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Peptidomic profiling of human milk with LC-MS/MS reveals pH-specific proteolysis of milk proteins

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Abstract

Human milk is a dynamic protein-protease system that delivers bioactive peptides to infants. The pH of milk changes from the mother's mammary gland to the infant's digestive tract. Although the release of human milk peptides has been studied during *in vivo* or *in vitro* digestion, these models did not explicitly vary nor observe the effect of pH. The objective of this research was to determine the effect of pH on the proteolysis of human milk. Using high-resolution accurate-mass Orbitrap mass spectrometry, profiles of endogenous human milk peptides before and after incubation at various pH levels have been mapped. Over 5000 peptides were identified. Comparative analyses classified 74 peptides that were consistently found independent of pH alterations, and 8 peptides that were released only at pH 4 or 5 (typical infant gastric pH). Results documented that the proteolysis of milk proteins, particularly β -casein, polymeric immunoglobulin receptor, and α -lactalbumin, is pH-dependent.

Keywords

Peptidomics; Milk peptides; Bioactive peptides; Human milk; Protein digestion; Proteolysis; Mass spectrometry

1. Introduction

Under selective pressure throughout mammalian evolution, milk has been instrumental as a nourishing diet in the survival of offspring. Milk proteins have diverse biological functions beyond serving as nutritional sources of amino acids. A subset of the milk proteins consists of substrates, proteases, protease activators, and protease inhibitors, forming a dynamic

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Conflict of interest

None.

Appendix. Supplementary material

proteolytic system (Dallas, Murray, & Gan, 2015). With the application of modern analytical tools to biological research, the release of human milk peptides has been followed from the mother's milk to the infant's digestive tract *in vivo* (Dallas, Guerrero, Khaldi, Borghese, Bhandari, Underwood, et al., 2014). Bioinformatic analyses have shown that milk-derived peptides generated in the infant stomach correspond with the cleavage patterns of proteases in human milk rather than pepsin in the infant's stomach (Holton, Vijayakumar, Dallas, Guerrero, Borghese, Lebrilla, et al., 2014). Scientific discoveries on the dynamic proteolytic system delivered to infants through mothers' milk are providing new insights into the biological process of selective proteolysis.

Peptides are important in almost all biological systems (Boonen, Creemers, & Schoofs, 2009; Petsalaki & Russell, 2008; Zasloff, 2002). Antimicrobial peptides are evolutionarily ancient weapons for both plants and animals in their defense against a broad range of microbes (Zasloff, 2002). Signaling peptides bind to protein domains and mediate cellular processes, particularly biochemical pathways (Petsalaki & Russell, 2008). Furthermore, bioactive peptides in signaling systems often coordinately function as a network, and cells respond to the integrated peptidome instead of a single peptide (Boonen, Creemers, & Schoofs, 2009). Therefore, the consortium of peptides delivered through milk may function in unison to regulate processes such as the metabolism of different cells and tissues in infants, and to mediate the development of the entire body at multiple sites within the regulatory hierarchy.

The diverse biogeography of the digestive tract affects the landscape of proteins and peptides in each location. The cellular and tissue environment changes dramatically from oral cavity to stomach and intestine, and pH varies significantly along the digestive tract. During ingestion, the environment of milk changes from neutral to acidic when entering the infant's stomach, and then the pH increases in the intestine (Bourlieu, Menard, Bouzerzour, Mandalari, Macierzanka, Mackie, et al., 2014; Gan, Bornhorst, Henrick, & German, 2018). The typical gastric pH in a newborn infant's stomach is around 4, but the pH varies from 2 to 6 depending on individual conditions, such as term or preterm birth, developmental stage, and health status (Agunod, Yamaguch, Lopez, Luhby, & Glass, 1969; He, Chen, Li, & Deng, 2017; Maffei & Nobrega, 1975; Mason, 1962; Sondheimer, Clark, & Gervaise, 1985). Fluctuation in pH may affect the ionization state of charged molecules, the structural stability of proteins, the activity of milk enzymes, the microstructure of milk compartments, and the substrate-enzyme molecular interactions within human milk. Therefore, we hypothesized that pH changes the dynamics of the proteolytic system of human milk and mediates the selective proteolysis of milk proteins to release specific peptides. Although recent studies characterized human milk peptides during infant digestion *in vivo* or *in vitro* (Dallas, et al., 2014; Su, Broadhurst, Liu, Gathercole, Cheng, Qi, et al., 2017; Wada & Lonnerdal, 2015), these models involved many variables and did not explicitly vary nor observe the effects of pH on human milk proteolysis.

The objective of this study was to determine how pH affects the proteolysis of human milk. Fresh human milk samples were incubated at various levels of physiologically relevant pH, and peptides were mapped using high-resolution liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Identifying how human milk proteins are changed

and what peptides are released upon pH alteration is important for understanding the complex biological functions of human milk and guiding innovations to improve infant health.

2. Materials and methods

2.1. Human milk collection

This study was approved by the University of California Davis Institutional Review Board. Human milk samples were provided by four healthy mothers (at 5 days, 2 months, 10 months, and 12 months of lactation, respectively) after informed consent. Each sample was expressed by an electric breast pump, collected into a sterile container, and transported to the laboratory on ice. A fraction of human milk was used immediately for pH adjustment, incubation, and proteolytic bacteria assay without freeze-thaw cycles. The remainder of milk was stored at -80°C for compositional analysis.

2.2. Compositional analysis of human milk

Composition of the four human milk samples was analyzed by the Fourier transform mid-infrared spectroscopy (Delta Instruments LactoScope FTIR Advanced, Advanced Instruments, Norwood, MA, USA). Prior to sample analysis, the instrument was calibrated using previously validated methods (Smilowitz, Gho, Mirmiran, German, & Underwood, 2014). Human milk samples were thawed at 4°C overnight, warmed to 38°C in a water bath and vortexed for 20 seconds to ensure homogenization for the analysis. Three measurements were taken for each sample.

2.3. Proteolytic bacteria assay

To evaluate and control for the impact of proteolytic bacteria in the experiments described below, the presence of proteolytic bacteria in the milk was tested using skim milk agar (Criterion, Hardy Diagnostics, Santa Maria, CA, USA). 100 μL of human milk was spread over the surface of skim milk agar and incubated at 37°C for 24 to 48 hours. Proteolytic bacteria were indicated by clean zones around the colonies (Storrs & Hull, 1956).

