Bioactive peptides released from *in vitro* digestion of human milk with or without pasteurization

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BACKGROUND: Pasteurized donor human milk (HM) serves as the best alternative for breast-feeding when availability of mother's milk is limited. Pasteurization is also applied to mother's own milk for very low birth weight infants, who are vulnerable to microbial infection. Whether pasteurization affects protein digestibility and therefore modulates the profile of bioactive peptides released from HM proteins by gastrointestinal digestion, has not been examined to date.

METHODS: HM with and without pasteurization (62.5 °C for 30 min) were subjected to *in vitro* gastrointestinal digestion, followed by peptidomic analysis to compare the formation of bioactive peptides.

RESULTS: Some of the bioactive peptides, such as caseinophosphopeptide homologues, a possible opioid peptide (or propeptide), and an antibacterial peptide, were present in undigested HM and showed resistance to *in vitro* digestion, suggesting that these peptides are likely to exert their bioactivities in the gastrointestinal lumen, or be stably transported to target organs. *In vitro* digestion of HM released a large variety of bioactive peptides such as angiotensin I-converting enzyme-inhibitory, antioxidative, and immunomodulatory peptides. Bioactive peptides were released largely in the same manner with and without pasteurization.

CONCLUSION: Provision of pasteurized HM may be as beneficial as breast-feeding in terms of milk protein-derived bioactive peptides.

Human milk (HM) is highly recommended as the optimal mode of infant feeding for the first several months of life. It serves as a source of nutrients with excellent bioavailability as well as multiple bioactive substances, contributing to growth, development, and health. Recent studies have made it more evident that breast-feeding may reduce the risk of metabolic disease later in life (1–3). When the mother's own milk is limited for several reasons, donor HM is advocated over infant formulas as HM is superior to infant formula in terms of immunoprotection and gastrointestinal development (4). Donor HM is commonly pasteurized at 62.5 °C for 30 min with a holder (i.e., low-temperature long-time

treatment) in order to avoid HM-transmitted bacterial and viral infections (4). Pasteurization is also applied to mother's own milk at some neonatal intensive care units for very low birth weight infants, who are highly vulnerable to cytomegalovirus in HM (5,6). However, this pasteurization may have negative effects on many of the nutrients and bioactive compounds. Pasteurization is accompanied by inactivation of bile salt-stimulated lipase, which may impair absorption of human milk fat and limit infant growth (6). Pasteurization may reduce antioxidants such as ascorbic acid and vitamin E, thereby possibly increasing the susceptibility of the recipient infant to oxidative stress (7). Pasteurization may also reduce immunomodulatory proteins, such as secretory immunoglobulin A and lactoferrin (LF), thereby attenuating the host defense potential (8). Further, metabolic hormones such as adiponectin and insulin also decrease after pasteurization, which may impact metabolic trajectories of infants later in life (9).

Proteins in HM primarily serve as a source of essential amino acids to infants, but they also exert various kinds of bioactivities beneficial to infant health (10). Some of these bioactivities are provided by peptides which are "encrypted" in their parental proteins, and they are exerted only after these peptides are released by the action of gastrointestinal digestive enzymes, membrane peptidases, and/or peptidases in the circulation (11). Although heat treatment is generally considered to affect protein digestibility (12), it is still equivocal whether formation of milk-protein derived bioactive peptides is affected by pasteurization of HM. The aim of this study was to use a peptidomic technique to compare *in vitro* formation of bioactive peptides released from proteins in HM and pasteurized HM. We have previously developed an *in vitro* digestion model, taking into account the somewhat higher stomach pH (4.0) of infants (13). Both HM and pasteurized HM were applied to *in vitro* digestion with porcine pepsin and pancreatin followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis, and the released peptides from major proteins, such as caseins (CNs), α -lactalbumin (α -LA), LF, and osteopontin (OPN), were searched for bioactivities using established databases.

