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Peptidome analysis of human skim milk in term and preterm milk

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ABSTRACT

The abundant proteins in human milk have been well characterized and are known to provide nutritional, protective, and developmental advantages to both term and preterm infants. Due to the difficulties associated with detection technology of the peptides, the expression of the peptides present in human milk is not known widely. In recent years, peptidome analysis has received increasing attention. In this report, the analysis of endogenous peptides in human milk was done by mass spectrometry. A method was also developed by our researchers, which can be used in the extraction of peptide from human milk. Analysis of the extracts by LC–MS/MS resulted in the detection of 1000–3000 Da peptide-like features. Out of these, 419 peptides were identified by MS/MS. The identified peptides were found to originate from 34 proteins, of which several have been reported. Analysis of the peptides' cleavage sites showed that the peptides are cleaved with regulations. This may reflect the protease activity and distribution in human body, and also represent the biological state of the tissue and provide a fresh source for biomarker discovery. Isotope dimethyl labeling analysis was also used to test the effects of premature delivery on milk protein composition in this study. Differences in peptides expression between breast milk in term milk (38–41 weeks gestation) and preterm milk (28–32 weeks gestation) were investigated in this study. 41 Peptides in these two groups were found expressed differently. 23 Peptides were present at higher levels in preterm milk, and 18 were present at higher levels in term milk.

1. Introduction

Breast is the most important food for the newborn, because it contains unique nutrient composition, immune components, anti-infective factors, and metabolic enzymes. These are all contribute to meet the critical needs for growth and development during a humans early life. It has bioactive properties that can facilitate a newborn to adapt from the intra-uterine to the extra-uterine environment, by stimulating the development of the brain, digestive tract, and immune system [1]. Several studies have shown that breast-feeding is associated with a lower incidence of obesity, diabetes, and cardiovascular disease later in life [2–5]. Human milk proteins are particularly important for premature infants. These proteins not only provide a digestible source of amino acids for preterm infants, but also confer immunological protection and perform developmental and regulatory functions, which can exert both long and short-term benefits compared to formula feeding [6,7].

According to the stage of lactation, the milk is classified as colostrum, produced upto 72 h after the birth on average; or mature milk, secreted after 72 h from the birth[7]. Significantly, the protein composition in milk of preterm mothers is different from that of term mothers. The concentration of total protein(s) is typically higher in preterm milk[8]; however, while some individual proteins are expressed at higher levels in preterm milk, others are present at lower concentrations [9–12]. A comprehensive understanding of the protein profile in breast milk is expected to contribute not only to our understanding of milk biogenesis and functions which are provided to the newborn but may also provide guidance for formula milk industries to produce better breast-milk like products.

Peptidomics, which is defined as the comprehensively qualitative and quantitative analysis of peptides in biological samples, is an area of increasing interest for the biomarker discovery [13,14]. The low molecular weight (LMW) fraction of proteome (which was usually considered as biological trash) has attracted increasing attention recently [15–17]. Circulating protein fragments generated in the body fluid or tissues may reflect the biological events

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and provide a rich bank for diagnostic biomarkers. The profiling of peptides generated in serum has being developed for cancer diagnosis [18–21]. Villanueva et al. [21] used MALDI-TOF MS-based peptides profiling to distinguish prostate, bladder, and breast cancer patients from healthy persons successfully. A few recent studies have identified a large number of endogenous peptides in the milk [22–24]. The endogenous peptides play crucial roles in the respiratory, cardiovascular, endocrine, inflammatory, and nervous systems. However, there is minimal information reported thus far on human milk peptidome analysis.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is mostly used for identification of peptides due to its high detection sensitivity and high throughput. The objective of this study was 2-fold. First, we aimed to further characterize the peptidome of term and preterm colostrums, using a ultracentrifugation method to compress the dynamic range of the samples prior to analysis by LC–MS/MS. Second, we sought to quantitatively examine whether there are differences in peptide expression between term and preterm milk that reflect the physiological and metabolic effects of preterm delivery upon the mammary gland.

