Basic nutritional investigation

In vivo endogenous proteolysis yielding beta-casein derived bioactive beta-casomorphin peptides in human breast milk for infant nutrition

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A B S T R A C T

Objective Beta-casein is a major protein in breast milk and an important source for several bioactive peptides that are encrypted within the sequence. Beta-casomorphins (BCMs) are short-chain proteolytic peptides that are derived from the beta-casein protein and have opioid effects in newborns. Human milk is known to contain naturally occurring milk-protein-derived bioactive peptides but the identification of naturally occurring beta-casein-derived BCMs in human breast milk has been limited due to difficulties in the detection of BCM peptides, which are small and circulate in low concentrations. **Methods** The present study aimed to identify the naturally occurring BCM peptides from beta-casein in human breast milk using liquid chromatography-tandem mass spectrometry. The BCM peptides identified in the breast milk were analysed to predict the milk proteases responsible for the cleavage patterns using a computational tool EnzymePredictor. **Results** In-depth peptidomics analysis of breast milk samples that were collected at different lactation stages during human lactation revealed the presence of BCMs including BCM-8, -9, -10, and -11 as well as precursors and truncated forms of the original peptide, which suggests that milk protease activity in the mammary gland generates biologically relevant BCMs. **Conclusions** To our knowledge, this is the first report to describe the presence of naturally occurring human BCM-10 and -11 in breast milk. Our study provides evidence of beta-casein-derived BCM peptides in human milk before infant digestion. Proteases that are present in milk are likely specific in their proteolysis of beta-casein. The identified bioactive BCM-8, -9, -10, and -11 as well as the precursor peptides meet the structural requirements to elicit opioid, immunomodulatory, antioxidative, and satiety functions in newborns.

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Introduction

More than 166 million years ago, the key mammalian characteristic of copious milk secretion evolved to provide nutrition, immunological protection, and developmental programming for newborns [1–3]. The role of milk is to feed newborns with nutritional proteins, lipids, and carbohydrate as well as minerals, vitamins, and water [4,5]. In addition to its role in nutrition, milk also has important biological functions including digestion, regulation, and uptake of other nutrients [6,7], protection against pathogenic bacteria [8], opioid-like activity [9], cognitive development [10], and immunomodulation [11,12].

Recent mass spectrometry-based proteomics demonstrated that hundreds of peptides (i.e., short-chain protein molecules) are also present in human milk. These peptides are released from human milk proteins after proteolytic activity within the mammary gland before ingestion by the infant [8]. Peptides that are released from *in vivo* digestion of milk proteins in the mammary gland are known to possess multiple health-promoting functions in the infant's gastrointestinal tract [13,14].

Recently, an *in vivo* human milk peptidome study identified more than 300 milk peptides that originate from caseins and whey proteins [8]. Interestingly, 59% of the peptides were derived from beta-casein, with a few peptides from α_{s1}-casein and the remaining from whey proteins [8]. In another proteomic study, peptides that were released from intact human milk (i.e., before infant digestion)

and infant gastric aspirates after breast feeding were compared and around 200 and 649 milk peptides were detected, respectively [13]. Most of the identified milk peptides were derived from beta-casein in both the intact milk and gastric samples and the results suggest that casein and whey protein digestion is sequence-specific. The results also suggest that this process begins in undigested breast milk and continues in the infant stomach [13].

A comparison of peptide content in human milk from mothers of preterm and term infants at birth revealed that preterm milk contains significantly increased numbers of milk peptides compared with term milk [15]. The majority of milk peptides in both preterm and term milk samples were derived from beta-casein. Interestingly, a comprehensive milk peptidomics analysis concluded that most of the peptides that were generated from beta-casein are derived primarily from the N- and C-terminal domains and fewer are generated from the middle region [16]. Proteomic studies that were carried out via bioinformatics analysis demonstrated that these milk peptides are released from corresponding proteins by the action of proteases that are naturally present in human milk [17,18]. Human milk contains a number of proteases including plasmin, elastase, cathepsin D, thrombin, kallikrein, cytosol aminopeptidase, and carboxypeptidase B2 [15–19]. The milk protease expression differs between preterm and term milk [20].

The major component of human milk casein fraction is beta-casein [21,22]. Upon proteolytic digestion, beta-casein releases domain-specific bioactive peptides: The N-terminal domain generates peptides that function as calcium carriers [11] and the C-terminal domain generates peptides that exhibit antibacterial properties [23]. The middle region of beta-casein is the site from which a group of peptides called beta-casomorphins (BCMs) are released [24] by milk proteolytic activity, either during *in vivo* gastrointestinal digestion or *in vitro* digestion [13,25].

The region that comprises of amino acids 51 to 62 (residues; Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-Tyr-Gly-Phe-Leu) has been defined as the BCM strategic zone of human beta-casein [25–27]. Specifically, in human beta-casein, BCMs that correspond to amino-acid fragments 51 to 54, 51 to 55, 51 to 56, and 51 to 57 have been identified and named BCM-4, -5, -6, and -7, respectively. These BCMs are obtained by successive C-terminal amino-acid cleavage of the 51 to 57 fragment [25,28,29]. BCMs have been shown to display important physiological roles and opioid activity via the μ opioid receptor [28,30].