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Received 1 August 2014; accepted 8 October 2014; advance online publication 4 February 2015. doi:10.1038/pr.2015.10

RESULTS

Peptides in undigested HM were analyzed prior to the analysis of digesta as background data (Figure 1), because some of the protein-derived peptides are potentially present in undigested HM (14,15). As can be seen, a large number of peptides were observed. In agreement with previous observations, most of the peptides present in undigested HM were derived from CNs, and no peptide was identified from major whey proteins such as α -LA and LF. The most abundant peptides are derived from β -CN, reflecting its high susceptibility to plasmin-mediated proteolysis as well as the high content of this casein subunit in HM (14,16). Focusing on bioactive peptides in undigested HM, as shown in Table 1 (14,15,17–30), two caseinophosphopeptide homologues, a possible opioid peptide or propeptide, and an antibacterial peptide were identified.

In vitro digestion of HM and pasteurized HM released a multitude of peptides from CNs and whey proteins (Figure 1); both milk samples released peptides largely in the same way as exemplified by β -casein (Figure 2). This similarity also holds true for the patterns of bioactive peptides (Table 1). Notable bioactive peptides, released only after *in vitro* digestion, include angiotensin I-converting inhibitory (ACEi) peptides, antioxidative peptides, and immunomodulatory peptides.

It should be noted that we also extensively searched for bioactive peptides derived from OPN, a multifunctional protein involved in many physiologic processes such as regulation of immune function (31), but no peptide was found that corresponds to any known bioactive peptides (Table 2).

DISCUSSION

Pasteurization is a common procedure for treating donor HM and mother's own milk of very low birth weight infants in order to prevent HM-transmitted microbial infection. However, pasteurization may be accompanied by adverse effects such as reduction in fat absorption, loss of vitamins, and attenuation of bioactive compounds (6,7,9), although it seems equivocal whether these changes are of significance for neonatal health (6,32). Aiming to further characterize "risks and benefits" of

pasteurization, we compared the patterns of bioactive peptides released from HM and pasteurized HM after *in vitro* digestion.

The release of bioactive peptides from major milk proteins after *in vitro* digestion was generally the same for HM and pasteurized HM. We therefore suggest that holder-pasteurization of HM does not affect formation of bioactive peptides to a significant extent, and that the minor differences observed may be attributed to small variations in conditions for *in vitro* digestion. This result may be corroborated by previous studies, including our own, that high-temperature-short-time-pasteurization of cow's milk results in a digestibility similar to that of raw milk (13). It should be noted, though, that the peptidomic technique used in this study has optimum sensitivity to determine peptides with medium length (~5–20 amino acid residues), which encompasses most bioactive peptides reported to date. Detection of shorter peptides is not compatible with this method because these peptides are hardly trapped on the reverse phase column in LC and they give poorer fragmentation in MS/MS (33). Thus, this study inevitably misses the information whether pasteurization of HM affects formation of bioactive peptides with 2–4 amino acid residues. However, there are only a limited number of peptides of this size and few kinds of bioactivities (e.g., ACEi peptides) have been described for them.

Bioactive peptides derived from major human milk proteins are discussed below.

β -CN-Derived Bioactive Peptides

Although the proportion of whey proteins exceeds that of CNs in HM, CNs still constitute a considerable part of proteins and β -CN is dominant among them (16), also serving as the greatest source of bioactive peptides (11).

