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Bioactive peptides released by *in vitro* digestion of standard and hydrolyzed infant formulas

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ABSTRACT

Hydrolyzed infant formulas serve as appropriate nutritional sources for infants afflicted with cow's milk allergy, and milk proteins in hydrolyzed formulas are industrially hydrolyzed extensively or partially. To investigate whether industrial hydrolysis may modulate the digestive trajectory of milk proteins, thereby releasing different profiles of bioactive peptides compared with standard formulas, both standard and hydrolyzed formulas were subjected to *in vitro* digestion and formation of bioactive peptides were compared. One standard, one extensively hydrolyzed, and one partially hydrolyzed infant formula were digested *in vitro* with pepsin and pancreatin, taking into account the higher gastric pH of infants, and the digesta were subjected to peptidomic analysis. The standard formula released a larger variety of bioactive peptides than from the hydrolyzed formulas, indicating that industrial hydrolysis of milk proteins may generally attenuate their indigenous bioactivities such as antibacterial, immuno-regulatory, and anti-oxidative activities. Conversely, industrial hydrolysis may facilitate the formation of bioactive peptides from hydrophobic proteins/regions such as β-LG and the "strategic zone" of β-CN, which encrypt bioactive peptides including a dipeptidyl dipeptidase-4-inhibitory, hypocholesterolemic, and opioid peptides. Infants fed hydrolyzed infant formulas may be influenced by milk protein-derived bioactive peptides in a manner different from those fed standard formula.

1. Introduction

Infant formulas (IFs) are frequently used when availability of mother's milk is limited. Most IFs are formulated using cow's milk protein because of its high nutritional value. However, for infants allergic to cow's milk protein, hydrolyzed IFs (HIFs) are used instead of standard IF [1]. Furthermore, there is increased use of HIFs for infants without allergic issues, as treatment of non-specific gastrointestinal problems as well as for prophylaxis of milk protein allergy [2]. Milk proteins in HIFs are hydrolyzed extensively or partially using industrial proteases such as alcalase, pronase, and papain, as well as gastrointestinal digestive enzymes such as pepsin, trypsin and chymotrypsin [3]. Industrial proteases may hydrolyze milk proteins in a way different from gastrointestinal digestive enzymes. The degree of hydrolysis and the composition of peptides formed also depends upon the digestive conditions such as temperature, digestion time, enzyme/protein ratio, *etc* [3]. Thus, industrial hydrolysis may modify the digestive trajectory of milk proteins in the gastrointestinal tract in different ways. As milk proteins 'encrypt' a variety of bioactive peptides [4], it is likely that infants fed HIFs will be influenced by milk protein-derived bioactive peptides differently from those fed standard IFs. To the best of our knowledge, there are no studies comprehensively comparing gastrointestinal release of bioactive peptides from standard IFs and HIFs.

The objective of this study was to compare the release of bioactive peptides after *in vitro* gastrointestinal digestion of 3 types of infant formulas, using the same peptidomic technique as we recently reported for bioactive peptides released from human milk (HM) [5]. A standard formula, an extensively hydrolyzed casein (CN) formula, and a partially hydrolyzed whey protein (WP) formula were subjected to *in vitro* digestion, taking into account the higher gastric pH of infants, using porcine pepsin and pancreatin, followed by liquid chromatography conjugated with tandem mass spectrometry (MS), and released bioactive peptides from major milk proteins, such as α-lactalbumin (α-LA), β-lactoglobulin (β-LG), and CNs, were probed using established databases.

Abbreviations: IF, infant formula; HIF, hydrolyzed IF; HM, human milk; CN, casein; WP, whey protein; MS, mass spectrometry; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; eHIF, extensively-hydrolyzed IF; pHIF, partially-hydrolyzed IF; ACE, angiotensin I-converting enzyme; DPP4, dipeptidyl dipeptidase-4.

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Table 1
Bioactive peptides present in undigested hydrolyzed infant formulas.