BCMs appear to be circulatory in human biological systems [31,32]. Human BCM-8 has been identified in the brain stem of infants [33] and human BCM-7 in the plasma of breast milk-fed infants [34]. Interestingly, Dallas et al. [13] identified eight human beta-casein-derived BCMs and precursor peptides (but lack the typical consensus first tyrosine residue) in the gastric aspirates of infants who are fed with breast milk but not in intact milk, which highlights that infant gastric proteases are involved in the digestion of the BCM strategic zone on the beta-casein protein to release shorter BCM peptides. However, the same researchers identified a larger BCM precursor peptide (Gln[19]-Tyr[59]) in term intact milk [15]; thus, this leader precursor peptide is presumably available to an infant's gastrointestinal proteases to release BCM-4 to -9 peptides.

More recently, Wada et al. [35] identified human beta-casomorphin (BCM-9; residues 51-59) in undigested human milk, which was also identified after *in vitro* digestion and pasteurization. Ferranti et al. [24] found many naturally occurring beta-casein-derived peptides in intact human milk including BCM-8. Using immunoassay techniques, human BCM-5 and -7 have also been detected in human milk before infant gastric digestion with higher concentrations of both in colostrum than in mature milk [14].

Elevated levels of human BCM-8 has also been found via radioimmunoassay in breast milk samples that were collected from breast-feeding women with mastitis [36], which suggests that increased proteolytic activity in mastitic milk results in elevated levels of BCMs cleavage. Despite these numerous studies, an exhaustive characterization of BCM peptide release across human lactation has never been studied *in vivo*.

Evidence is emerging that proteolytic enzymes in human milk precisely control the cleavage of human BCM peptides before and during an infant's gastrointestinal digestion [13], from early to late lactation [14], and also in healthy human milk compared with mastitic human milk [36]. Human breast milk composition is finely tuned to the requirements of the infant, its composition changes dynamically over the course of lactation [37], and beta-casein concentration also increases during the transition from colostrum to mature milk [38]. Accordingly, a BCM precursor peptide (position 19–59: QKVEKVKHEDQQQGEDEHQDKIYPSFQPQPLI**YPFVEPI**PY) concentration is reported to change significantly over full-term lactation [15].

BCMs are nutropioids (i.e., food-derived opioid peptides) [39] and a detailed identification and characterization of individual BCMs across human lactation is important to understand their biological role in infant nutrition or mammary gland. The aim of the present study was to identify and characterize the human beta-casein-derived BCM peptidome in human breast milk, using a combination of reverse-phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Using this technique, various lengths of naturally occurring BCM peptides were identified. The BCM peptides that are found in breast milk are likely to promote bioactivities such as sleep induction, mucosal development, immunomodulatory, antioxidative, satiety, and gastrointestinal functions. We suggest that breast milk is the source of these bioactive BCM peptides that could be transferred to the neonatal gut through breastfeeding and may exert effects in the gastrointestinal tract as well as in the whole body of neonates.

Methods

Breast milk sample collection

Breast milk samples were collected from 10 mothers using commercial breast pumps at home. The samples were either immediately frozen at −20°C and then transferred to −80°C for long-term storage or transported on ice for up to 1 h and then frozen at −80°C. All participants were healthy and gave birth to healthy infants. None of the 10 mothers had clinical signs of mastitis on sampling day. The collection date was recorded as well as the mother's age, infant's birth date, lactation day, blood group, gestational age, and infant sex (Table 1). The studies were approved by the Deakin University human research ethics committee (2011-104).

Sample preparation for mass spectrometry analysis

The samples were removed from the freezer, thawed on ice, and then vortexed for 1 min. To obtain the skim fraction, 1 mL of each breast milk sample was centrifuged at 7000 × g for 10 min at 4°C and the skim milk fraction was removed from beneath the fat layer by pipette. Centrifugation was repeated on the skim fraction to remove any remaining visible lipid layer.

Peptides were precipitated by tricholoroacetic acid (TCA) with the addition of 1 mL of 200 g/L TCA [8]. Samples were vortexed briefly and then centrifuged at 3000 g for 10 min at 4°C and the peptide contained supernatant was collected, leaving the precipitated protein. Subsequently, the peptide supernatant was subjected to two separate methods for peptide enrichment and purification. In the first method, a centrifugal filtration with 7 kDa molecular weight cutoff (MWCO) filter (Thermo Scientific, Scoresby, Australia) was applied to isolate small MW (<7 kDa) peptide fractions followed by solid phase extraction (SPE) for the peptide analysis. With the second method, the peptide supernatant was subjected to a SPE method. Salts, TCA, oligosaccharides, and lactose were then removed from the peptides through SPE with 500 mg bed C18 columns (Supelco). Peptides were eluted from columns with 0.8 mL of 80% acetonitrile (ACN)-containing 0.1% trifluoroacetic acid and collected in 1.5 mL Eppendorf tubes. These were speedvac dried for 20 min to reduce the concentration of ACN and lyophilized at −65°C