Four known bioactive peptides (residues 1–18, 1–23, 51–59, and 185–211) were identified in undigested HM, and they were preserved even after *in vitro* digestion of HM and pasteurized HM. Except residues 51–59, the presence of these peptides in undigested HM has been observed previously (14,15). Two peptides of residues 1–18 and 1–23 are considered human counterparts of caseinophosphopeptides because they have the "acid motif" SerP-SerP-SerP-Glu-Glu, essential for the mineral-carrier potential (11). Both of the peptides were confirmed in digested HM and pasteurized HM, suggesting that these peptides would be stable following gastrointestinal digestion and be functional for facilitating mineral uptake. To the best of our knowledge, a peptide of residues 51–59 has not been reported present in undigested HM. This peptide includes the entire sequences of human β -casomorphins (residues 51–54, 51–55, 51–57, and 51–58) (20), as well as it meets the structural requirements to exert such activity, i.e., a tyrosine residue at the N-terminus followed by another aromatic residue at the third or fourth position, possibly exerting multiple functions in neonates such as gastrointestinal function, mucosal development, and sleep induction (11). This longer peptide would be opioidergic, and/or a pro-opioid peptide like bovine β -casomorphins; their longer precursor peptides are considered to be absorbed and then they are excised in the blood

Figure 1. Number of peptides derived from major human milk (HM) proteins. Exclusive peptide counts from β -casein (β -CN), α_{s1} -CN, κ -CN, α -lactalbumin (α -LA), lactoferrin (LF), and osteopontin (OPN) are shown. Open bars, bars in gray, and closed bars indicate counts in undigested HM, digested HM, and digested pasteurized HM, respectively.

Table 1 Bioactive peptides released from proteins in human milk and pasteurized human milk

Parental protein	Residue	Amino acid sequence ^a	HM	HM	Past HM	Function ^b	Reference
			Undig	Dig	Dig		
β-CN	1–18	RETIESL SS EEESITEYK	Y	Y	Y	CPP homologue (1–18)	14
	1–23	RETIESL SS EEESITEYKQKVEK	Y	Y	Y	CPP homologue (1–23)	14
	48–52	PLIYP	N	Y	N	ACEi (48–52)	17
	50–53	IYPF	N	Y	Y	AO and PEPi (50–53)	18,19
	50–58	IYPFVEPIP	N	Y	Y	PEPi (50–58) and OP (51–58, etc.)	19,20
	51–59	YPFVEPIPY	Y	Y	Y	PEPi (50–58) and OP (51–58, etc.)	19,20
	52–59	PFVEPIPY	N	Y	Y	PEPi (52–59)	19
	53–59	FVEPIPY	N	N	Y	PEPi (53–59) and Immunomodulatory (54–59)	19,21
	54–59	VEPIPY	N	N	Y	Immunomodulatory (54–59)	21
	54–60	VEPIPYG	N	N	Y	Immunomodulatory (54–59)	21
	56–59	PIPY	N	Y	Y	PEPi (56–59)	19
	105–117	SPTIPFFDPQIPK	N	Y	Y	Immunomodulatory (105–117)	15
	121–131	<i>LENLHLPLPL</i>	N	Y	Y	PEPi (122–131) and ACEi (125–129, etc.)	17,22,23
	122–129	ENLHLPLP	N	Y	Y	ACEi (122–129)	17,22
	122–131	ENLHLPLPL	N	Y	Y	PEPi (122–131) and ACEi (125–129, etc.)	17,22,23
	123–132	<i>NLHLPLPLQ</i>	N	Y	Y	PEPi (122–131) and ACEi (125–129, etc.)	17,22,23
	125–130	HLPLPL	N	N	Y	ACEi (125–129) and PEPi (125–130)	19,22
	154–160	WSVPQPK	N	Y	Y	AO (154–160)	18
	167–173	QVVPYPQ	N	Y	Y	AO (167–173)	18
	185–211	QELLNPTHQIYPVTQPLAPVHNPISV	Y	N	Y	Antibacterial (185–211)	24
	206–209	HNPI	N	Y	N	AO (206–209)	25
α _{s1} -CN	5–11	<i>PLRYPER</i>	N	Y	N	ACEi (8–11)	26
	136–142	<i>YYPQIMQ</i>	N	Y	Y	ACEi (136–143)	26
	160–170	<i>NYEKNVMLQW</i>	N	Y	N	ACEi (164–170)	26
κ-CN	100–103	<i>IPPK</i>	N	Y	Y	Antithrombotic (101–103)	27
	101–104	<i>PPKK</i>	N	Y	Y	Antithrombotic (101–103)	27
α-LA	51–59	<i>GLFQISNK</i>	N	Y	Y	Immunomodulatory (51–53)	21
	104–108	WLAHK	N	Y	Y	ACEi (104–108)	28
LF	566–573	<i>DLKLADFA</i>	N	Y	Y	ACEi (567–569) and AO (570–572)	29,30
	566–574	<i>DLKLADFAL</i>	N	Y	N	ACEi (567–569) and 572–574) and AO (570–572)	29,30
	567–572	<i>LKLADF</i>	N	Y	Y	ACEi (567–569) and AO (570–572)	29,30