2. Materials and methods

2.1. Sample collection

Term and preterm colostrums samples were obtained from healthy lactating mothers at Nanjing Maternal and Child Health Hospital, China. All donors gave written informed consent for their donations to be used in this research, and this study was approved by Nanjing Medical University, Human Research Ethics Committee and Nanjing Maternal and Child Health Hospital. Lactating mothers collected the milk with mechanical breast pumps (Ameda-Egnel Lincolnshire, Illinois) over the first week. Milk was stored on dry ice during transport to the laboratory (upto 1 h), and centrifuged (2000*g*, 20 min, 4 °C) to remove the lipid layer and cell debris pellet. The aqueous phase (skim milk) was then aliquoted with protease inhibitor (Complete mini EDTA-free, Roche Applied Science) and stored at −80 °C.

2.2. Sample treatment

All milk samples were thawed, pooled, and centrifuged at 10,000*g* for 15 min twice to remove the cream layer. Samples were then centrifuged at 120,000*g* at 4 °C for 60 min, and the supernatant was collected. Protein concentrations of all the samples were determined by the bicinchonic acid method (BCA) (Pierce), using BSA as a standard. In the ultrafiltration method, 20 μL of milk samples were diluted in a denaturing and reducing solution (7 M urea, 2 M thiourea, and 20 mM DTT), transferred to centrifugal filter devices. Molecular weight cut-off (MWCO) filters (Millipore, USA) of 10 and 30 kDa were washed with 0.5 ml H_2O prior to use. The milk samples were centrifuged through the filters according to the manufacturer's recommendations. The filtrates were then desalted and concentrated by C18 solid phase extraction (SPE) (Strata C18-E, 55 μm, 760A, 100 mg/mL, Phenomenex), and finally lyophilised. The derived peptides of the different samples are then labeled in-solution with isotopomeric dimethyl labels [25]. The labeled samples are mixed and simultaneously analyzed by LC–MS/MS whereby the mass difference of the dimethyl labels is used to compare the peptide abundance in the different samples.

2.3. SDS–PAGE

Pre-cast Novex Tricine Gels (Invitrogen, USA) were used with Tricine SDS Runing Buffer. The thawed protein samples were mixed 1:1 (vol) with 2× sample buffer (100 mM Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, and 0.01% bromophenol blue in ultrapure water; 5% DTT was added just before use) and heated for 5 min at 95 °C. The protein loaded on the gel was about 10 μL of protein per well. The gel was run for 60 min at 120 V. The proteins were stained for 4 h using the Colloidal Blue Staining Kit (Invitrogen, USA), and destained overnight in ultrapure water.

2.4. LC–MS/MS

Two different mass spectrometers were used to identify the labeled samples. The freezing dried sample was dissolved in 0.1% formic acid and filtered with a 0.45-μm membrane before injection. Peptides were loaded onto a trap column (Acclaim PepMap100 C18, 75 μm × 20 mm, LC Packings) and separated with a C18 column (Acclaim PepMap C18, 75 μm × 150 mm, LC packings) on an Ultimate 3000 nano-LC system (Dionex). The mobile phase was composed of: (A) 0.1% formic acid dissolved in water and (B) 0.1% formic acid dissolved in acetonitrile. A linear gradient from 0% to 20% B delivered at 0.2 ml/min over 50 min was used and the eluates were automatically transferred to the MS system.

The samples were directly injected into a MALDI TOF/TOF (Ultraflextreme, Bruker Daltonics, Bremen) instrument operated in the positive ion mode. Full scan analysis was performed over the *m*/*z* range 600–5000 at 2 spectra/s. The capillary voltage and cone voltage were maintained at 3.9 kV and 40 V, respectively. For MS analysis, 2000 single shot spectra were accumulated from 10 random positions on each sample, irradiating each position with 200 laser pulses. From the MS analysis, compounds with S/N. 15, were selected for MS/MS analysis. For MS/MS analysis 2000 single shot spectra were recorded of the precursor ions and 4000 of the fragment ions. All data were collected, transformed, and analyzed using MassLynx™ software (version 4.1, Waters Corporation).