Table 1
Details of breast milk samples collected from mothers

Mother	Mother's age (y)	Lactation stage (d)	Mother's blood group	Problems with pregnancy	Gestational diabetes	Infant gestational age at birth (wk)	Infant sex (M/F)	Infant health problems
Preterm milk samples								
M1	26	10	A+	N	N	33	M+M (congenital twins)	None
M2	30	20	A+	N	N	33	M	None
Term milk samples								
M3	30	37	O+	N	N	40	M	None
M4	29	55	B+	N	N	39	F	None
M5	26	68	O+	N	N	40	F	None
M6	30	90	O+	N	N	37	M	None
M7	26	163	B+	N	N	39	F	None
M8	35	190	A+	N	N	41	M	None
M9	42	254	O+	N	N	40	F	None
M10	31	459	B+	N	N	40	F	None

F, female; M, male.

overnight in a freeze dryer (Virtis, SP Scientific). Freeze-dried peptide samples were resuspended in 100 μL of 3% ACN with 0.1% formic acid (FA) for mass spectrometry injection.

Estimation of peptide concentration

The total milk peptide concentration was measured with the bicinchoninic acid assay method (Thermo Scientific). The microplate procedure was employed to measure peptide contents of each extracted milk peptide sample [40]. The absorbance at 562 nm was measured using an ultraviolet-visible recording spectrophotometer (Bio-Rad, Gladesville, Australia).

Human beta-casomorphin peptide synthesis

All human BCM-standard peptides with a minimum purity of 98% were synthesized based on possible BCM peptides that were derived from bovine beta-casein [41,42] at the Australian Biobest Biotechnology Service, Australia (Table 2).

Synthetic beta-casomorphin peptides amino acid analysis

An amino acid analysis was performed to measure the qualitative and quantitative amino acid content of synthetic human BCM peptides (Suppl. Table 1). The amino acid analysis was performed by the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia). We analyzed both *in vivo*-derived BCM peptides and their synthetic counterparts by LC-MS/MS to confirm the human milk-derived BCM peptide mass, retention time, and size of peak.

Liquid chromatography-tandem mass spectrometry analysis of peptide samples

LC-MS/MS was carried out on a QExactive plus Orbitrap mass spectrometer (Thermo Scientific) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000). The liquid chromatography system was equipped with an Acclaim Pepmap nano-trap column (Dinoex-C18, 100 Å, 75 μm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dinoex-C18, 100 Å, 75 μm x 15 cm). The peptide samples were injected into the enrichment column at

an isocratic flow of 5 μL/min of 3% v/v CH$_3$ CN containing 0.1% v/v formic acid for 5 min and applied before the enrichment column was switched in-line with the analytical column.

The eluents were 0.1% v/v formic acid (solvent A) and 100% v/v CH$_3$ CN in 0.1% v/v formic acid (solvent B). The flow gradient was: (i) 0 to 5 min at 3% B; (ii) 5 to 28 min, 3 to 25% B; (iii) 28 to 30 min, 25 to 40% B; (iv) 30 to 32 min, 40 to 85% B; (v) 32 to 34 min, 85 to 85% B; and (vi) 34 to 34.1 min 85 to 3% and 34.1 to 40 min at 3% B. The QExactive mass spectrometer was operated in the data-dependent mode, whereby full MS1 spectra were acquired in positive mode (resolution 70,000) an with an automatic gain control target of 3 e6. Ten of the most intense peptide ions with charge states $\geq$2 were isolated and fragmented using normalized collision energy of 26 and resolution of 17500 with an automatic gain control target of 5 e5. The dynamic exclusion duration was set at 30 sec.

Database construction

A human milk peptide library was constructed using publicly available data from previous peptide studies in human milk [16,24] and comprised a total of 689 milk peptides (Suppl. Table 2). Specifically, data were downloaded from the supplementary information of Guerrero et al. [16] and manually mapped from Ferranti et al. [24]. A milk-specific peptide library was employed rather than using the entire human protein library.

Mass spectrometry data process and peptide analysis

Peptide identification was performed using the Mascot search engine, which provides an analysis pipeline that processes the raw files generated by the QExactive plus Orbitrap mass spectrometer. The Mascot generic format files were searched against human milk-derived peptide sequences as previously described with some modifications [8]. The search was set up for no enzyme with no missed cleavage sites. Search parameters specified an initial mass spectrometry precursor mass tolerance of 20 ppm and a tandem mass spectrometry fragment tolerance of 0.2 Da. Peptides that were identified with a Mascot search were

Table 2
Synthesis of human beta-casein-derived beta-casomorphin peptides

Physiologic properties of designed and reference human beta-casomorphin peptides			
S. No	Peptide	Amino acid sequence	Monoisotopic theoretical mass
1	hBCM-4	Y P F V Tyr-Pro-Phe-Val	525.27
2	hBCM-5	Y P F V E Tyr-Pro-Phe-Val-Glu	654.31
3	hBCM-7	Y P F V E P I Tyr-Pro-Phe-Val-Glu-Pro-Ile	864.45
4	hBCM-8	Y P F V E P I P Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro	961.50
5	hBCM-9	Y P F V E P I P Y Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-Tyr	1124.56
6	hBCM-11	Y P F V E P I P Y G F Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-Tyr- Gly-Phe	1328.65

BCM, beta-casomorphin; hBCM-4, human BCM-4; hBCM-5, human BCM-5; hBCM-7, human BCM-7; hBCM-8, human BCM-8; hBCM-9, human BCM-9; hBCM-11, human BCM-11.