^aIn the amino acid sequence column, peptides in italic indicate possible precursors for bioactive peptides, which include bioactive sequences. As for the amino acid sequences of caseinophosphopeptide (CPP) homologues, letters underscored indicate phosphorylated sites, and bold letters represent the "acid motif."^bIn the function column, the abbreviations ACEi, AO, PEPi, and OP denote angiotensin I-converting enzyme-inhibitory, antioxidative, prolyl endopeptidase-inhibitory, and opioid peptide, respectively. Dig, digested; HM, human milk; Past, pasteurized; Undig, undigested.

circulation. It should be added that this peptide also inhibits prolyl endopeptidase (PEP) *in vitro* and is possibly involved in learning and memory processes (19), although its role in neonatal physiology has not been studied. While the peptide of residues 51–59 was still present after *in vitro* digestion of HM and pasteurized HM, shorter peptides such as an immunomodulatory peptide (residues 54–59) (21) and PEP-inhibitory

(PEPi) peptides (residues 52–59, 53–59, and 56–59) (19) were identified only after digestion. Thus, gastrointestinal digestion would be required for the formation of these shorter bioactive peptide and they would be released from intact β-CN as well as the precursor peptide (residues 51–59). The last bioactive peptide in undigested HM was a peptide of residues 185–211, known as an antibacterial peptide (24). This peptide was also

Figure 2. Digestive patterns for β -casein (β -CN) in human milk (HM) and pasteurized HM. Sequences of β -CN-derived peptides released from HM and pasteurized HM were compared after *in vitro* digestion. The top line bar with numbers represents the whole β -CN sequence with amino acid residue numbers. Bars in light gray, medium gray, and dark gray indicate peptides specific to HM, peptides in both HM and pasteurized HM, and peptides specific to pasteurized HM, respectively.

identified after digestion of pasteurized HM (though not observed in digested HM), suggesting that this peptide should be considerably resistant to gastrointestinal digestion, and possibly being involved in prevention of infection in the gastrointestinal tract.

In vitro digestion released a multitude of bioactive peptides from β -CN, and one of the noteworthy regions may be residues 121–132. This region provides a PEPi peptide, previously investigated as an anticancer peptide (residues 122–131) (23), an ACEi peptide (residues 122–129) (17) and another PEPi peptide, studied in the context of learning and memory processes (residues 125–130) (19). While their roles *in vivo* have scarcely been investigated, all these peptides would also be precursors for a potent ACEi peptide of residues 125–129 (HLPLP) (22). A Caco-2 cell study has demonstrated that a longer peptide (residues 124–129) is hydrolyzed to residues 125–129 in the course of transport across the intestinal epithelium, and the residues 125–129 exhibited high resistance to proteolysis in human plasma (34). Despite the limitation in our knowledge of their mechanism of action, milk protein-derived ACEi peptides as well as their precursors may be involved in the modulation of cardiovascular disease later in life (11). Other notable bioactive peptides are antioxidative, such as residues 50–53, 154–160, 167–173, and 206–209 (18,25). Although they have

rarely been investigated *in vivo*, they may protect newborn infants from diseases mediated by oxidative stress.