2.4. pH adjustment and incubation

Each human milk sample was divided into five aliquots of 500 μL . The first aliquot was kept on ice to serve as a control without pH adjustment or 37°C incubation; 500 μL of 2X protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added to prevent proteolysis in the control group. The four treatment groups were incubated for 2 hours at 37°C under different pH conditions. One treatment group remained at its natural pH of approximately 7. For the other three treatment groups, 1M HCl was used to adjust the pH to 5 (5.0 ± 0.1), 4 (4.0 ± 0.1) and 2 (2.0 ± 0.1), respectively. After incubation, 500 μL of 2X protease inhibitor cocktail was added to stop proteolysis. Mixtures of 1M NaOH and 1M HCl were then added to neutralize the samples and adjust for salt variations. The final volume and amount of materials in each tube were the same after neutralization (500 μL milk, 500 μL protease inhibitor cocktail, 20 μL of 1M HCl, and 20 μL of 1M NaOH) in order to minimize the effect of pH or ion strength on the subsequent peptide extraction and analysis. The experimental procedure is depicted in Fig. 1.

2.5. Peptide extraction

Samples were centrifuged at 15000 x g for 15 min at 4°C to separate the fat and precipitate the cells in human milk. The aqueous layer was transferred to a new tube without retrieving the cream layer or disturbing the cell pellet. An equal volume of 20% (w/v) trichloroacetic acid (TCA) was added to each sample, and the mixture was kept at 4°C for 10 min to precipitate proteins. After centrifugation at 15000 x g for 10 min at 4°C, the supernatant was purified by C18 solid-phase extraction. The C18 96-well plate (Glygen Corp, Columbia, MD, USA) was activated with 60% acetonitrile in 0.1% formic acid and equilibrated with 0.1% formic acid. Samples were loaded on the plate, and then washed with 0.1% formic acid to remove salts and carbohydrates. Peptides were eluted with 60% acetonitrile in 0.1% formic acid. Eluted peptides were dried in a centrifugal vacuum concentrator (Genevac MiVac Quattro concentrator, Genevac Ltd., Ipswich, UK) for Orbitrap analysis.

2.6. Peptide quantification

Following extraction, peptide concentration of each sample was measured in duplicate with the Pierce Quantitative Fluorescent Peptide Assay (Thermo Scientific, Rockford, IL, USA). Peptides were labeled by an amine-reactive fluorescent detection reagent, and the fluorescence intensity was detected at Ex390nm/Em475nm with a microplate reader (SpectraMax M5, Molecular Devices, San Jose, CA, USA).

2.7. Peptidomic profiling by LC-MS/MS

LC-MS/MS analysis was performed based on previously published methods for milk samples with slight modifications (Beck, Weber, Phinney, Smilowitz, Hinde, Lonnerdal, et al., 2015). Specifically, samples were resuspended in nanopure water containing 2% acetonitrile and 0.1% formic acid. One microgram of peptides was loaded onto a 100 μm \times 25 mm Magic C18 100Å 5U trap column before being separated on a 75 μm \times 150 mm Magic C18 200Å 3U reverse phase column. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in 80% acetonitrile. Peptides were separated over 120 min with a gradient from 0 to 100% solvent B at a flow rate of 3 $\mu\text{L}/\text{min}$. Eluted peptides were analyzed with an ESI source and a Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific). Mass spectra were collected in data-dependent mode with one precursor scan followed by 15 MS/MS scans. Full-scan MS spectra were acquired with a scan range of 350 to 1600 m/z, resolution of 70000, and target of 1×10^6 ions or maximum injection time of 30ms. MS/MS spectra were acquired with a scan range of 200 to 2000 m/z, resolution of 17500, and target of 5×10^4 ions or maximum injection time of 50ms. MS/MS fragmentation was performed using higher-energy collision-induced dissociation with normalized collision energy of 27. Precursors with unassigned, 1 or >4 charge states were excluded from fragmentation.

2.8. Peptide identification

The sequences of peptides were identified from MS/MS spectra using X!Tandem (Craig & Beavis, 2004) online advanced search against the SwissProt *Homo sapiens* (Human) proteome (May 2017, 20201 entries). A non-specific enzyme cleavage pattern was defined, and 50 missed cleavage sites were allowed. No complete modifications were set. Oxidation

of methionine, deamidation of asparagine and glutamine, as well as phosphorylation of serine and threonine were selected as potential modifications. Mass error tolerance was 40 ppm for precursor ions and 20 ppm for fragment ions. For individual spectra, a peptide match was accepted if the e-value was below 0.01.

2.9. Data analysis

Outputs from X!Tandem were processed computationally using a custom Python script. The program extracts data from multiple X!Tandem xml files and exports as a csv file. Comparison of peptides in different experimental groups and statistical summarization of results were performed in R (version 3.3.3). Peptide concentration of 2-hour incubation samples was normalized by that of the corresponding 0-hour control sample; the normalized results were analyzed by ANOVA for Randomized Block Design, with pH as the treatment factor and milk as the blocking factor. Monoisotopic molecular weight of proteins was computed by ExPASy Compute pI/Mw tool (https://web.expasy.org/compute_pi/); the information was used in a bubble chart to visualize the pattern of proteolysis. Functional classification of identified proteins was achieved with the PANTHER (protein annotation through evolutionary relationship, version 12.0) database (Mi, Huang, Muruganujan, Tang, Mills, Kang, et al., 2017). Sequences of peptides were searched on bioactive peptide databases, including EROP-Moscow (Endogenous Regulatory OligoPeptide knowledgebase, release of Nov 2016, 14511 entries) (Zamyatnin, Borchikov, Vladimirov, & Voronina, 2006), BIOPEP (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008), and MBPDB (Milk Bioactive Peptide Database) (Nielsen, Beverly, Qu, & Dallas, 2017).

3. Results and discussion

3.1. Compositional analysis and proteolytic bacteria assay of the human milk samples

The composition of human milk is well studied, and typical macronutrient concentrations are documented; thus, analyses were conducted to verify that the samples used in these experiments were within normal ranges. Results for the four human milk samples were $0.983 \pm 0.426\%$ (Mean \pm SD) protein, $5.659 \pm 5.118\%$ fat, and $6.941 \pm 0.674\%$ lactose, which are similar to data from previous studies (Ballard & Morrow, 2013).

