} -casein, and osteopontin peptide abundance and count, and lower polymeric immunoglobulin count. Only lactoferrin peptide release did not differ by fortification status. These results are notable, as every bioactive peptide identified from human milk was identified from either β -casein, α_{s1} -casein, lactoferrin, or κ -casein (which also had significantly lower count in fortified infants, results not shown).²² These differences likely did not impact total protein nutrition to fortified infants, as bovine milk peptides accounted for the difference in protein digestion. However, the impact to infant health and development could potentially arise from the reduced presence of human milk protein-derived bioactive peptides in fortified infants.

3.3. Bioactive Peptides in the Stomach

Milk proteins have been a highly studied source of bioactive peptides due to milk's evolution as a source of nutrition for mammals. Bioactive peptides have been shown to be released from milk proteins by natural milk proteases,⁸ bacterial proteases,^{31,32} and in vitro digestive proteases,^{17,18,33} but few studies have measured the bioactivity of peptides released by in vivo human digestive proteases. The purpose of this study was not to determine the bioactivity of gastric milk peptides, but it does serve as a launch pad for future studies of bioactive peptides. Homology search of total identified peptides revealed 92

unique peptides identical to a known bioactive peptide, and 678 unique peptides with 80% sequence homology (770 total) (Table S2). Of the 770 matching peptides, 127 were human milk protein-derived, 631 were bovine milk protein-derived, and 12 could be sourced to either species. Most of the potential bioactive peptides identified were bovine milk peptides, and thus only present in fortified infants. This finding is despite the fact that there were fewer total bovine peptides than human peptides, and is most likely a result of our use of a homology search for identification of potential bioactivity. As bovine milk peptides have been studied far more than human milk peptides, a larger pool of bioactive bovine milk peptides is available for comparison. Continued progress in human milk peptide discovery will increase the number of homologous matches that can be identified in milk digestive samples.

Table 3 lists the breakdown of proteins from which the peptides were found and the types of bioactive function they were identified with. One hundred forty-nine peptides were matched to more than 1 function: 32 had 4 different functions, 32 had 3 functions, and 85 had 2 functions. A range of potential bioactivities was determined in peptides across all sample types. However, it is likely that only a few of the peptides may be functional in the stomach. Opioid-receptors in the stomach can bind milk-derived opioid peptides and delay gastric emptying time,³⁴ and increase mucin production.³⁵ Some bacteria susceptible to antimicrobial peptides have been found in the stomach, although whether these bacteria inhabit the stomach or are merely passing through remains unknown.³⁶

Peptides homologous to bioactive peptides in the MBPDB increased over time of gastric digestion. The average abundance of all 80% homologous peptides significantly increased from milk to 1 h of gastric digestion (Figure 5A). Homologous peptide abundance tended to increase after two and 3 h of gastric digestion, but changes were not significant.

Homologous peptides in the gastric samples represented 18.9%, 18.1%, and 19.9% of the total abundance of 1, 2, and 3 h gastric samples, respectively. Homologous peptides in milk only represented 3.4% of total milk peptide abundance. Peptide count followed a similar pattern of increase as abundance (Figure 5B). Milk contained an average of 52 ± 9.2 homologous peptides, which significantly increased to 129 ± 14.5 in 1 h gastric samples, 147.9 ± 16.7 in 2 h samples, and 158.2 ± 18.3 in 3 h samples. Unlike for total peptide abundance, homologous peptides did not continue to increase across gastric digestion. The only significant increase occurred from milk to 1 h gastric digestion. It seems that the initial introduction of milk proteins to gastric conditions catalyzed the majority of potentially bioactive peptide release, with only small increases thereafter.

3.4. Structural Characteristics of Peptide Survivability

Though the relative abundance and count of bioactive peptides increased over digestion, a remaining question was whether bioactive milk peptides were more likely to survive gastric proteolysis than nonbioactive peptides. Consideration of these factors is important, as many identified bioactive peptides are functional for enterocytes specifically,^{37,38} or against bacteria that primarily inhabit the gut,³¹ whereas others are functional only when they are absorbed across the intestinal membrane.³⁹ Peptides that survive longer in gastric proteolytic conditions are more likely to reach the intestine and be biologically relevant. We therefore

identified factors that may contribute to a peptide's survival in the stomach by comparing peptides that survived further digestion after their appearance with peptides that were absent from one or more time point after their appearance. However, as gastric emptying is continuous during digestion,²⁴ it is possible that some of the differences were due to peptides progressing into the intestine early rather than being further cleaved.