α_{s1} -CN-Derived Bioactive Peptides

While bovine α_{s1} -CN constitutes a majority of bovine milk proteins, the amount of the human counterpart is very small (16), and bioactive peptides derived from human α_{s1} -CN have received limited attention. Formation of three ACEi peptides has been reported after *in vitro* tryptic digestion of recombinant α_{s1} -CN (residues 8–11, 136–143, and 164–170) (26), and we identified two longer and one shorter sequences including/overlapping the above-mentioned ACEi peptides in digested HM and pasteurized HM (residues 5–11, 136–142, and 160–170). These peptides may thus serve as ACEi peptides and/or propeptides.

κ -CN-Derived Bioactive Peptides

κ -CN, a minor CN subunit, serves to stabilize casein micelles in milk because of its highly glycosylated C-terminus, and this glycosylated region also functions as a prebiotic and an inhibitor of infection (11). While we identified no notable fragment derived from κ -CN that completely matches known bioactive peptides, both HM and pasteurized HM released two peptides of residues 100–103 and 101–104, which are longer than an antithrombotic peptide (PPK) by one amino acid residue (27).

Table 2. Peptide sequences derived from osteopontin

Residue	Amino acid sequence	HM	HM	Past HM
		Undig	Dig	Dig
1–26	IPVKQADSGSSEKQLYNKYPDAVAT	Y	Y	Y
3–26	VKQADSGssEEKQLYNKYPDAVAT	Y	N	N
24–28	VATWLNPDPSQKQNL	N	Y	Y
26–35	TWLNPDPSQK	N	Y	Y
26–37	TWLNPDPSQKQN	N	Y	Y
26–38	TWLNPDPSQKQNL	N	Y	Y
27–35	WLNPDPSQK	N	Y	Y
27–36	WLNPDPSQKQ	N	Y	Y
27–37	WLNPDPSQKQN	N	Y	Y
27–38	WLNPDPSQKQNL	N	Y	Y
27–40	LNPDPSPQKQNL	N	Y	Y
27–42	WLNPDPSQKQNLAPQ	N	Y	Y
28–38	LNPDPSPQKQNL	N	Y	Y
153–170	SKSKFRRPDIQYPDATD	Y	N	N
153–176	SKSKFRRPDIQYPDATDEDITSH	Y	N	N
155–170	SKKFRRPDIQYPDATD	Y	N	N
157–170	KFRRPDIQYPDATD	Y	N	N
157–176	KFRRPDIQYPDATDEDITSH	N	Y	Y
157–185	KFRRPDIQYPDATDEDITSHmESEELNGA	N	Y	Y
157–186	KFRRPDIQYPDATDEDITSHmEsEELNGAY	Y	N	N
158–170	FRRPDIQYPDATD	Y	N	N
158–171	FRRPDIQYPDATDE	N	Y	Y
158–176	FRRPDIQYPDATDEDITSH	N	Y	Y
158–177	FRRPDIQYPDATDEDITSHm	N	Y	Y
158–185	FRRPDIQYPDATDEDITSHmEsEELNGA	N	Y	Y
158–186	FRRPDIQYPDATDEDITSHmESEELNGAY	Y	N	N
159–170	RRPDIQYPDATD	Y	N	N
159–174	RRPDIQYPDATDEDIT	Y	N	N
159–176	RRPDIQYPDATDEDITSH	Y	Y	Y
159–177	RRPDIQYPDATDEDITSHm	N	Y	N
159–184	RRPDIQYPDATDEDITSHmEsEELNG	Y	N	N
159–185	RRPDIQYPDATDEDITSHmESEELNGA	Y	N	N
159–186	RRPDIQYPDATDEDITSHmEsEELNGAY	Y	N	N
159–187	RRPDIQYPDATDEDITSHmESEELNGAYK	Y	N	N
160–166	RPDIQYP	Y	N	N
160–168	RPDIQYPDA	Y	N	N
160–170	RPDIQYPDATD	Y	N	N
160–171	RPDIQYPDATDE	N	Y	Y
160–173	RPDIQYPDATDEDI	Y	N	N
160–174	RPDIQYPDATDEDIT	Y	N	N
160–175	RPDIQYPDATDEDITS	Y	N	N
160–176	RPDIQYPDATDEDITSH	Y	Y	Y
160–177	RPDIQYPDATDEDITSHm	N	Y	Y
160–185	RPDIQYPDATDEDITSHmEsEELNGA	Y	Y	Y
160–186	RPDIQYPDATDEDITSHmEsEELNGAY	Y	Y	Y
160–187	RPDIQYPDATDEDITSHmEsEELNGAYK	Y	N	N
160–190	RPDIQYPDATDEDITSHmESEELNGAYKAIP	Y	N	N
161–176	PDIQYPDATDEDITSH	Y	N	N
161–187	PDIQYPDATDEDITSHmEsEELNGAYK	Y	N	N
162–176	DIQYPDATDEDITSH	Y	N	N