To demonstrate the effects of endogenous human milk proteolytic enzymes, contamination by exogenous bacteria that produce proteolytic enzymes needs to be controlled. In this study, milk samples were examined for proteolytic bacteria with skim milk agar. In contrast to raw cow's milk with high bacterial counts (Storrs & Hull, 1956), freshly expressed human milk from healthy mothers has low viable bacterial counts on agar as detected on non-selective agar media (Jost, Lacroix, Braegger, & Chassard, 2013). No clear zones were observed surrounding the bacterial colonies on skim milk agar in this study, so the milk samples were suitable for addressing the research question.

Milk samples used for compositional analysis were stored at -80°C prior to analysis (Smilowitz, Gho, Mirmiran, German, & Underwood, 2014). However, freeze-thaw cycles may affect milk structure, bacterial survival, and enzyme activity; in addition, proteolysis

may occur during thawing. Therefore, fresh milk samples without freeze-thaw cycles were used for the proteolytic bacteria assay and peptide analysis.

3.2. Effects of pH on the amount and profile of human milk peptides

To investigate the effects of pH on the human milk peptides, we measured the concentration of total peptides and mapped the profile of these peptides after incubation at various pH levels. Peptide concentrations in samples after 2-hour incubation were normalized by the corresponding 0-hour control before comparison (Fig. 2A). ANOVA results indicate that the total amounts of peptides between pH treatment groups were not significantly different ($p>0.05$).

Peptidomic profiling was employed with the goal of comprehensive identification of endogenous peptides in milk samples. Using high-resolution accurate-mass Orbitrap detection, we have identified a total of 5847 peptides among all 19 samples. There were 2757 peptides identified in the samples without incubation, and 5496 peptides in the samples after 2 hours of incubation. To reduce the influence of individual variation and focus on the effect of pH, peptides found from all biological replicates in each treatment group were analyzed (Table S1). The number of these common peptides ranged from 158 to 230, which was about 5 – 11% of the total peptides identified in the respective groups (Fig. 2B). One sample of the pH 2 group (collected at 5 days postpartum) had a pH higher than 2.1 after pH adjustment; thus, this sample was excluded from data analysis. Due to this missing data point, the number of peptides found at least once in the pH 2 group was lower than that of other groups.

MS/MS spectra were analyzed by database searching against the human proteome, and all the individual spectrum-to-sequence assignments were validated with statistical confidence (expectation value <0.01). The length of peptides was limited within the range of 6 to 50 amino acid residues for reliable sequence assignments. The current experimental design and acquisition settings of mass spectrometry focused on qualitative characterization of peptides with high confidence and sensitivity, facilitating the discovery of targeted peptides for further quantitation.

3.3. Conserved peptides found in all samples

In the analyzed samples, 74 peptides were found consistently in samples before and after incubation and from all biological replicates (Table 1). These conserved peptides are likely produced in the mammary gland, delivered to infants through milk, and maintaining their structure despite the pH change. To determine whether this number of conserved peptides was substantially lowered by a single influential sample, analyses were performed on the dataset with one sample removed each time, and the number of conserved peptides was calculated for the remaining samples. The average value for conserved peptides with one sample removed was 76 ± 2.49 , indicating that the diversity in conserved peptides was similar across samples.

Among the 74 conserved peptides, sequences that matched entries in the bioactive peptide databases were mainly related to antimicrobial activities. The peptide YPERLQNPSESEPIP from α_{S1} -casein (residues 23–38) in this dataset is part of the

antimicrobial peptide RPKLPLRYPERLQNPSESSEPIP (residues 16–38), which was produced by chymosin treatment of casein and active against *Staphylococcus aureus* (Lahov & Regelson, 1996). Peptides at the C-terminal end of human β -casein LLNPTHQIYPVTQPLAPVHNPISV (residues 203–226), LNPTHQIYPVTQPLAPVHNPISV (residues 204–226), NPTHQIYPVTQPLAPVHNPISV (residues 205–226), and PTHQIYPVTQPLAPVHNPISV (residues 206–226) are part of QELLLNPTHQIYPVTQPLAPVHNPISV (residues 200–226). This peptide was produced by hydrolysis of human sodium caseinate with a partially purified protease of *Lactobacillus helveticus* PR4, and has shown a broad spectrum of inhibition against Gram-positive and Gram-negative bacteria (Minervini, Algaron, Rizzello, Fox, Monnet, & Gobetti, 2003). The unmatched peptides may exert biological functions that have not been explored at present.

3.4. Unique peptides released at infant gastric pH

Eight unique peptides with 12 to 29 amino acid residues were found in samples at pH 4 and 5 (typical infant gastric pH) that did not appear in the samples before incubation or after incubation at other pH levels (Table 2). The criteria to screen these unique peptides were strict in this study. First, identical modifications on the amino acid residues were required; second, the occurrence of peptides in samples at the corresponding pH condition was 100% across biological replicates; third, these peptides were not identified in any samples under other conditions. These conservative criteria were set to obtain a high certainty that the release of these peptides is related to pH, and to reduce the probability of false positives.

During ingestion, the environment of milk shifts from neutral to acidic. This alteration in pH may change the structure of milk and activate specific proteolytic enzymes, which could subsequently trigger proteolytic processing of other proteins, leading to changes in their structure and function. Development of gastric secretion takes years in early life; the pH in an infant's stomach is generally higher than that of adults (Agunod, Yamaguch, Lopez, Luhby, & Glass, 1969; Klumpp & Neale, 1930). The unique peptides that are released only at infant-relevant pH have potential health implications. For example, the sequence PVTQPLAPVHNPIS (residues 212–225) from β -casein had an approximate match in all three bioactive peptide databases with QELLLNPTHQIYPVTQPLAPVHNPISV (residues 200–226), which is an antimicrobial peptide discussed above. No exact matches were found for these peptides among current entries in the bioactive peptide databases; further analyses are needed to explore their functions.

3.5. Source proteins of the identified peptides

The 5847 peptides identified in this study were derived from 516 proteins. The molecular functions of these proteins and their coding genes based on the PANTHER classification system are shown in Fig. 3. Common peptides found in all biological replicates of each group (Table S1) were derived from 29 proteins. The PANTHER analysis results of these 29 proteins are organized in Table S2. Among the 29 proteins, binding (GO:0005488) was annotated for 9 proteins, and transporter activity (GO:0005215) was annotated for 3 proteins.