With milk considered to be digestion time = 0 h, 153 of the 770 homologous peptides were present in at least 1 infant at all time points, 242 were present after 1 h of gastric digestion onward, 80 from 2 h onward, and 46 only appeared after 3 h of digestion. The remaining 244 peptides were not identified in any infant for at least 1 of the time points after their initial appearance, thus likely being further digested after release. In the total peptidome, 7210 peptides survived digestion after their appearance time and 4402 did not. A slightly larger percentage of homologous peptides survived compared with total peptides (67.9% and 62.1%, respectively).

Homologous peptide amino acid composition had more similarities with surviving peptides than nonsurviving peptides (Figure 6A). Surviving peptides had significantly higher percentages of F, L, M, P, Q, V, W, and Y residues, and lower D, E, R, and S residues than nonsurviving peptides. Surviving peptides were thus more likely to be hydrophobic and contain more proline residues. Though pepsin has low amino acid specificity with slight preference for bulky hydrophobic side-chains,⁴⁰ these bulky side-chains made up a larger percentage of surviving peptides than nonsurviving peptides, indicating an alternate determining factor for survivability. Proline, due to its nitrogen being covalently bound into a ring structure, forms bends in a protein's secondary structure.⁴¹ These bends might be enough to prevent efficient interaction of the active site of pepsin and other proteases with substrate peptide bonds. Proline has been known to reduce protease efficiency when substituted for other amino acids;^{42,43} thus, the relatively higher percentage of proline in surviving peptides could be a contributing factor to their survival. The high ratio of proline in homologous peptides could indicate their tendency to survive further gastric digestion.

As with amino acid composition, bioactive peptide post-translational modification (PTM) rates more closely resembled surviving peptide modification rates than those of nonsurviving peptides (Figure 6B). Surviving peptides had fewer oxidized Met and phosphorylated Ser and Thr residues than nonsurviving peptides. There are examples of substrate phosphorylation enhancing proteolytic activity of proteases or marking substrates for digestion,^{44,45} but phosphorylation can also impart proteolytic resistance to the substrate.⁴⁶ The present study only examined oxidation and phosphorylation as potential PTMs. Future identification of other types of PTMs in peptidomic data can allow further determination of the effect of specific PTMs on peptide resistance or susceptibility to proteolysis. As homologous peptide PTMs more closely resembled surviving peptide PTMs, it is possible that bioactive peptides are more likely to survive gastric digestion.

In addition to analyzing factors that contributed to overall peptide survivability, we also examined rates of peptide release over time across the sequence of three human milk proteins. β -Casein (Figure 7A), α_{s1} -casein (Figure 7B), and lactoferrin (Figure 7C) released the most abundant human milk peptides. Regions of protein digestion appeared to be

consistent over time within the protein sequences. For β -casein and α_{s1} -casein, the N-terminal regions (β -casein f(1–42) and α_{s1} -casein f(1–36)) were most highly abundant in milk, then decreased during gastric digestion, whereas the peaks in regions β -casein f(52–146), β -casein f(181–211), α_{s1} -casein f(97–126), and α_{s1} -casein f(150–167) consistently increased in abundance as digestion progressed. Though lactoferrin had major abundance peaks at f(65–93) and f(271–299), abundance was only distinctly different between milk and gastric samples, and not over time.

We identified seven regions in these three proteins that released multiple peptides with similar bioactivity based on homology. The regions β -casein f(35–52), β -casein f(90–103), and β -casein f(107–133) were antihypertensive, β -casein f(179–211) and lactoferrin f(269–288) were antimicrobial, β -casein f(102–120) was cell-proliferative, and lactoferrin f(660–667) was mucin-stimulatory peptides. Four of these regions corresponded to peaks of peptide release that increased during digestion, suggesting their release may be controlled inside the stomach.