Table 2. Continued on next page

Table 2. Continued

Residue	Amino acid sequence	HM	HM	Past HM
		Undig	Dig	Dig
162–177	DIQYPDATDEDITSHm	Y	N	N
162–187	DIQYPDATDEDITSHmESEELNGAYK	Y	N	N
163–176	IQYPDATDEDITSH	Y	N	N
163–187	IQYPDATDEDITSHmESEELNGAYK	Y	N	N
164–176	QYPDATDEDITSH	Y	N	N
170–186	DEDITSHmESEELNGAY	Y	N	N
170–187	DEDITSHmEsEELNGAYK	Y	N	N
171–187	EDITSHmESEELNGAYK	Y	N	N
172–186	DITSHmESEELNGAY	Y	N	N
172–187	DITSHmESEELNGAYK	Y	N	N
183–200	NGAYKAIPVAQDLNAPSD	Y	N	N
184–200	GAYKAIPVAQDLNAPSD	Y	N	N
186–193	YKAIPVAQ	N	Y	Y
186–194	YKAIPVAQD	N	Y	Y
186–194	YKAIPVAQDLNA	N	Y	Y
186–204	YKAIPVAQDLNAPSDWDSR	N	Y	Y
186–214	YKAIPVAQDLNAPSDWDSRGKDSYETSQ	N	Y	Y
187–204	KAIPVAQDLNAPSDWDSR	N	Y	Y
187–214	KAIPVAQDLNAPSDWDsRGKDsYETSQ	N	Y	Y
188–204	AIPVAQDLNAPSDWDSR	N	Y	Y
188–213	AIPVAQDLNAPSDWDSRGKDSYETSQ	N	Y	Y
189–199	IPVAQDLNAPS	Y	N	N
189–200	IPVAQDLNAPSD	Y	N	N
190–200	PVAQDLNAPSD	Y	N	N
190–216	PVAQDLNAPSDwDSRGKDSYETSQ	N	Y	Y
194–204	DLNAPSDWDSR	N	Y	Y
200–230	DWDsRGKDsYETSQ	Y	N	N
205–230	GKDsYETSQ	Y	N	N
208–230	SYETsQLDDQsAETHSHKQSRLY	Y	N	N
209–230	YETSQ	Y	N	N
210–230	ETSQ	Y	N	N
213–230	QLDDQsAETHSHKQSRLY	Y	N	N
215–230	DDQsAETHSHKQSRLY	Y	N	N
216–230	DQsAETHSHKQSRLY	Y	N	N
232–254	RKANDEsNEHSDVIDsQELSKVS	Y	N	N
233–254	KANDEsNEHSDVIDsQELSKVS	Y	N	N
235–254	NDESNEHSDVIDsQELSKVS	Y	N	N
236–251	DESNEHSDVIDsQELS	Y	N	N
358–274	HsHEFHsHEDmLVVDPK	Y	N	N
268–279	mLVVDPKSKEED	Y	N	N
269–282	LVVDPKSKEEDKHL	N	N	Y
269–283	LVVDPKsKEEDKHLK	N	Y	Y
275–287	SKEEDKHLKFRIS	Y	N	N
275–298	SKEEDKHLKFRISHELDSASsEVN	Y	N	N
277–287	EEDKHLKFRIS	Y	N	N
284–298	FRISHELDSASsEVN	Y	Y	Y
285–298	RISHELDSASsEVN	Y	Y	Y
286–298	ISHELDSASsEVN	Y	Y	N
286–298	ISHELDSASsEVN	Y	N	N
287–298	SHELDSASsEVN	Y	N	N