To elucidate the effect of pH on the proteolysis of individual human milk proteins, unique peptides from each protein were counted for every sample. The peptide counts from the 3 biological replicates of mature milk present in all treatment groups were averaged, and the top 10 proteins with highest averaged peptide count were plotted in a bubble chart (Fig. 4). Most of the identified peptides originated from β -casein, polymeric immunoglobulin receptor, and osteopontin. Our data show that the size of source proteins did not correlate with the occurrence of peptides. Instead, pH alteration changed the profile of peptides released from these proteins. Particularly, pH 4 incubation enhanced the diversity of peptides derived from β -casein and polymeric immunoglobulin receptor.

The dominance of β -casein derived peptides has been reported in undigested human milk as well as after *in vitro* and *in vivo* digestion (Dallas, et al., 2014; Su, et al., 2017; Wada & Lonnerdal, 2015). One might speculate a correlation between the number of peptide sequences and the abundance of their source protein. However, our data show that the abundance of a source protein was not a major determinant of the count of its peptides. The most abundant proteins in human milk are β -casein (0.04 to 4.42 g/L), α -lactalbumin (2.75 to 3.72 g/L), lactoferrin (0.97 to 2.91 g/L), secretory IgA (0.22 to 0.61 g/L), and lysozyme (0.03 to 0.35 g/L) (Ballard & Morrow, 2013; Huang, Kailemia, Goonatilleke, Parker, Hong, Sabia, et al., 2017; Ke, Chen, Pan, Zhang, Mo, & Ren, 2016; Liao, Weber, Xu, Durbin-Johnson, Phinney, & Lonnerdal, 2017). Interestingly, peptides from the second most abundant human milk protein α -lactalbumin were not significantly released except for the pH 4 treatment group in this study. These results indicate that pH is a factor that regulates the dynamics of the proteolytic system in human milk and controls the release of peptides from α -lactalbumin.

Polymeric immunoglobulin receptor was the second major source of the released peptides in human milk. Following proteolytic processing, this protein yields a secretory component that mediates the epithelial transport of secretory IgA and the immune responses at mucosal surfaces (Kaetzel, 2005). The conserved peptides AVADTRDQADGSRASVDSGSSEEQGGSSRA (residues 610–639) and AVADTRDQADGSRASVDSGSSEEQGGSSR (residues 610–638) identified in our study were also found in human reflex tear fluid (Hayakawa, Landuyt, Baggerman, Cuyvers, Lavigne, Luyten, et al., 2013). It is likely that these peptides are important in mucosal immunity, interacting with the mucosal immune system in the oral cavity and the gastrointestinal tract.

Osteopontin was the third major source for human milk peptides. Christensen *et al.* have shown the milk proteases plasmin and cathepsin D are able to cleave osteopontin near its integrin-binding motifs (Christensen, Schack, Klaning, & Sorensen, 2010). Those cleavage sites account for many of the osteopontin-derived sequences identified in our study.

3.6. Discussion on the study model

Lactation is essential for the success of mammalian evolution, and the role of milk proteins in the regulation of growth and development of the infant is beyond simply providing nutrients (Lefèvre, Sharp, & Nicholas, 2010). Peptides released from milk proteins have been proposed to be intermediate products during protein digestion that further break down

to serve as sources of amino acids; they can also be the products of proteolytic processing that are critical for physiological regulation (Neurath, 1989). In previous research studies, discoveries of bioactive peptides often involved the explicit hydrolysis of proteins using experimentally administered exogenous enzymes to yield peptides for subsequent screening. Here, this study provides a novel strategy to interrogate the release of peptides due to the protease-protein interactions within the milk system. Without the addition of exogenous enzymes, it becomes possible to discover the products of controlled proteolysis within milk and to reveal their natural functions in biology.

This study was designed to explicitly investigate the effect of pH variation on the proteolysis of the human milk system. A limitation of the study model is that the simplified incubation conditions could not represent all physiological conditions during *in vivo* digestion. The proteolysis of human milk during infant digestion is affected by many factors, such as dynamic changes of pH, amounts and activities of digestive enzymes, diverse biogeography along the digestive tract, as well as developmental stage and health status of the infant. Currently, this process is not well understood, and variables that are important for the regulation of proteolytic processing in human milk may have yet to be identified. Characterization of key controlling factors is needed to improve our understanding of the regulation process.

4. Conclusions

Peptides are revealing diverse functions in biology. Human milk is a dynamic system containing proteins and proteases, and is a vehicle that delivers numerous functional peptides to infants. During the ingestion of human milk, pH is a critical environmental change that affects the proteolysis of milk proteins. Our data showed that pH alteration did not significantly change the total concentration of peptides in milk, but did change the profiles of peptides released from specific proteins, such as β -casein, polymeric immunoglobulin receptor, osteopontin, and α -lactalbumin. Results illustrated that the effect of pH on the proteolytic system of human milk is related to selected proteolysis of targeted proteins to release specific peptides, rather than assisting protein breakdown in general for nutritional purposes. Comparative analyses of treatment groups classified unique collections of peptides with potential bioactivities. These findings have extended the scientific knowledge on the diversity of bioactive peptides and begun the difficult task of interrogating the regulation of proteolysis of human milk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Human milk contains a dynamic proteolytic system.
- Selective proteolysis of milk proteins into specific peptides was pH-dependent.
- Seventy-four human milk peptides were consistently found despite pH alterations.
- Eight peptides were released only at pH 4 or 5 (typical infant gastric pH).
- Total concentration of peptides was not significantly changed by pH alterations.