Table 3
Enzyme cleavage specificity rules used with the EnzymePredictor tool to predict enzyme activity

Enzyme	P1	P1'
Cathepsin D	A,V, L, I, *P*, M, F,W	A, V, L, I, *P*, M, F
Elastase	A, V, I, L, G or R	G, *P*, A, L or F
Plasmin	K or R	Any
Thrombin	R	G
Proline-endopeptidase	*P*	not *P*

P1 is the amino acid directly before the cleavage site (protein *N*-terminal side on the left). P1' is the amino acid directly after the cleavage site (protein *C*-terminal side on the right).

manually verified for each spectrum. Peptide matches were accepted if the MASCOT score e-values were ≤0.01, which corresponds to a 99% confidence level.

Prediction of proteases

The online tool EnzymePredictor (http://bioware.ucd.ie/~enzpred/Enzpred.php) was used to predict which proteases are most likely responsible for the digestion of BCMs from beta-casein in human milk [43]. EnzymePredictor requires the input of unique peptides that were identified in the sample and their associated protein (UniProt accession number). The tool was built by including 35 enzymes. The EnzymePredictor comprises of a range of enzymes, some of which are not specific to human milk and some are from bacteria. These enzymes were removed from the prediction output results. The enzyme specificity patterns that were used in the EnzymePredictor algorithm to evaluate cleavages are shown in Table 3. Enzyme cleavage site-specific information was used to identify the amino acids

that are located in the P4-P3-P2-P1|P1'-P2'-P3'-P4' positions of both the N- and C-terminal cleavage sites of each peptide.

Results

Identification of in vivo released, beta-casomorphin peptides in human milk

We compared two peptide isolation methods to isolate milk peptides for BCM peptidomic analysis by LC-MS/MS. In the first method, we applied a centrifugal filtration method to isolate a native and small MW (<7 kDa) peptide fraction from milk samples (2 preterm, 8 full-term). In the second method, we applied the standard solid phase extraction (SPE) method. Both methods yielded similar BCMs and BCM precursor peptides by LC-MS/MS analysis. A total of 14 unique, naturally occurring BCM and BCM precursor peptides were identified in the human milk samples from 10 mothers (2 preterm; 8 full term). Figure 1 shows an image of the 14 unique BCM peptides that were identified in the milk. Using mass spectrometry, naturally occurring BCM, precursors, and truncated peptides around the strategic zone in beta-casein (residues 51-61) from human milk were detected (Table 4). The BCM peptides and precursors that were identified ranged from 8 to 35 amino acids and mass ranged from 961.50 to 3833.13 Da.

BCM-8 was identified in day 20, 90, 163, and 190 lactation samples. BCM-9 was identified in day 10, 90, and 254 lactation samples.
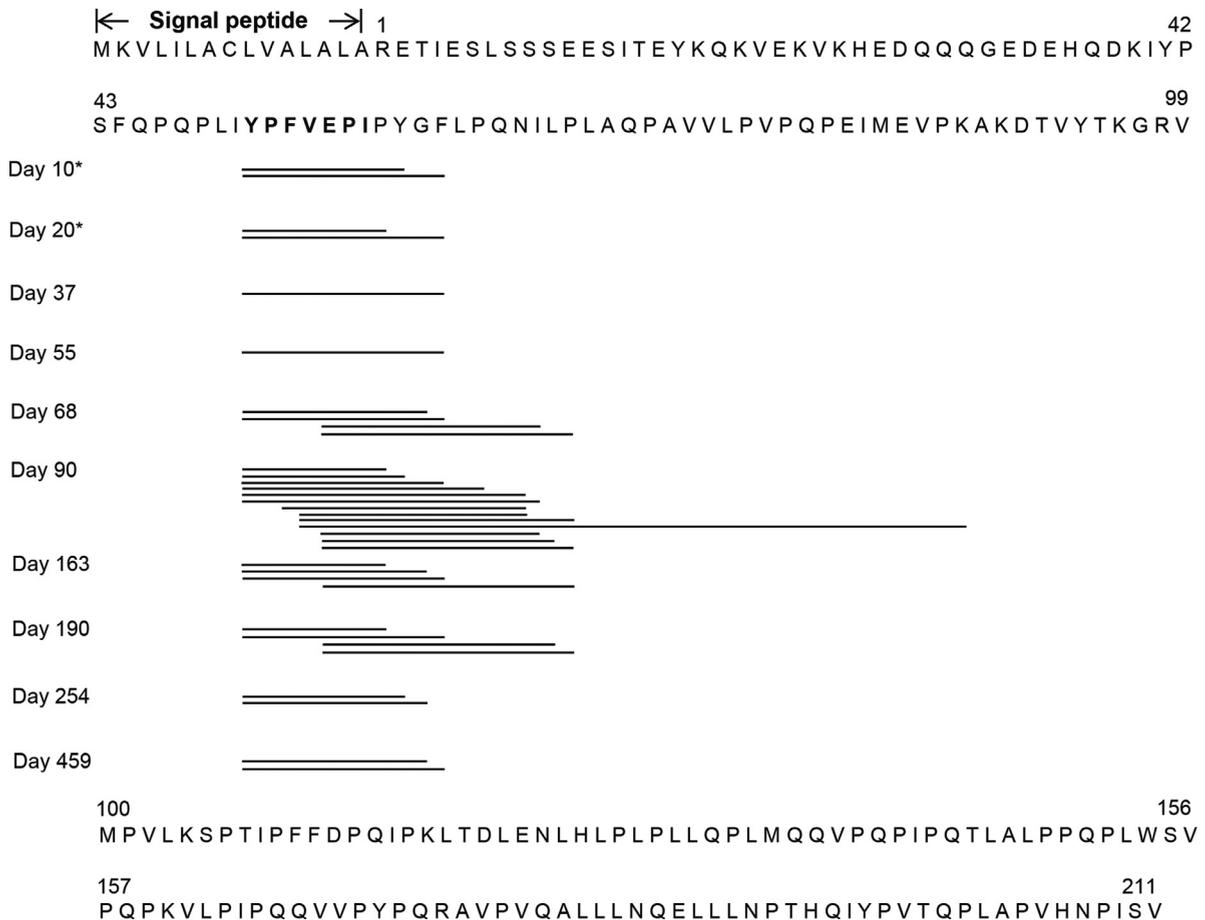
Fig. 1. Human beta-casein derived proteolysed beta-casomorphin (BCM), precursors, and truncated peptides detected by mass spectrometry analysis in human milk in relation to the beta-casein sequence. The identified BCM peptides in all 10 milk samples aligned against the human beta-casein protein sequence (subsequent lines) and each line represents a unique peptide. The BCM-7 residues are in bold and preterm milk samples are indicated with an asterisk.