2.5. Bioinformatics

All the acquired data were processed using the ProteinScape software (Bruker Daltonics). The MS/MS data were searched using the Mascot search engine (Matrix science) against the SwissProt sequence database with the Homo sapiens subset considering the following variable modifications: phosphorylation, amidation, deamidation, pyroglutamic acid, oxidation, acetylation, sulfation, and oxidized as well as reduced cysteines. The error tolerance for precursor ion masses was set to 15 ppm and for fragment ion masses, 0.6 Da. All included peptides in the search were at least 7 amino acids long. A lower peptide score threshold of 15 was used. Protein hits with at least one peptide with ion score >27 were retrieved. For these, all peptide matches were manually validated. The search results were compiled into a protein list using ProteinScape. All MS/MS spectra considered for protein identification were manually validated using the BioTools software (Bruker, Bremen, Germany) according to the following rules: (a) prominent cleavage N-terminally to proline, (b) b and y ion pairs, (c) ion series continuity, (d) major peaks identified as b or y ions, (e) resemblance of fragment ion patterns in peptide hits that cover partially the same amino acid sequence in a protein, (f) abundance of y/b ions according to position of basic amino acids in the sequence and (g) abundant cleavage C-terminally to aspartic acid and glutamic acid if an arginine is present in the sequence. In order to categorize the identified peptides, the results were analyzed using the software program IPA (Ingenuity Databases) and the UniProt Database release 2013_5 (http://www.uniprot.org/).

3. Results

3.1. Sample preparation

The peptidome constitutes only a minor fraction of the total protein contents of human milk. Because of the limited loading capacity of LC columns (<1 μg), it is necessary to enrich the peptide fraction. Ultrafiltration using MWCO filters provides a simple means to achieve this. Filters of different cut-off sizes (10 kDa, 30 kDa) were evaluated. 30 kDa filters resulted in permeability of few amounts of caseins, serum, and milk fat globule membrane (MFGM). While 10 kDa filters efficiently removed these proteins (Fig. 1), they retained a large part of the peptides in the mass range of interest.

From studies in plasma and CSF it is well known that many peptides bind to larger proteins and may therefore be retained in the ultrafiltration step [26–28]. To improve the recovery of such peptides we investigated the effect of pretreating milk samples with denaturing and reducing solution (7 M urea, 2 M thiourea, and 20 mM DTT), in order to dissociate the peptides from the carrier proteins prior to the ultrafiltration step. Salts had been removed by C18 solid phase extraction after ultrafiltration.

3.2. Peptide identification

The extracted peptides from human milk were directly analyzed by LC–MS/MS. In this way, from the six individuals, 419 peptides, originating from 34 protein precursors were identified with extremely high stringency and manual verification (Table 1). We first analyzed broad features of the milk peptidome. Peptides identified from human milk, all spread over a broad range of MW and pI, as shown in Fig. 2, and some distribution differences can be observed. In addition, the MW versus pI distribution of the identified peptides in human term and preterm milk, is depicted in Fig. 2C. It is obvious that the points of the identified peptides clustered into three groups in the acidic and basic pH range. As a result of limitations in instrumentation and data processing, the MWs of the peptides identified by LC–MS/MS are less than 3 kDa, which is supported by previous reports [29].

Gene ontology analysis was then used to investigate whether the peptides precursors was biased for proteins from any compartments or protein classes. The 419 peptides were categorized by both their subcellular location and their functions, according to their annotations in the UniProt Database release 2013_5 (http://www.uniprot.org/). None of the categories were considerably different, demonstrating that the ultrafiltration preparation method is universal in that it does not lead to preferential extraction of proteins from specific cellular compartments or with specific functions. The majority of the proteins found in the present study were cytoplasmic (45%) or extracellular space proteins (38%) (Fig. 3A). Functionally, the majority of the proteins identified were involved in either in the immune response (19%) or in cellular metabolism and cellular growth (28%) (Fig. 3B).