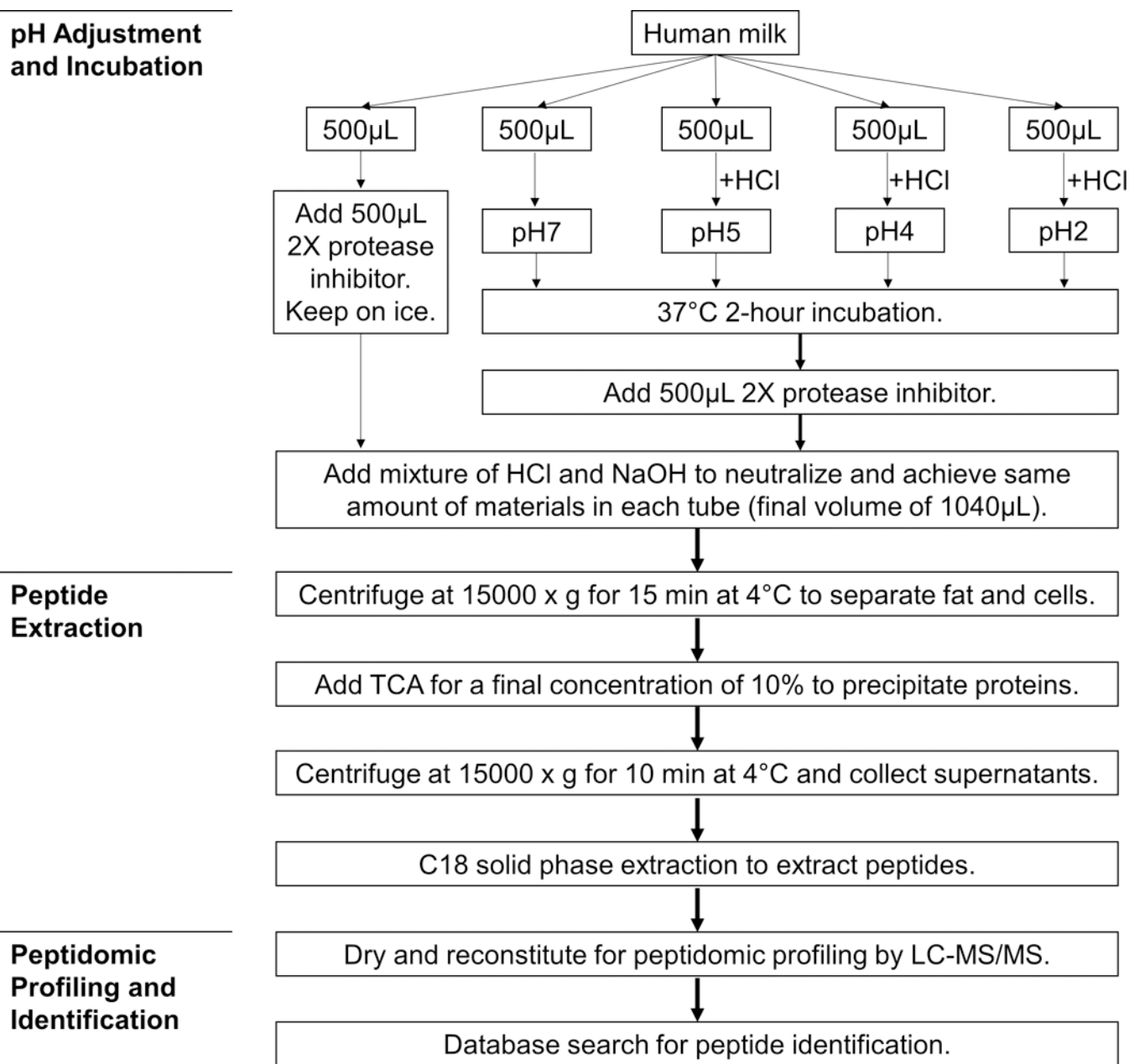


Fig. 1. Flowchart depicting the experimental procedure of pH adjustment of human milk and peptide analysis by mass spectrometry. Human milk sample collected from each subject served as a biological replicate. The experimental procedure was applied to each biological replicate.

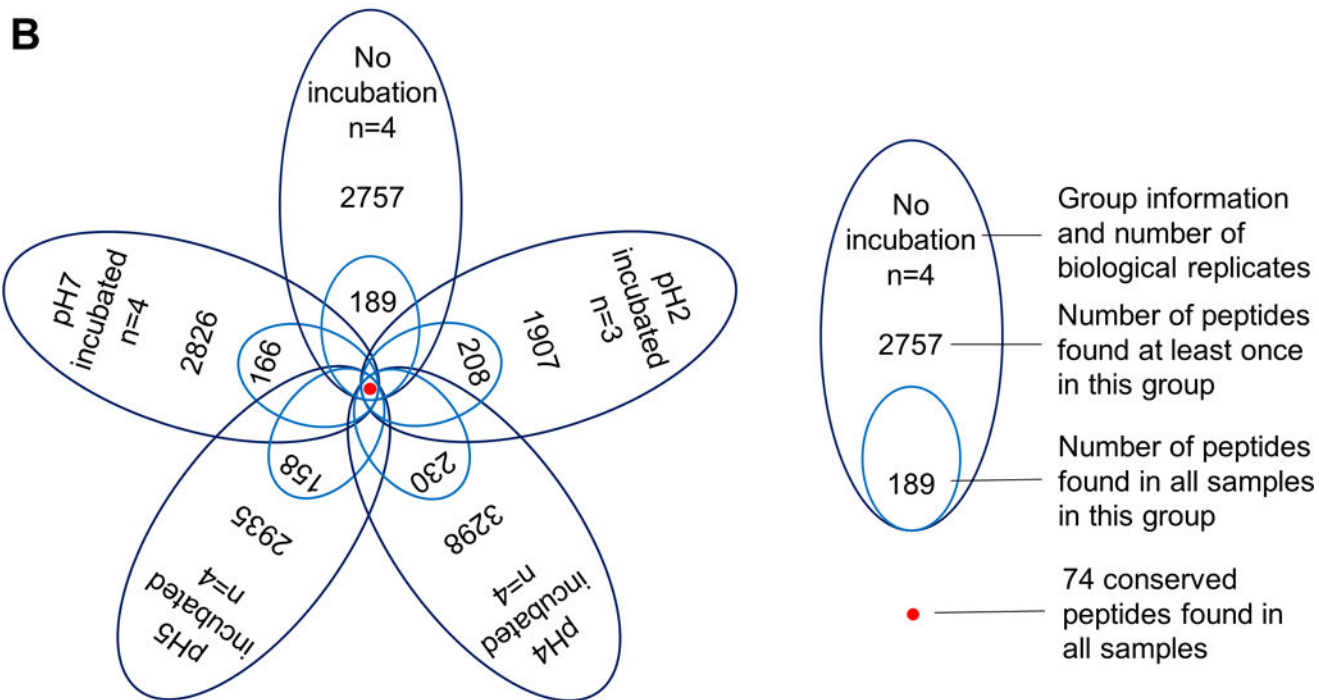
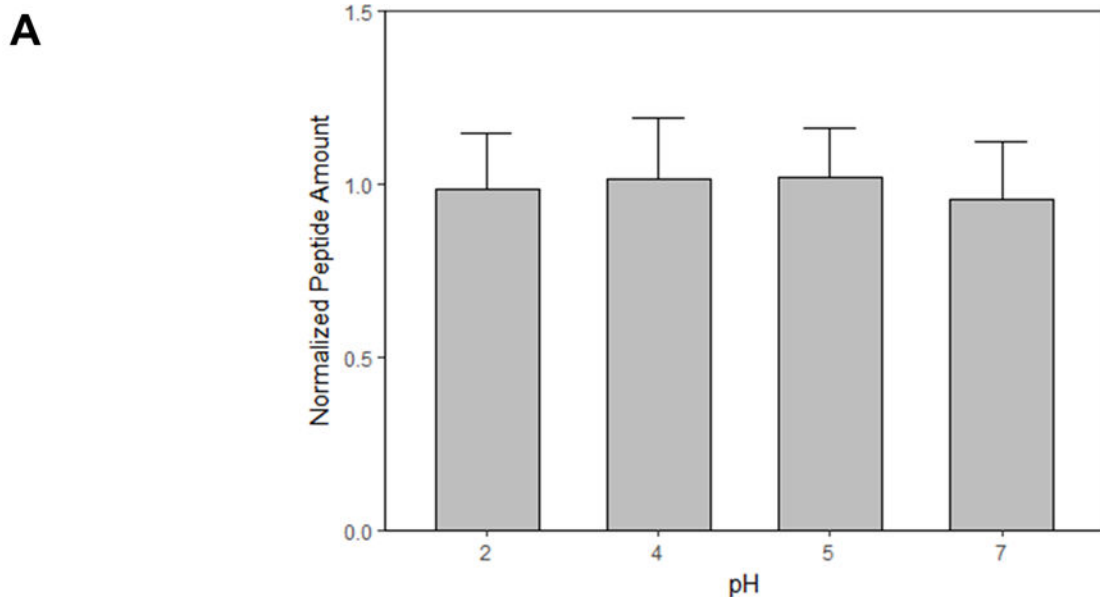