Table 4
Beta-casomorphins, precursors, and truncated peptides identified by mass spectrometry in human breast milk

Lactation day	Peptide sequence	Peptide name	Position in human β-casein	Monoisotopic theoretical mass	Observed experimental m/z	Peptide identified using mass spectrometry in others study (Reference)
10*	**YPFVEPI**PY	BCM-9	51 to 59	1124.56	562.79	Wada et al. (2015)
	YPFVEPIPYGF	BCM-11	51 to 61	1328.65	664.83	
20*	**YPFVEPI**P	BCM-8	51 to 58	961.50	961.51	Ferranti et al. (2004)
	YPFVEPIPYGF	BCM-11	51 to 61	1328.65	664.83	
37	**YPFVEPI**PYGF	BCM-11	51 to 61	1328.65	664.83	
55	**YPFVEPI**PYGF	BCM-11	51 to 61	1328.65	664.83	
68	**YPFVEPI**PYG	BCM-10	51 to 60	1181.58	591.30	
	YPFVEPIPYGF	BCM-11	51 to 61	1328.65	664.83	
	EPIPYGFLPQNI	Truncated	55 to 66	1387.72	694.37	
	EPIPYGFLPQNILP	Truncated	55 to 68	1597.86	799.43	
90	**YPFVEPI**P	BCM-8	51 to 58	961.50	481.25	Ferranti et al. (2004)
	YPFVEPIPY	BCM-9	51 to 59	1124.56	562.79	Wada et al. (2015)
	YPFVEPIPYGF	BCM-11	51 to 61	1328.65	664.83	
	YPFVEPIPYGFLP	Precursor	51 to 63	1538.79	769.90	
	YPFVEPIPYGFLPQN	Precursor	51 to 65	1780.89	890.95	
	YPFVEPIPYGFLPQNI	Precursor	51 to 66	1893.97	947.49	
	FVEPIPYGFLPQN	Truncated	53 to 65	1520.77	760.89	
	VEPIPYGFLPQN	Truncated	54 to 65	1373.71	687.36	
	VEPIPYGFLPQNILP	Truncated	54 to 68	1696.93	848.97	
	VEPIPYGFLPQNILPLAQPAVVLPVPQPEIMEVPK	Truncated	54 to 88	3833.13	959.04	
	EPIPYGFLPQNI	Truncated	55 to 66	1387.72	694.37	
	EPIPYGFLPQNIL	Truncated	55 to 67	1500.80	750.91	
	EPIPYGFLPQNILP	Truncated	55 to 68	1597.86	799.44	
163	**YPFVEPI**P	BCM-8	51 to 58	961.50	961.51	Ferranti et al. (2004)
	YPFVEPIPYG	BCM-10	51 to 60	1181.58	1181.59	
	YPFVEPIPYGF	BCM-11	51 to 61	1328.65	1328.66	
	EPIPYGFLPQNILP	Truncated	55 to 68	1597.86	799.43	
190	**YPFVEPI**P	BCM-8	51 to 58	961.50	481.26	Ferranti et al. (2004)
	YPFVEPIPYGF	BCM-11	51 to 61	1328.65	664.83	
	EPIPYGFLPQNIL	Truncated	55 to 67	1500.80	750.91	
	EPIPYGFLPQNILP	Truncated	55 to 68	1597.86	799.43	
254	**YPFVEPI**PY	BCM-9	51 to 59	1124.56	562.79	Wada et al. (2015)
	YPFVEPIPYG	BCM-10	51 to 60	1181.58	591.30	
459	**YPFVEPI**PYG	BCM-10	51 to 60	1181.58	591.30	
	YPFVEPIPYGF	BCM-11	51 to 61	1328.65	664.83	

BCM, beta-casomorphin.
* Preterm breast milk samples. Bold letters indicate the amino acid residues match to the BCM-7 bioactive peptide.