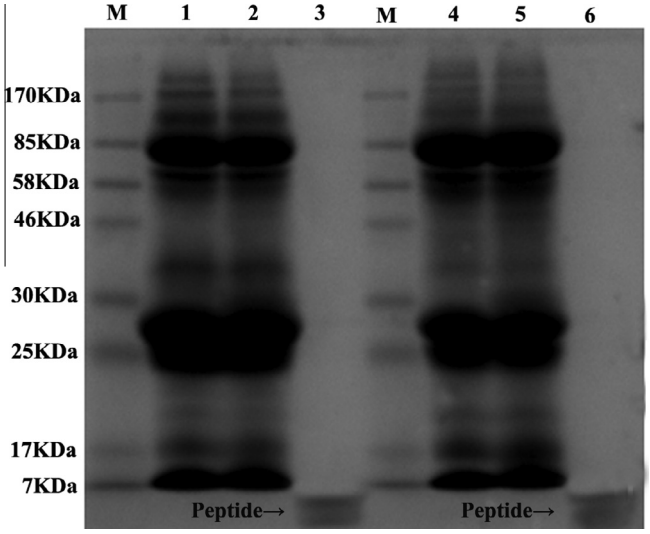

Fig. 1. SDS–PAGE analysis of human milk samples before and after ultrafiltration treatment. Lane 1, row term colostrums without any treatment; lane 2, skim term colostrums; lane 3, peptides from term colostrums after ultrafiltration; line 4, row preterm colostrums without any treatment; line 5, skim preterm colostrums; line 6, peptides from preterm colostrums after ultrafiltration. M = Protein marker.

3.3. Peptides cleavage pattern

With further analysis of the identified peptides, many of the peptides from one protein come from the same segment. The generation of peptide ladder was not random. The enzymes in tissue cleaved the protein according to their rules. Protein of Beta-casein generated the highest number of peptides (279 peptides) in our case, which meant it was most easily cleaved by the endogenous enzymes. With further analyzed cleavage site distribution, the obtained results for the four cleavage sites were shown in Fig. 4. From the graph, leucine (L) was the most hit cleavage site for C-terminal amino acid of the preceding peptide, while proline (P) dominated the cleavage site of C-terminal amino acid of the identified peptide. Leucine (L) is the most cleavaged site of N-terminal amino acid of the identified peptide and N-terminal amino acid of the preceding peptide.

3.4. Quantitative comparison of term and preterm milk

SDS–PAGE analysis revealed that levels of Beta-casein and α-lactalbumin were similar in the term and preterm (Fig. 1). But the peptide concentration of the pooled term and preterm milk samples were different after ultrafiltration (0.505 μg/mL and 0.235 μg/mL, respectively). In the isotope dimethyl labeling analysis of the ultrafiltration-treated pooled term and preterm milk samples, 419 peptides were identified. All of the 419 peptides were found in both the term and preterm milk samples and in each duplicate. The $\log_2$ scale was used and $\log_2(1) = 0$. The ratios that are greater than zero indicate up-regulation of the peptide, while ratios less than zero indicate down-regulation. As shown in Table 1, 23 peptides constituted a significantly greater proportion of the peptide content in the preterm milk (abundance changed more than 3-folds), and 18 peptides were present at a significantly higher level in the term milk (abundance changed more than 3-folds). All 41 peptides showed statistically significant changes ($p < 0.05$). The remaining 378 peptides did not differ in abundance within the two samples.

4. Discussion

Centrifugal ultrafiltration with accurate MW cutoff is a method most widely used for peptide enrichment based on a size-exclusion mechanism [30,31]. 10 kDa filters were found optimal for our application, achieving efficient removal of caseins, serum, and milk fat globule membrane (MFGM), the three most abundant milk proteins, without compromising peptide recovery (Fig. 1). The extracted peptides were directly analyzed by LC–MS/MS three times. About 215 peptides can be identified in a single run, and totally, 419 peptides (from 34 proteins) were identified in all three runs (Table 1). A remarkable trait of the identified peptides is that 279 of them (67%) are from Beta-casein, which meant it was most easily cleaved by the endogenous enzymes. All the identified

Table 1
Part peptides that are differentially expressed between term colostrums and preterm colostrums.