Modifications of peptide side chains such as phosphorylation are omitted here.
Dig, digested; HM, human milk; Past, pasteurized; Undig, undigested.

The peptide MAIPPK derived from bovine κ -CN also contains PPK and exerts antithrombotic activity (27), and it is therefore plausible that the above two peptides would also be antithrombotic, possibly involved in modulating risk of cardiovascular disease later in life.

α -LA-Derived Bioactive Peptides

Whey protein comprises ~60% of human milk protein, and the major component is α -lactalbumin (16). α -Lactalbumin is a rich source of essential amino acids and of central importance to the rapidly growing neonate (35), and it has also been reported to provide various bioactive peptides (11). *In vitro* digestion of HM and pasteurized HM released an ACEi peptide (residues 104–108) (28). The formation of this fragment has also been confirmed by *in vitro* digestion of a standard infant formula and of a protein hydrolysate formula (Y. Wada and B. Lönnerdal, unpublished data), suggesting that considerable resistance to proteolysis may guarantee its functionality *in vivo*.

LF-Derived Bioactive Peptides

LF is another abundant whey protein in HM (16). This protein possesses several health promoting functions, and it also encrypts unique bioactive peptides, such as lactoferricin and prebiotic peptides (11). However, these peptides were not detected in this study, presumably due to presence of disulfide bonds within these peptides, making it difficult to identify them with this analytic method. Although the three peptides of residues 566–573, 566–574, and 567–572 contain ACEi peptides (residues 567–569 and 572–574) and an antioxidative peptide (residues 570–572), which was originally identified from sardine and soybean protein (29,30), information about these peptides is scarce and it is therefore uncertain whether they may be active *in vivo*.

OPN-Derived Peptides

OPN is a heavily glycosylated and phosphorylated protein and is also present in a relatively high concentration in human milk (16,36). It is a multifunctional protein involved in many physiologic processes such as regulation of immune function (31). However, bioactive peptides derived from it have not been investigated to date, and no peptide was found that corresponds to any known bioactive peptides.

Peptides were present in undigested HM, and most of them are vulnerable to proteolysis as they disappeared after *in vitro* digestion. Other peptides were released after *in vitro* digestion and showed quite similar profiles in HM and pasteurized HM. Interestingly, there was no peptide identified which was released from residues 29–153, and Dallas *et al.* also reported a similar observation (15). One possible explanation can be attributed to five glycosylation sites of OPN in human milk, all of which are located in residues 29–153 (36); glycosylated peptides are possibly released but lack of information regarding exact oligosaccharide structures makes them impossible to be identified by this peptidomic method. As OPN isoforms seem to exert different bioactivities depending on their glycosylation moieties (R. Jiang and B. Lönnerdal, unpublished data),

structural elucidation would be warranted for identifying and characterizing novel glycopeptides from OPN. It is also probable that glycosylation moieties may block the access of digestive enzymes, thereby hindering the release of peptides.

In conclusion, pasteurized HM may be as beneficial as breastfeeding in terms of providing bioactive peptides, such as ACEi, antibacterial, caseinophosphopeptides, and immunomodulatory peptides as discussed above. These peptides may contribute to the growth, development and health of neonates. However, *in vivo* investigations will be required to substantiate the above conclusions, as well as to verify which of the observed bioactive peptides are of biological significance for infants.