Fig. 2. Effects of pH on the total amount (A) and profile (B) of human milk peptides.

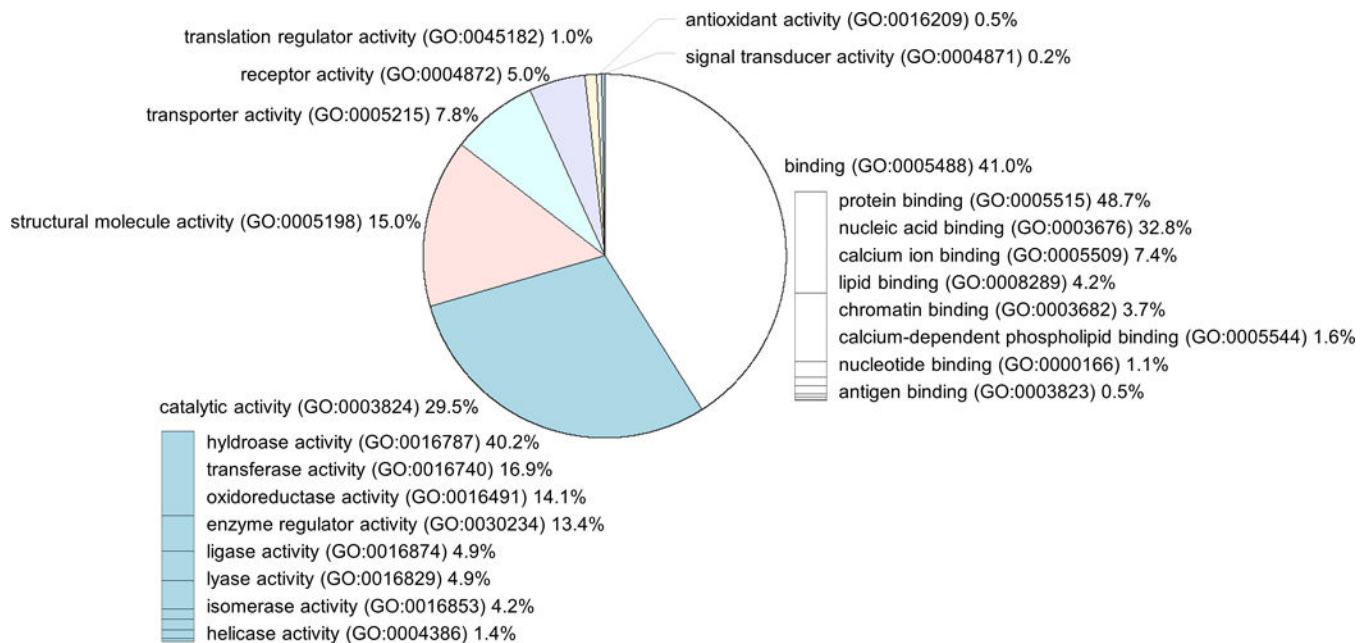


Fig. 3.
Molecular function of source proteins.