Sequence	Mass	Protein names	log2 (L/H)	*p*-value
Peptides down-regulated in human preterm milk (>3-folds)				
VLPIPQQVVPYPQRAVPVQALL	2424.4	Beta-casein	−9.5	5.3E−05
LLLNQELLLNPTHQIYPV	2117.2	Beta-casein	−6.2	7.4E−03
VLPIPQQVVPYPQRAVPVQALLLNQ	2779.6	Beta-casein	−4.4	9.8E−16
VLPIPQQVVPYPQRAVPVQALLL	2537.5	Beta-casein	−4.2	2.8E−02
HLPLPLLQPLMQQVPQPIPQT	2387.3	Beta-casein	−4.2	4.2E−03
LKSPTIPFFDPQIPKLTDLEN	2412.3	Beta-casein	−4.1	2.5E−02
VEPIPYGFLPQNILPLA	1880	Beta-casein	−4.0	2.5E−05
LGSAMQNTQNLLQMPY	1807.9	Alpha-2-macroglobulin	−4.0	5.3E−03
LWSVPQPKVLPIPQQV	1828.1	Beta-casein	−3.8	4.9E−02
DLENLH	739.4	Beta-casein	−3.8	2.7E−03
VEPIPYGFLPQNILPLAQP	2105.2	Beta-casein	−3.7	4.5E−03
DPDAPLQPVTPLQL	1502.8	C4A variant protein	−3.5	1.1E−09
LLLNQELLLNPTHQIYPVTQPLAP	2724.5	Beta-casein	−3.5	1.1E−12
FVEPIPYGFLPQNILP	1843	Beta-casein	−3.5	4.8E−17
VEPIPYGFLPQNILP	1695.9	Beta-casein	−3.3	8.4E−05
IPFFDPQIPKLTDLEN	1886	Beta-casein	−3.3	2.5E−06
WGRLTWRKMCRKLLDMTFSS	2571.3	vWF-cleaving protease	−3.3	1.4E−06
TIPFFDPQIPKLTDLEN	1987	Beta-casein	−3.3	1.5E−04
DLENLHLPL	1062.6	Beta-casein	−3.2	3.4E−04
LTDLENLHLPLPLLQP	1825	Beta-casein	−3.2	2.3E−02
DDPDAPLQPVTPLQLFEGRRN	2377.2	C4A variant protein	−3.1	7.6E−03
GRVMPVLKSPTIPFFDPQIPK	2366.3	Beta-casein	−3.1	1.5E−02
LLQPLMQQVPQPIPQTLALPPQP	2546.4	Beta-casein	−3.1	3.1E−04
Peptides up-regulated in human preterm milk (>3-folds)				
DLENLHLPLPLLQPLM	1855	Beta-casein	3.1	3.7E−06
YPFVEPIPYGFL	1440.7	Beta-casein	3.6	3.3E−05
ASQLMGENRTMTIHNGMFFST	2372.1	Fibrinogen beta chain	3.6	1.3E−07
LVNERWVLTAA	1270.7	Kallikrein-7	3.7	2.4E−03
LWSVPQPKVLPIP	1472.9	Beta-casein	3.9	2.5E−04
DSGSSEEKQLYNKYPDAVAT	2201	Secreted phosphoprotein 1	4.0	3.4E−04
AVVLPVPQPEIMEVPKAKDTVYT	2523.4	Beta-casein	4.0	1.4E−04
WRKMCRKLLDMTFSSKTNTLVVR	2869.5	vWF-cleaving protease	4.2	5.5E−05
QPLMQQVPQPIPQTLALPPQP	2320.3	Beta-casein	4.3	2.4E−05
LAQPAVVLPVPQPEIMEVPK	2154.2	Beta-casein	4.4	8.4E−05
ATSSLCSVTNTSMMTSE	1805.7	Ascites sialoglycoprotein	4.4	2.5E−06
LQPLMQQVPQPIP	1487.8	Beta-casein	4.6	1.4E−06
SIQLPTTVRDIMNRW	1829	Membrane alanine aminopeptidase variant	4.9	1.5E−04
AVPVQALLLNQ	1164.7	Beta-casein	5.5	3.4E−04
APVHNPISV	932.5	Beta-casein	5.5	2.5E−03
MKFISTSLLLMLLVSSLS	1982.1	B cell-attracting chemokine 1	5.7	2.7E−03
LLNPTHQIYPVT	1394.8	Beta-casein	6.0	1.1E−19
EDLIDEDDIPVRSFFP	1905.9	C4A variant protein	6.6	3.7E−11

L = Peptide intensity (term colostrums).
H = Peptide intensity (preterm colostrums).