3.5. Conclusions

Several limitations of this study include the use of mass spectrometry to measure peptide release and protein digestion over time. Peptide abundance is an incomplete estimation of protein digestion as the ionization efficiency of peptides differs based on amino acid side chains and PTMs.^{47,48} Furthermore, the parameters of this study's mass spectrometry analysis did not allow for identification of low m/z peptides, which may significantly contribute to the total peptide content. The identification of these peptides will be necessary in future studies to complete the peptide profile of milk protein digestion. This study also lacked information on the protein composition of the milk and fortifier, which would improve the analysis of relative peptide release from individual milk proteins. Human milk proteins can vary by the individual, so it will be important to understand how initial milk protein composition affects peptide release.⁴⁹

The results from the present study contribute to a comprehensive view of milk protein digestion and bioactive peptide release from milk in the stomach of infants. Some remaining questions that future studies will need to address include how protein digestion and peptide release differ between term and preterm infants, the degree of peptide release in the infant intestinal tract, and what bioactive peptides remain undiscovered from the less well-studied regions of the milk proteome, particularly those known to release a large amount of milk peptides. In addition, questions related to clinical practice that deserve study include the impact of released peptide profiles on measures of intestinal health, including inflammation, permeability, motility, the composition and functional capacity of the microbiota, and on gut-related health outcomes such as sepsis and necrotizing enterocolitis. It will also be valuable to determine the impact of continuous vs bolus feeding on milk peptide release and to determine whether some infants would benefit from exogenous proteases (e.g., infants with short gut syndrome or other forms of protein malabsorption).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACN	acetonitrile
TFA	trifluoroacetic acid
FA	formic acid
GA	gestational age
PTM	post-translational modification

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Figure 1.
Venn diagram of the unique peptides identified in each sample and combination of samples.

Figure 2.

Boxplots of the abundance (A) and count (B) of total peptides from all infants by sample type. Boxes are bordered by the first and third quartiles. The center bar represents the median, $n = 14$. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. Volcano plot (C) depicting the fold change in peptide intensity (3 h gastric divided by milk) vs the FDR-corrected p -value between peptides found in both milk and 3 h gastric digests. Filled circles indicate significant ($q < 0.05$) peptides >10-fold higher or lower in gastric samples.

Figure 3.

Comparison of average peptide abundance (A) and count (B) between fortified and nonfortified infants, grouped by milk protein source species. Data are shown as means $\pm$ SE, fortified $n = 6$ infants, nonfortified $n = 5$ infants. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. M, milk samples; G1, G2, and G3 are gastric samples after 1, 2, and 3 h of digestion, respectively.

Figure 4.

Comparison of average peptide abundance (A) and count (B) between fortified and nonfortified infants, grouped by human milk proteins. Data are shown as means $\pm$ SE, fortified $n = 6$ infants, nonfortified $n = 5$ infants. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. M, milk samples; G1, G2, and G3 are gastric samples after 1, 2, and 3 h of digestion, respectively; CASB, human β -casein; CASA1, human α_{s1} -casein; TRFL, human lactoferrin; LALBA, human α -lactalbumin; OSTP, human osteopontin; PIGR, human polymeric immunoglobulin receptor.

Figure 5. Boxplots of the abundance (A) and count (B) of peptides homologous to bioactive peptides by sample type. Boxes are bordered by the first and third quartiles. The center bar represents the median, $n = 14$. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$.

Figure 6. Comparison of peptide characteristics. (A) Average percentage of amino acids in surviving, non-surviving, and bioactive peptides. Data are means $\pm 95\%$ CI, surviving $n = 7210$ peptides, non-surviving $n = 4402$, bioactive $n = 770$. Asterisks (*) indicate significant differences. (B) Percentage of total peptides with modifications for surviving, non-surviving, and bioactive peptides; surviving $n = 7210$ peptides, non-surviving $n = 4402$, bioactive $n = 770$. Asterisks (*) indicate significant differences. O, oxidation; P, phosphorylation.

Figure 7.

Abundance of peptides released from human milk β -casein (A), α_{s1} -casein (B), and lactoferrin (C) mapped to the protein sequence by amino acid. Results are shown as means, $n = 14$. Bracketed lines indicate regions of homologous bioactive peptides. i, antihypertensive (20 peptides); ii, antihypertensive (8); iii, cell-proliferative (20); iv, antihypertensive (31); v, antimicrobial (46); vi, antimicrobial (4); vii, mucinstimulatory (2).

Table 1.