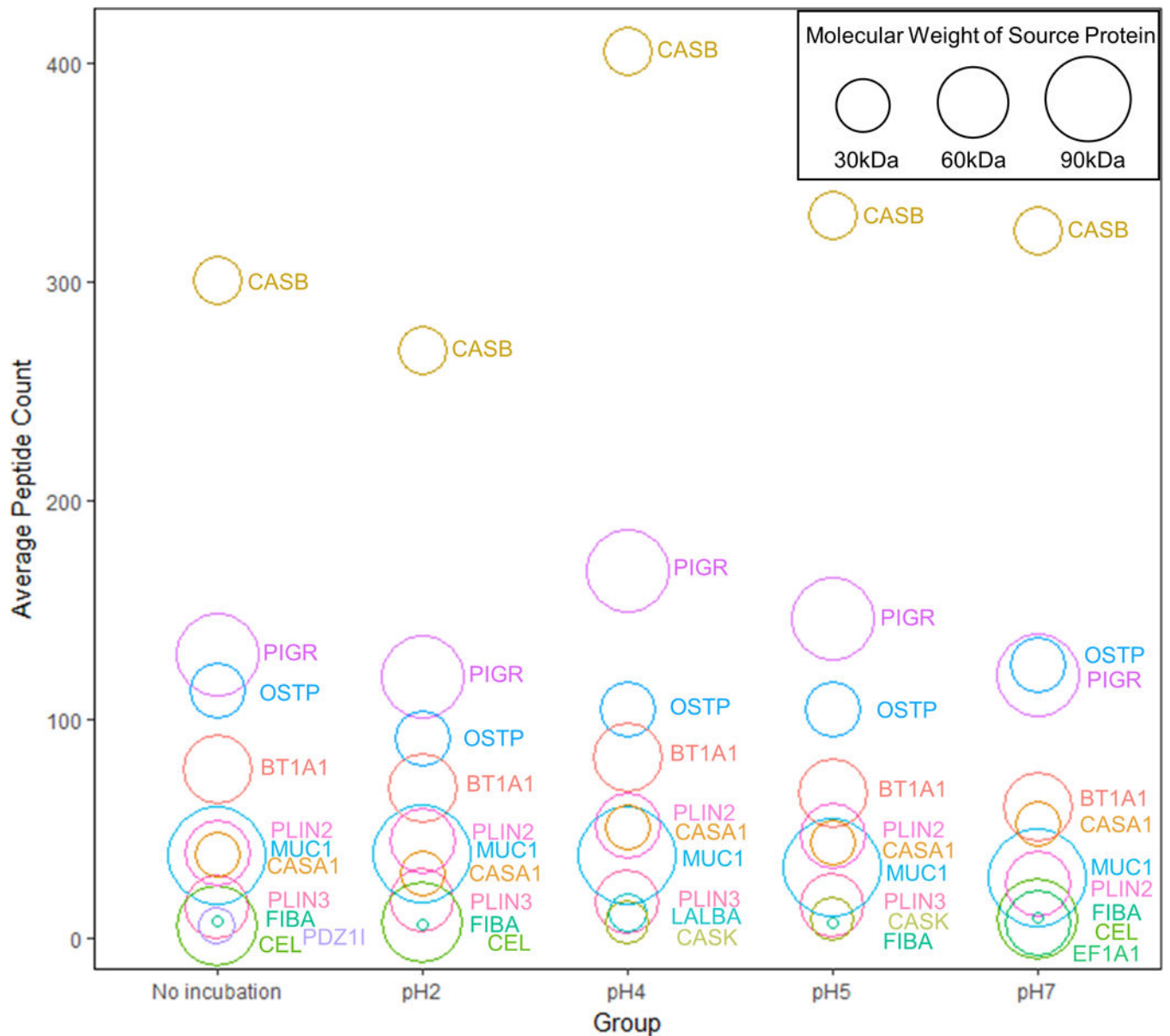


Fig. 4. Bubble chart of average peptide count for source proteins. Each circle represents a protein (BT1A1: Butyrophilin subfamily 1 member A1; CASA1: Alpha-S1-casein; CASB: Beta-casein; CASK: Kappa-casein; CEL: Bile salt-activated lipase; EF1A1: Elongation factor 1-alpha 1; FIBA: Fibrinogen alpha chain; LALBA: Alpha-lactalbumin; MUC1: Mucin-1; OSTP: Osteopontin; PDZ11: PDZK1-interacting protein 1; PIGR: Polymeric immunoglobulin receptor; PLIN2: Perilipin-2; PLIN3: Perilipin-3). The position of the circle indicates the average count of unique peptides released from this protein among biological replicates (n=3) in each group. The area of the circle is proportional to the size (molecular weight) of the protein.

Table 1.

Conserved peptides found in all samples.

Protein	UniProt AC	Peptide	Pre ^a	Start ^b	End ^c	Post ^d		
Polymeric immunoglobulin receptor	P01833	AEEKAVADTRDQADGSR	PRLF	606	622	ASVD		
		AVADTRDQADGSRASVDSG	AEEK	610	628	SSEE		
		AVADTRDQADGSRASVDSGSSEEQGGSS	AEEK	610	637	RALV		
		AVADTRDQADGSRASVDSGSSEEQGGSSR	AEEK	610	638	ALVS		
		AVADTRDQADGSRASVDSGSSEEQGGSSRA	AEEK	610	639	LVST		
		AVADTRDQADGSRASVDSGSSEEQGGSSRALVST	AEEK	610	643	LVPL		
		DGSRASVDSGSSEEQGGSSRA	RDQA	619	639	LVST		
		DQADGSRASVDSGSSEEQGGSSR	ADTR	616	638	ALVS		
		DQADGSRASVDSGSSEEQGGSSRA	ADTR	616	639	LVST		
		DQADGSRASVDSGSSEEQGGSSRA [Q+Deamidated;]	ADTR	616	639	LVST		
		DQADGSRASVDSGSSEEQGGSSRALVST	ADTR	616	643	LVPL		
		DSGSSEEQGGSSRALVST	RASV	626	643	LVPL		
		DTRDQADGSRASVDSGSSEEQGGSSRA	KAVA	613	639	LVST		
		SGSSEEQGGSSRALVST	ASVD	627	643	LVPL		
		SRASVDSGSSEEQGGSSRA	QADG	621	639	LVST		
		SVDSGSSEEQGGSSRA	GSRA	624	639	LVST		
		TRDQADGSRASVDSGSSEEQGGSSRA	AVAD	614	639	LVST		
		VADTRDQADGSRASVDSGSSEEQGGSSR	EEKA	611	638	ALVS		
		VADTRDQADGSRASVDSGSSEEQGGSSRA	EEKA	611	639	LVST		
		VADTRDQADGSRASVDSGSSEEQGGSSRA [Q+Deamidated;]	EEKA	611	639	LVST		
		Fibrinogen alpha chain	P02671	DSGEGDFLAEGGGVR	AWTA	21	35	GPRV
		Beta-casein	P05814	EKVKHEDQQQGEDEHQDK	KQKV	37	54	IYPS
ESLSSSEESITEYK	RETI			20	33	QKVE		
ETIESLSSSEESITEYK	ALAR			17	33	QKVE		
ETIESLSSSEESITEYK [S+Phospho;]	ALAR			17	33	QKVE		
ETIESLSSSEESITEYK [S+Phospho;S+Phospho;]	ALAR			17	33	QKVE		
ETIESLSSSEESITEYKQK	ALAR			17	35	VEKV		
ETIESLSSSEESITEYKQKVEK [S+Phospho;]	ALAR			17	38	VKHE		
EVPKAKDVTYTK	PEIM			100	111	GRVM		
IESLSSSEESITEYK [S+Phospho;]	ARET			19	33	QKVE		
KVEKVKHEDQQQGEDEHQDK	EYKQ			35	54	IYPS		
LLNPTHQIYPVTQPLAPVHNPISV	NQEL			203	226]		
LNPTHQIYPVTQPLAPVHNPISV	QELL			204	226]		
LNQELLLNPTHQIYPVTQPLAPVHNPISV	QALL			198	226]		
LSSSEESITEYK [S+Phospho;]	TIES			22	33	QKVE		
LSSSEESITEYKQKVEK [S+Phospho;]	TIES			22	38	VKHE		
NPTHQIYPVTQPLAPVHNPISV	ELLL			205	226]		
NQELLLNPTHQIYPVTQPLAPVHNPISV	ALLL			199	226]		