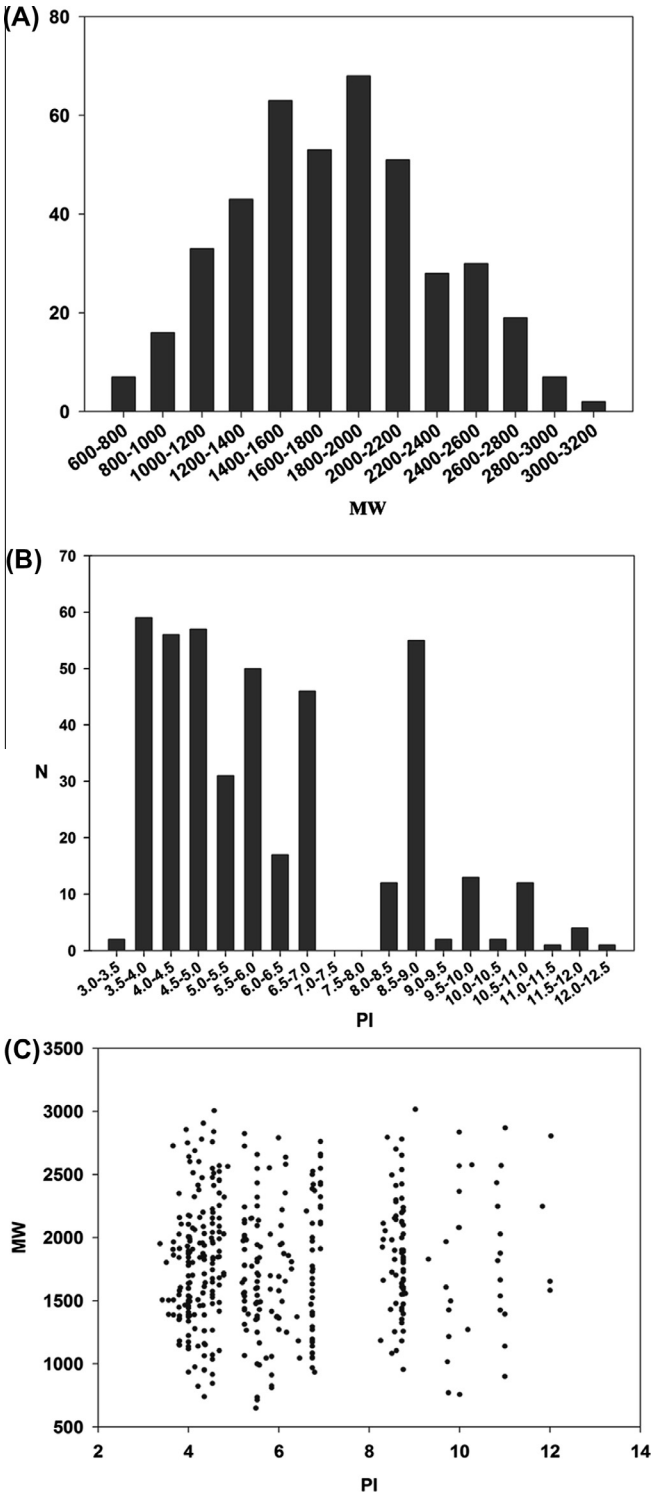

Fig. 2. (A) Distribution of molecular weight (MW) of peptides. (B) Distribution of isoelectric point (pI) of peptides. (C) Scatter plot of MW versus pI distribution of the peptides.

peptides had the MW below 3000 Da, and MW of most peptides was at around 1400–2200 Da (Fig. 2). Because it is beyond the ability of MS/MS for identification of peptides with a molecular weight over 3000 Da [29].