BCM-10 was identified in day 68, 163, 254, and 459 lactation samples. BCM-11 was the most abundant peptide that was released as observed via spectral count. Milk that was collected on day 68 of lactation contained two shortened BCM fragments (55−66 and 55−68), which were missing up to four amino acids at the N-terminus. The total number of BCM peptides that were found in the 90-day lactation sample included three precursors (51-63, 51-65, 51-66, and 7 truncated fragments 53-65, 54-65, 54-68, 54−88, 55−66, 55−67, and 55−68). In the 190-day lactation sample, two shortened peptides (55-67 and 55-68) that were missing the Tyr-Pro-Phe-Val residues were detected. The specific BCM-8, -9, and -11 proteolytic-products tandem mass spectrometry fragmentation spectrum was matched with the synthetically generated human BCM-8, -9, and -11 peptide sequences using LC-MS/MS analysis. Figure 2 shows the LC-MS/MS fragmentation of the single charged BCM-11 peptide sequence YPFVEPIPYGF, that was identified *in vivo* and a good fragmentation that matched with the synthetic counterpart.

Computational prediction of proteases in human milk

The mass spectrometry analysis revealed that milk contains variable lengths of naturally occurring BCMs. To determine the milk proteases that are responsible for the cleavage of BCMs and BCM precursor peptides in milk, we used the online software EnzymePredictor [43]. The results indicate that BCM and BCM precursor peptides were generated by proteases that are known to be present in human milk including, cathepsin D, elastase, plasmin, proline endopeptidase, and thrombin (Fig. 3). Human milk also contains kallikrein, cytosol aminopeptidase, and carboxypeptidase B2 in active form but in our analysis, cleavage sites for kallikrein, cytosol aminopeptidase, and carboxypeptidase B2 were not detected because these enzymes were not present in the EnzymePredictor database.

Discussion

Beta-casein is the major source of naturally occurring bioactive peptides in human milk [8,13,15,16]. Many of these beta-casein-derived, biologically active peptides in human milk are absent in commercially available cow's milk-based infant formula [8,35]. Previous studies suggest that human beta-casein undergoes enzymatic hydrolysis within the mammary gland by plasmin, trypsin, cathepsin D, and elastase [16−18]. Recent human milk studies have characterized the global peptidome and reported on the presence of BCMs in the human milk peptide repertoire [15,35] and their levels during lactation [14]. However, how the BCM family of peptides (e.g., human BCM-4, -5, -7, -8, -9, and -11) are formed throughout human lactation and how the individual BCMs appear relative to one another is unknown. Previous research on the endogenous BCM family peptides in human milk has been limited to the identification of BCM-4 and -5 using chromatography [25]
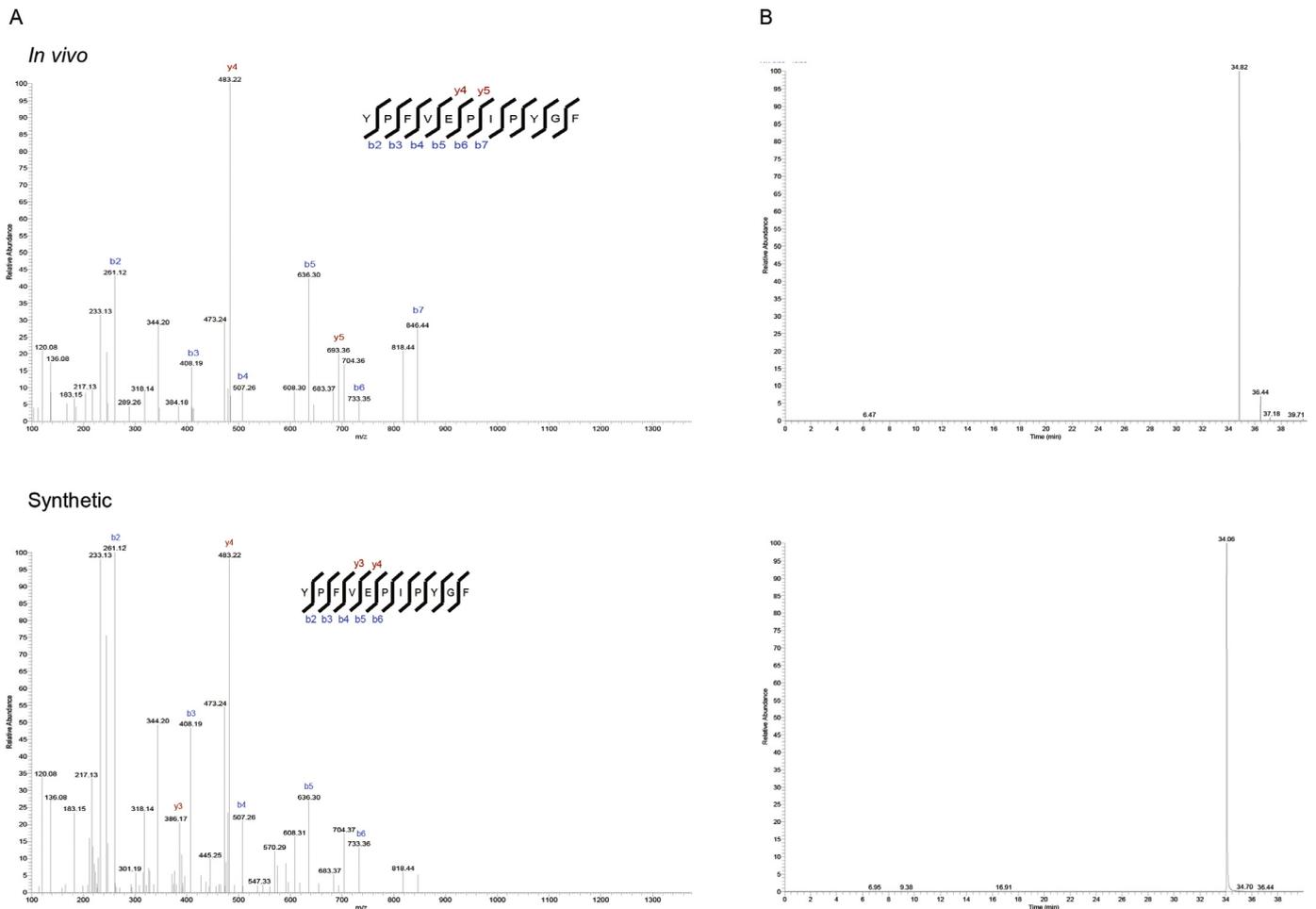
Fig. 2. Mass spectrometry identification and verification of beta-casomorphin (BCM)-11 peptide from human milk. (A) Liquid chromatography coupled with tandem mass spectrometry spectra of a single charged naturally occurring endogenous BCM-11 peptide (YPFVEPIPYGF) from human milk (top) and the synthetic BCM-11 peptide that corresponds to the *in vivo* peptide sequence (bottom). (B) Extracted ion chromatograms of the *in vivo* derived BCM-11 peptide (top) and the synthetic BCM-11 peptide that bears the same peptide sequence (bottom). Spectral differences in endogenous and synthetic BCM peptide spectra may be due to co-elution of peptides in the highly complex milk peptide sample.