Protein	UniProt AC	Peptide	Pre ^a	Start ^b	End ^c	Post ^d
		PTHQIYPVTQPLAPVHNPISV	LLLN	206	226]
		QKVEKVKHEDQQQGEDEHQD [Q+Ammonia-loss;]	TEYK	34	53	KIYP
		QKVEKVKHEDQQQGEDEHQDK [Q+Ammonia-loss;]	TEYK	34	54	IYPS
		QKVEKVKHEDQQQGEDEHQDK [Q+Deamidated;Q+Ammonia-loss;]	TEYK	34	54	IYPS
		QKVEKVKHEDQQQGEDEHQDKIYP [Q+Ammonia-loss;]	TEYK	34	57	SFQP
		RETIESLSSSEESITEYK	LALA	16	33	QKVE
		RETIESLSSSEESITEYK [S+Phospho;]	LALA	16	33	QKVE
		RETIESLSSSEESITEYK [S+Phospho;S+Phospho;]	LALA	16	33	QKVE
		RETIESLSSSEESITEYKQK [S+Phospho;]	LALA	16	35	VEKV
		RETIESLSSSEESITEYKQKVE [S+Phospho;S+Phospho;]	LALA	16	37	KVKH
		RETIESLSSSEESITEYKQKVEK	LALA	16	38	VKHE
		RETIESLSSSEESITEYKQKVEK [S+Phospho;]	LALA	16	38	VKHE
		RETIESLSSSEESITEYKQKVEK [S+Phospho;S+Phospho;]	LALA	16	38	VKHE
		SEESITEYK	SLSS	25	33	QKVE
		SLSSSEESITEYK [S+Phospho;]	ETIE	21	33	QKVE
		SLSSSEESITEYKQKVEK	ETIE	21	38	VKHE
		SLSSSEESITEYKQKVEK [S+Phospho;]	ETIE	21	38	VKHE
		SSEESITEYKQK [S+Phospho;]	ESLS	24	35	VEKV
		SSSEESITEYK	IESL	23	33	QKVE
		SSSEESITEYKQKVEK [S+Phospho;]	IESL	23	38	VKHE
		TIESLSSSEESITEYK [S+Phospho;]	LARE	18	33	QKVE
		TIESLSSSEESITEYK [S+Phospho;S+Phospho;]	LARE	18	33	QKVE
		VKHEDQQQGEDEHQDK	KVEK	39	54	IYPS
		VKHEDQQQGEDEHQDKIYPSFQPQP	KVEK	39	63	LIYP
Osteopontin	P10451	DIQYDPATDEDITSH	FRRP	178	192	MESE
		DQSAETHSHKQSRLY [S+Phospho;]	SQLD	232	246	KRKA
		SHELDSASSEVN [S+Phospho;]	KFRI	303	314]
Mucin-1	P15941	NPAVAATSANL	LSYT	1245	1255]
		TNPAVAATSANL	SLSY	1244	1255]
Bile salt-activated lipase	P19835	EGGFVEGVNKK	AVYT	29	39	LGLL
Alpha-S1-casein	P47710	EKQTDEIKDTR	NILR	58	68	NEST
		RLQNPSESSEPIPLESREEYMNGMN [M+Oxidation;M+Oxidation;]	RYPE	26	50	RQRN
		YPERLQNPSESSEPIPI	LPLR	23	38	LESR
Butyrophilin subfamily 1 member A1	Q13410	DADTLHSKLIPTQPSQGAP	SAPR	508	526]
		DGREQEAEQMPEYR [M+Oxidation;]	LVHR	79	92	GRAT
		DGREQEAEQMPEYRG [M+Oxidation;]	LVHR	79	93	RATL

^a Amino acid residues before the starting position

^b Starting position of peptide in the complete protein sequence

^c Ending position of peptide in the complete protein sequence

^d Amino acid residues after the ending position

Table 2.

Unique peptides released at pH 4 and 5.

Condition	Protein	UniProt AC	Peptide	Pre ^a	Start ^b	End ^c	Post ^d
pH4	Alpha-lactalbumin	P00709	HTSGYDTQAIVENNESTEYGL	CTMF	51	71	FQIS
			IVENNESTEYGL	DTQA	60	71	FQIS
	Beta-casein	P05814	SVPQPKVLPQPQVVPYPQRAVPVQALLL	QPLW	170	198	NQEL
	Osteopontin	P10451	SYETSQLDDQSAETHSHKQSR [T+Phospho;]	RGKD	224	244	LYKR
pH5	Beta-casein	P05814	LSSSEESITEYKQK [S+Phospho;]	TIES	22	35	VEKV
pH4&pH5	Apolipoprotein A-I	P02647	LSALEEYTKKLNTQ	KVSF	254	267]
			SALEEYTKKLNTQ	VSFL	255	267]
	Beta-casein	P05814	PVTQPLAPVHNPIIS	HQIY	212	225	V]

^a Amino acid residues before the starting position^b Starting position of peptide in the complete protein sequence^c Ending position of peptide in the complete protein sequence^d Amino acid residues after the ending position