One of the challenges associated with peptidome mapping experiments is processing and applying the large amounts of information generated within a particular biological context. One way in which the expanded peptidome of human milk may be useful is if the precursors proteins of peptide identified reveal any information about either the metabolism or function of the mammary gland. In the present study, proteins involved in cellular metabolism/cellular and immune response comprise 47% of the total proteins, representing key functions of peptides precursors (Fig. 3A). 59 peptides precursors were identified to be involved in lipid metabolism and transport (Fig. 3B). This may be caused by dietary fat intake during infancy is very high, because of their high-energy requirement and beneficial effects of fat on growth and development of the brain and vision. Approximately 50% of the total

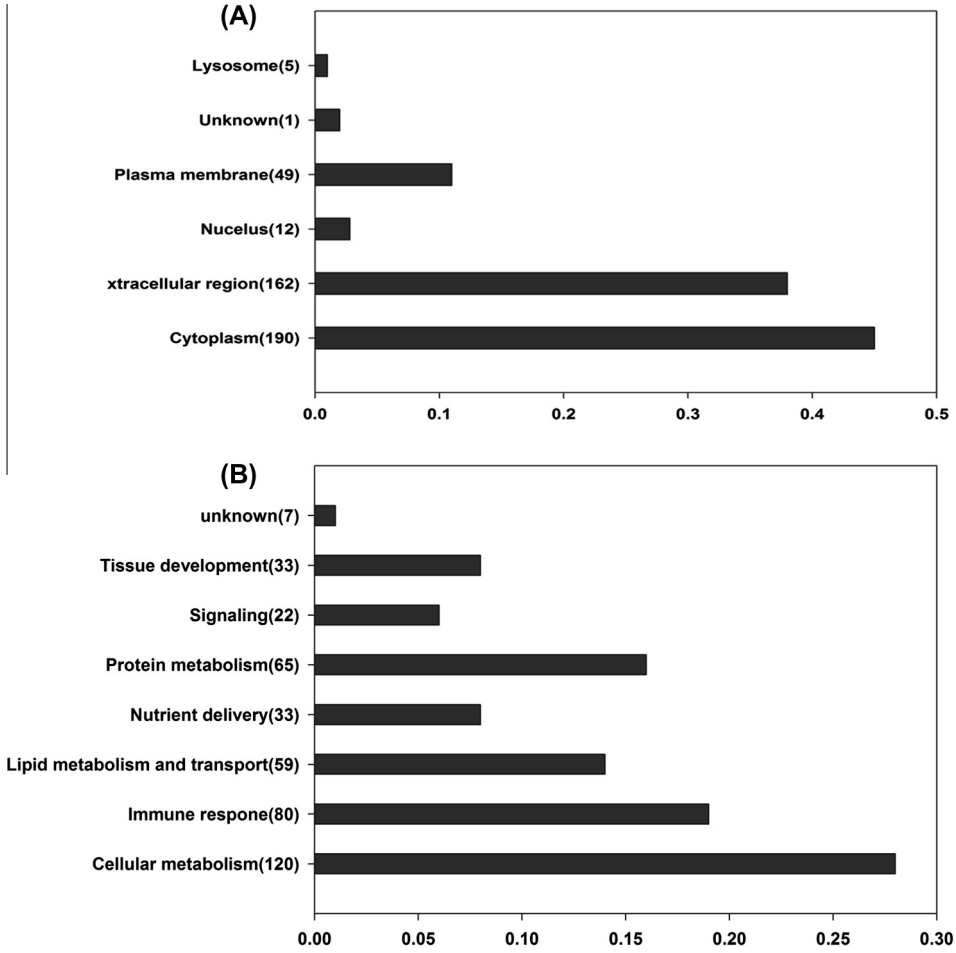

Fig. 3. Peptides precursors classifications. (A) Subcellular location of all 419 peptides precursors identified in the LC–MS/MS analyses. (B) Functional categorization of 419 peptides precursor identified in LC–MS/MS analyses. The number of proteins in each category is indicated in parentheses.

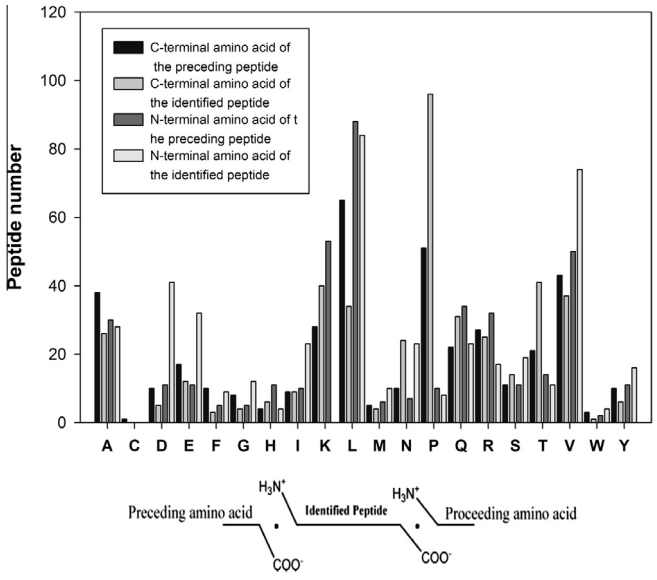

Fig. 4. Distribution of the four cleavage sites in the identified peptides.