and BCM-5 and -7 using high-performance liquid chromatography [14], BCM-7 using enzyme-linked immunosorbent assay [44], BCM-8 using mass spectrometry [24] and radioimmunoassay [31], BCM-9 by mass spectrometry [35], and a few precursor peptides from specific lactation stages or pooled breast milk samples [15,16,24].

In this study, we have identified for the first time that BCM-10 and BCM-11 peptides are naturally present in breast milk before infant digestion. The BCM-10 and -11 may be intact until they reach the infant gut and play a beneficial physiological role in newborns. We have also identified various lengths of BCM peptides in human milk. One BCM precursor peptide with residues Val^{54}-Lys^{88} that was found in this study partially overlaps with the BCM precursor peptide (Ile^{40}-Lys^{88}) that was identified in day 90 breast milk by Guerrero et al. [16].

Our results are consistent with other proteomic studies of BCM release in human milk that identified BCM-8 and -9 in breast milk [24,35]. Ferranti et al. [24] found BCM-8 in intact breast milk and Wada et al. [35] detected BCM-9 in intact breast milk and also after *in vitro* proteolytic digestion and pasteurization. BCM-9 showed resistance during *in vitro* proteolytic digestion and pasteurization [35]. The authors suggested that BCM-9 may exert potent biological activity in the infant gut [35]. The three BCM precursor peptides

(Glu^{35}-Ala^{73}, Pro^{48}-Ala^{73}, and Tyr^{51}-Ala^{73}) in both preterm and term milk from the Ferranti et al. [24] study, matched peptide fragments that were identified in the present study but were truncated (residues Tyr^{51}-Pro^{63}, Tyr^{51}-Asn^{65}, and Tyr^{51}-Ile^{66}). The present study identified BCM peptides that differ from previous studies of human milk peptidomics using mass spectrometry [15,16,24,35]. Although the same analysis method was used, this difference may have resulted from variations in mothers' gestational age, lactation stage, diet, and endogenous proteolytic activity in human milk.

Similar BCM peptides have been identified in beta-casein from bovine milk and milk-based products (e.g., cheese, yoghurt, and infant formulas) that corresponds to residues 60 to 66 [42,44–46]. A significant amount of information is available on the identification and characterization of bovine BCMs and many studies demonstrate activity *in vitro* and in animal models. Biological functions that are associated with bovine BCMs include opioid activity [47,48].