Infant Characteristics^a

infant	sex	GA at birth (week)	postconceptual age (week)	weight (kg)	length (cm)	fortification	twins with
1 ^b	F	23	28	0.95	30.5	None	
1 ^b	F	23	30	1.2	32	Gastric Only	
1 ^b	F	23	32	1.67	39	Gastric Only	
1 ^b	F	23	35	1.8	41.5	All	
1 ^b	F	23	37	2.3	42	All	
2	F	29	30	1.1	38.5	None	3
3	F	29	30	1.1	39.4 ^c	None	2
4	F	26	32	1.4	37.5	Gastric Only	
5	F	32	32	1.6	42.5	None	6
6	F	32	32	1.5	40	None	5
7	F	29	32	1.5	39	All	8
8	F	29	32	1.4	39	All	7
9	F	26	34	2	40.5	All	
10	M	26	34	2.3	43.8	All	

^aPostconceptual age is the time at which samples were collected, and includes infant GA plus infant lifespan. Weight and Length were measured at the postconceptual age.

^bSamples were collected from infant 1 at five different postconceptual ages.

^cLength value represents birth length rather than postconceptual age length.

Table 2.Proteins with the Highest Abundance and Count Averaged Across Relevant Samples^a

protein	average abundance $\pm$ SD	protein	average count $\pm$ SD
Human β -casein	$8.85 \times 10^{10} \pm 4.49 \times 10^{10}$	Human β -casein	285.4 ± 86.8
Bovine β -casein	$4.18 \times 10^{10} \pm 1.60 \times 10^{10}$	Bovine β -casein	185.2 ± 27.4
Human α_{s1} -casein	$2.34 \times 10^{10} \pm 1.63 \times 10^{10}$	Bovine α_{s1} -casein	138.9 ± 18.6
Bovine κ -casein	$2.30 \times 10^{10} \pm 1.50 \times 10^{10}$	Bovine κ -casein	90.2 ± 18.6
Bovine α_{s1} -casein	$2.17 \times 10^{10} \pm 1.28 \times 10^{10}$	Human α_{s1} -casein	88.8 ± 39.3
Human lactoferrin	$2.12 \times 10^{10} \pm 1.67 \times 10^{10}$	Human osteopontin	64.7 ± 35.5
Human α -lactalbumin	$2.06 \times 10^{10} \pm 1.65 \times 10^{10}$	Human PIgR	63.7 ± 19.5
Bovine GLYCAM1	$1.58 \times 10^{10} \pm 8.65 \times 10^9$	Human lactoferrin	61.2 ± 37.8
Bovine β -lactoglobulin	$1.54 \times 10^{10} \pm 1.47 \times 10^{10}$	Bovine β -lactoglobulin	57.2 ± 13.7
Human osteopontin	$1.33 \times 10^{10} \pm 1.25 \times 10^{10}$	Bovine α_{s2} -casein	54.3 ± 9.6
Human PIgR	$7.30 \times 10^9 \pm 3.77 \times 10^9$	Bovine GLYCAM1	43.8 ± 6.4
Bovine α_{s2} -casein	$5.94 \times 10^9 \pm 2.91 \times 10^9$	Bovine PIgR	32.4 ± 5.1
Human serum albumin	$5.19 \times 10^9 \pm 4.79 \times 10^9$	Human bile salt-activated lipase	31.8 ± 14.2
Human bile salt-activated lipase	$5.06 \times 10^9 \pm 4.56 \times 10^9$	Human tenascin	21.7 ± 21.7
Human κ -casein	$2.86 \times 10^9 \pm 2.13 \times 10^9$	Human κ -casein	20.4 ± 7.1

^aHuman protein $n = 14$; bovine protein $n = 6$.

Table 3.

Milk Proteins from Which Homologous Bioactive Peptides Were Identified

protein	homologous peptides	antihypertensive	antimicrobial	antioxidant	cell proliferative	immunomodulatory	mucin stimulatory	other
Bovine β -casein	227	150	73	21	21	23	22	84
Bovine α_{s1} -casein	146	71	64	5	–	6	5	15
Bovine β -lactoglobulin	118	29	75	5	11	–	–	41
Human β -casein	118	51	46	1	20	–	1	1
Bovine α_{s2} -casein	66	9	59	6	–	–	–	1
Bovine κ -casein	56	5	41	–	–	–	2	11
Both β -casein	12	12	–	–	–	–	–	1
Bovine α -lactalbumin	11	3	–	–	–	–	–	8
Bovine lactoferrin	7	–	2	–	5	–	–	–
Human lactoferrin	6	–	4	–	–	–	2	–
Human κ -casein	2	1	–	1	–	–	–	–
Human α -lactalbumin	1	–	–	–	–	–	–	1