energy intake is acquired from milk lipids during the first month after birth, and 35% of an infant's weight gain during the first 6 months consists of body fat [31,32]. The functional meaning of these peptides enrichment is unknown; however, peptidome profiling data shed some light on the mechanisms leading to the presence of these peptides in human milk as will be further studied in the future.

The enzymes in tissue cleaved the protein depends on their rules. For example, no peptide from serum albumin and one of the abundant proteins in mouse liver were detected by direct analysis of the extracted peptides. However, the peptides from serum albumin can be detected in the digests of HMW fractions. The enzymes in tissue cleaved the protein according to their rules. Protein of Beta-casein generated the highest number of peptides (279 peptides) in our case, which meant it was most easily cleaved by the endogenous enzymes. We further analyzed the cleavage site distribution, and the obtained results for the four cleavage sites were shown in Fig. 4. We can see that leucine (L) was the most hit cleavage site for C-terminal amino acid of the preceding peptide, while proline (P) dominated the cleavage site of C-terminal amino acid of the identified peptide; leucine (L) is the most cleavaged site of N-terminal amino acid of the identified peptide and N-terminal amino acid of the preceding peptide. It was postulated that the HMW peptides were produced by endoproteolytic cleavages of their progenitor proteins, and then other enzymes such as exoproteases of aminopeptidase and carboxypeptidase further cleaved the HMW peptides to produce the LMW peptides [33–35]. The cleavage sites' distribution of peptidome in human milk was different from that of serum peptidome [33]. This can be interpreted to mean that the proteases varied greatly in different tissue and body fluid. Proteases often represent a host response to the physiological states and can play a critical role in mediating the communication

between a biological event and its microenvironment. For example, the proteases regulate in different disease states will result in the difference of degradation pattern, and also the degradation fragments can be the biomarkers of cancer.

To investigate whether the human milk peptidome undergoes detectable changes at different stages of mammary gland development, isotope dimethyl labeling analysis was used to compare the peptide composition of term and preterm milk. 419 peptides were identified from the ultrafiltration-treated pooled term and preterm milk samples. We operate on a log2 scale and $\log 2(1) = 0$, ratiosgreater than zero indicate up-regulation of the peptide, while ratios less than zero indicate down-regulation. As shown in Table 1, 23 peptides constituted a significantly greater proportion of the peptide content in the preterm milk (abundance changed more than 3-folds), and 18 peptides were present at a significantly higher level in the term milk (abundance changed more than 3-folds). All 41 peptides showed statistically significant changes ($p < 0.05$). The remaining 378 peptides did not differ in abundance between the two samples.

In summary, the present study represents a comprehensive study to date of the human milk peptidome, identifying 419 peptides, as well as documenting changes in the relative abundance of peptides in term and preterm milk. Knowledge obtained from this characterization of the peptides in human milk will provide insights into the regulatory mechanism involved in the synthesis and secretion of human milk, particularly after preterm delivery, as well as identifying potential proteins in human milk responsible for the nutritional, immunological, and developmental advantages conferred onto the breastfed infant.

We investigated that MS-based technology coupling with ultrafiltration has already advanced sufficiently to allow not only detection of large numbers of endogenously processed peptides in biological fluids. Thus, MS can now be used to study these processing events resulting in cleavage or changing of the cleavage pattern which may allow linking them to specific physiological or pathophysiological events. In addition, isotope dimethyl labeling analysis was used to identify differentially expressed proteins between term and preterm milk, providing insights into metabolic differences in the mammary gland after preterm birth in comparison to term birth. We believe that the presented data set contains valuable unique information, which will enable interested researchers to identify novel human milk regulatory mechanisms.

Acknowledgments

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