The administration of BCMs in rats [49] and the guinea pig colon [50] showed a reduced gastrointestinal absorption of nutrients and prolonged gastrointestinal transit time. In rats, BCMs have been shown to induce mucin secretion [51]. Trompette et al. [52] found that after absorption, BCMs modulate intestinal mucus discharge through the activation of μ-opioid receptors in rat

Fig 3. Proteases involved in the digestion of beta-casomorphins (BCMs), BCM precursors, and truncated BCM peptides from beta-casein in human milk as predicted by the EnzymePredictor tool. The small blue numbers indicate the amino-acid position in the intact beta-casein of human milk. The BCM strategic-zone amino acids are in bold. The number of times a peptide has been identified in all milk samples is displayed at the right in red.

intestinal cells. BCMs also act as serotonin receptor antagonists [53]. Peritoneal injections of BCM in rats increased the plasma pro-lactin levels [54] and the oral administration of BCMs in dogs stimulated increased postprandial somatostatin [55]. In dogs, the administration of BCMs modulates the release of insulin [56]. Furthermore, BCMs have been shown to affect ion transport [57]. Therefore, BCMs appear to have a wide array of physiological bioactivity across a range of species.

Human BCMs are structurally similar but not identical to their bovine counterparts and share the same first three signature amino-acid residues Tyr-Pro-Phe [42]. Human BCM peptide shares high-sequence homology with bovine milk-derived BCM peptides [27,28]. Therefore, we hypothesize that the identified BCM-8, -9, -10, and -11 as well as the precursor peptides may potentially exert similar bioactive functions. BCMs can be absorbed into the blood and once absorbed, they can reach receptors in the brain and peripheral tissues where they exert calmness and sleeping actions [58]. Other bioactive functions include antioxidative activity for residues human IYPF 50−53 [59,60].

Recently, human beta-casein-derived BCM-7 opioid peptide signaling via μ-opioid receptors has been shown to regulate intestinal gluconeogenesis and satiety [39]. When BCM was shortened at the N-terminus by one, two, or three amino-acid residues, the resultant truncated fragments were reported to exert nonopioid functions such as immunomodulation for the VEPIPY (residues 54-59) peptide [61, 62] and in vitro prolyl endopeptidase inhibition for peptides (residues 52-59, 53-59, 56-59) and may also be involved in the learning and memory processes of infants [63].

Bioactives that were derived from mothers' milk were speculated to contribute to infant behavior because they interact with infant neurobiology [64]. We hypothesize that the identified BCM-8, -9, -10, and -11 as well as the precursors peptides have the first N-terminal essential residues that can trigger biological activity, possibly act on the infant brain by binding to μ-opioid receptors, and modulate infant behavior [65]. BCM-11 was identified in all samples except at day 254 of lactation, which could be due to a variety of reasons including detection limitations, mother's diet, or individual mother variations. We did not quantify the BCM

peptides in human milk and an absolute quantification of identified BCM peptides may provide critical clues related to interactions with μ-opioid receptors in the infant intestinal tract.

Human beta-casein intact protein carries encrypted BCM peptides [9] and, upon exposure to milk-derived proteases, undergoes site-specific hydrolysis of peptide bonds to release functional BCMs and other peptides [16,17,24]. The bioinformatics analysis predicted that the milk-associated proteases (cathepsin D, elastase, plasmin, proline endopeptidase, and thrombin) are most likely involved in the release of BCMs, BCM precursors, and truncated BCM peptides that are derived from the beta-casein in human milk. However, cathepsin D was deemed responsible for most of the cleavage within the BCM region. Elastase (serine protease cleaves Ala, Val, leu, Ile, Gly, and Ser amino acids) is responsible for N- and C-terminal BCM cleavages [66,67]. Plasmin has a preferential cleavage site on the C-terminal side of lysine or arginine amino-acid residues of BCMs [68]. Proline endopeptidase digests at 'Pro' amino acid of the N-terminal of BCMs [17]. The mechanism for human BCMs release by milk proteases before neonatal intake and further degradation into smaller fragments by the neonates' gastric proteases requires further study. A clarification of the BCMs release and function may contribute greatly to our understanding of BCMs bioactivity in newborns.

Reportedly, bovine BCMs can be released during digestion via *Pseudomonas putida* and *Streptococcus faecalis* [69] and *Lactococcus lactis* [70] bacterial enzymes that are found in bovine milk. Human milk also contains microbiota [71,72] such as *Pseudomonas, Staphylococcus, Streptococcus, Lactobacillus*, and *Bifidobacterium* [73,74]. We hypothesize that breast milk-derived bacteria might contribute to the release of BCM peptides.

Beta-casein has an elongated, flexible, and rheomorphic (nonfixed) structure [75], which allows for increased degradation by proteolytic enzymes, and the BCM strategic zone (middle region of beta-casein) to release peptides of various lengths. The longer BCM precursor peptides remain intact until they reach the neonates' gastrointestinal tract to exert their bioactive function. Ferranti et al. [24] suggest that the release of shorter peptides from casein degradation is an advantage for the immature digestive system of

newborns, because peptides in a partially hydrolyzed form can be easily absorbed by newborns. However, further studies are needed to quantify these peptides before and after infant gastric digestion and their localization over time.

Conclusions

Overall, these findings demonstrate that BCM peptide digestion from beta-casein begins within the mammary gland. Using a peptidomics approach, our findings demonstrate that naturally occurring BCM -10 and -11 are present in human breast milk. Future studies will need to examine the potential physiological roles of these BCMs and how they exert their affects in human newborns. These results support the continued investigation into the potential role of these peptides in infant physiology and the way mammary glands process milk.

Acknowledgments

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2018.05.011.

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