METHODS

Samples

This study procedure was approved by the Institutional Review Board at the University of California, Davis. HM samples were obtained from a volunteer mother, from whom informed consent was obtained, at different lactation days and were pooled. Part of the sample was pasteurized at 62.5 °C for 30 min in a water bath. Both HM and pasteurized HM were stored at –20 °C until further analysis.

In Vitro Digestion of Milk Samples

Milk samples were skimmed twice by centrifugation at 8,500g for 30 min and then subjected to *in vitro* digestion (13). After protein concentration was determined by the Bradford method (37), simulated gastric digestion was started with adjusting the pH to 4.0 (mimicking the stomach pH in infants) with 1 mol/l HCl. Porcine pepsin (Sigma-Aldrich, St Louis, MO; 2% in 1 mmol/l HCl) was added to the sample in a 1:12.5 ratio (pepsin:protein). Samples were placed in an incubating shaker (New Brunswick Scientific, Edison, NJ) at 140 rpm at 37 °C for 15 min. Then, after the pH of the samples was adjusted to 7.0 with 0.1 mol/l NaHCO₃, simulated intestinal digestion was performed. Pancreatin (Sigma-Aldrich; 0.4% in 0.1 mol/l NaHCO₃) was added to the samples in a 1:62.5 ratio (pancreatin:protein), and the samples were placed in the incubator shaker at 140 rpm at 37 °C for 5 min. After the incubation, the enzymes were inactivated in a water bath at 85 °C for 3 min.

Peptidomic Analysis

Peptidomic analysis was conducted at the Proteomic Core Facility at the University of California, Davis, using a similar method as described previously (13). Undigested HM, digested HM, and digested pasteurized HM samples were spun with a 10 kDa filter (Millipore, Bedford, MA). The collected filtrate was subject to solid phase extraction using Aspire Chromatography Tips (Thermo Scientific, San Jose, CA). The desalted peptides were reconstituted in 2% acetonitrile/0.1% trifluoroacetic acid and 10 μ l of each sample was loaded onto a 100 μ m $\times$ 25 mm Magic C18 100Å 5U reverse phase trap before being separated on a 75 μ m $\times$ 150 mm Magic C18 200Å 3U reverse phase column. Peptides were eluted using a gradient of buffer A (0.1% formic acid) and buffer B (100% acetonitrile) with a flow rate of 300 nl/min. A 60 min gradient was run with 5–35% B over 45 min, 35–80% B over 5 min, 80% B for 1 min, 80–5% B over 1 min, and held at 5% B for 8 min. Tandem mass spectra were extracted and charge states were deconvoluted and deisotoped. All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version CYCLONE (2013.02.01.2)). X! Tandem was set up to search the Human reference database (May 2013; 20,252 entries) plus an equal number of reverse sequences and 60 common laboratory contaminant proteins, assuming a non-specific digestion enzyme. X! Tandem was searched with a fragment ion mass tolerance of 20 ppm and a parent ion tolerance of 20 ppm. Glu->pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, gln->pyro-Glu of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine and tryptophan, and dioxidation of methionine and tryptophan were specified in X! Tandem as variable modifications. Scaffold (version Scaffold_4.2.1, Proteome Software, Portland,

OR) and SEQUEST (Proteome Discoverer 1.1; Thermo Scientific) were used to validate MS/MS based peptide and protein identifications with the criteria as described previously (38–41). Peptides derived from major HM proteins were compared with the database of BIOPEP (42), and the search results were further corroborated by browsing reference articles.

ACKNOWLEDGMENTS

We are grateful to Brett Phinney and Darren Weber, University of California, Davis, for performing the proteomic analysis.

Disclosure: The authors declare no conflict of interest.

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