Parental protein	Residue	Amino acid sequence[a]	eHIF[b]	pHIF[b]	Function[c]	Ref.
α-LA	75–80	(ISCDKF)	N	(Y)	Antibacterial (61–68/75–80)	[14]
	104–108	WLAHK	N	Y	ACEi (104–108)	[15]
β-LG	15–20	VAGTWY	N	Y	Antibacterial (15–20)	[14]
	78–82	IPAVF	N	Y	DPP4i (78–82)	[14]
	78–83	IPAVFK	N	(Y)	Antibacterial (78–83)	[14]
	92–100	VLVLDTDYK	N	Y	Antibacterial (92–100)	[14]
	102–105	YLLF	N	Y	OP (102–105)	[14]
	142–148	ALPMHIR	N	Y	ACEi (142–148)	[16]
$_{\alpha S1}$-CN	91–97	YLGYLEQ	N	(Y)	Anxiolytic (91–97)	[17]
	91–100	YLGYLEQLLR	N	(Y)	Anxiolytic (91–97, 91–100)	[17]
$_{\alpha S2}$-CN	174–179	FALPQY	N	(Y)	ACEi (174–179 or 181), AO (174–181)	[18,19]
	174–181	FALPQYLK	N	(Y)	ACEi, AO (174–181)	[18,19]
β-CN	1–25	RELEELNVPGEIVESL**SSSEE**SITR	N	(Y)	CPP (1–25)	[20]
	59–66	VYPFPGPI	N	Y	PEPi (59–66)	[21]
	59–67	VYPFPGPIP	N	Y	PEPi (59-66 or 67)	[21]
	59–68	VYPFPGPIPN	N	Y	ACEi (59–68), PEPi (59–66 or 67)	[16,21]
	60–63	YPFP	Y	N	OP (60–63, 64, 65, 66, 67, or 70)	[22]
	60–65	YPFPGP	Y	N	OP (60–63, 64, 65, 66, 67, or 70)	[22]
	60–68	(YPFPGPIPN)	N	Y	OP (60–63, 64, 65, 66, 67, or 70)	[22]
	63–68	PGPIPN	Y	Y	Immuno-regulatory (63–68)	[23]
	132–139	(NLHLPLPL)	N	(Y)	ACEi (133, or 134–138), PEPi (134–139)	[21,24]
	132–140	NLHLPLPLL	N	Y	ACEi (133, or 134–138), PEPi (132-140, 134–139)	[21,24,25]
	133–139	(LHLPLPL)	N	Y	ACEi (133, or 134–138), PEPi (134–139)	[21,24]
	134–138	HLPLP	Y	N	ACEi (134–138)	[24]
	134–139	HLPLPL	N	Y	ACEi (134–138), PEPi (134–139)	[21,24]
	170–176	VLPVPQK	N	Y	AO (170–176)	[26]
	177–183	AVPYPQR	N	(Y)	AO (177–183)	[26]
	193–201	(YQEPVLGPV)	Y	N	AO (193–196)	[27]
	193–202	(YQEPVLGPVR)	N	Y	AO (193–196)	[27]

[a] Peptides in parentheses indicate possible precursors for bioactive peptides, which include bioactive sequences. As for the amino acid sequences of caseinophosphopeptide (CPP), letters underscored indicate phosphorylated sites, and bold letters represent the "acid motif".
[b] The abbreviations eHIF and pHIF denote extensively hydrolyzed infant formula and partially hydrolyzed infant formula, respectively. "Y"s in parentheses show the bioactive peptides/precursors that were not detected after *in vitro* digestion.
[c] The abbreviations ACEi, AO, CPP, DPP4i, OP, and PEPi denote angiotensin I-converting enzyme-inhibitory, anti-oxidative, caseinophosphopeptide, dipeptidyl peptidase-4-inhibitory, opioid peptide, and prolyl endopeptidase-inhibitory, respectively.

2. Materials and methods

2.1. Samples

A standard IF (CN + WP-based, Similac Advance; Abbott Nutrition, Abbott Park, IL), an extensively hydrolyzed CN-based IF (Similac Expert Care Alimentum; Abbott Nutrition), and a partially hydrolyzed WP-based IF (Gerber Good Start Gentle; Nestlé Infant Nutrition, Florham Park, NJ) were purchased at a local supermarket. They were designated as standard IF, eHIF, and pHIF, respectively.

2.2. In vitro *digestion of formula samples*

Formula samples were subjected to *in vitro* digestion as reported previously [5]. Conditions for the *in vitro* digestion in general [6], digestion time [7], and pH of the gastric digestion [7–11], were reviewed carefully and selected accordingly. Shorter digestion times were chosen here than we routinely employ to investigate protein digestibility [12], aiming to obtain the profile of bioactive peptides present in the mid-course of gastrointestinal digestion.

Powdered standard IF, eHIF, and pHIF were reconstituted with Milli-Q water (Millipore, Billerica, MA), with reference to instructions on product labels. Reconstituted formulas were centrifuged at 8500 g for 30 min twice, and the upper fat layer was removed (we have previously confirmed that no protein loss is accompanied by this step [12]). The skimmed samples were subjected to *in vitro* digestion. Simulated gastric digestion was started by adjusting the pH to 4.0 (mimicking the stomach pH in infants) with 1 M HCl. Porcine pepsin (Sigma–Aldrich, St. Louis, MO; 2% in 1 mM 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 $NaHCO_3$) 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.

2.3. Peptidomic analysis

Undigested eHIF and pHIF, and the 3 digested IFs were subjected to peptidomic analysis as described previously [5]. Briefly, undigested eHIF & pHIF, and the 3 digested formulas were spun with a 10 kDa filter (Millipore). The collected filtrates were subjected 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 each sample was loaded onto a 100 μm × 25 mm Magic C18 100 Å 5U reverse phase trap before being separated on a 75 μm × 150 mm Magic C18 200 Å 3U reverse phase column. Peptides were eluted using a gradient of 0.1% formic acid and 100% acetonitrile. 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 Uniprot Bos taurus reference database (June, 2013; 23860 entries) plus an equal number of reverse sequences and 60 common laboratory contaminant proteins, assuming a non-specific digestion enzyme. Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) and SEQUEST (Proteome Discoverer 1.1; Thermo Scientific) were used to validate MS/MS based peptide and protein identifications. Peptides derived from major bovine milk proteins of interest were compared with the database of BIOPEP [13], and the search results were further corroborated by browsing reference articles.