REVIEW PAPER

On the trail of milk bioactive peptides in human and animal intestinal tracts during digestion: A review

Rachel Boutrou · Gwénaële Henry · Laura Sanchez-Rivera

Abstract Digestion of proteins leads to the release of numerous peptides in the gastrointestinal tract, among them several bioactive peptides. This review compiles, organises and critically analyses the information available on bioactive peptides present in vivo in the digestive tract of adult humans and animals. It focuses on milk proteins, which are recognised as one of the main sources of bioactive peptides with different biological functions. To date, few studies have been performed on the presence of milk-derived peptides in the intestinal tract in vivo. The diversity of these studies is remarkable in terms of the milk products that have been tested, the animal models selected and the segment of the digestive tract considered. Altogether, 69 bioactive peptides identified in vivo are reviewed. Most of them are released from casein, and especially from β-casein, to which 38 bioactive peptides are attributed. These sequences can be classified mainly as angiotensin-converting enzyme (ACE) inhibitors, opioids and antimicrobial peptides. Particular attention is paid to β-casomorphins and caseinomacropeptide-derived peptides. The function of caseinophosphopeptides as a mineral carrier is also discussed. Few bioactive peptides derive from whey proteins.

Keywords Milk · Bioactive peptides · Intestinal tract · In vivo

R. Boutrou (✉) · G. Henry
INRA, UMR1253, Science et Technologie du Lait et de l'Œuf, 35042 Rennes, France
e-mail: rachel.boutrou@rennes.inra.fr

R. Boutrou · G. Henry
AGROCAMPUS OUEST, UMR1253, Science et Technologie du Lait et de l'Œuf, 35042 Rennes, France

L. Sanchez-Rivera
Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), C/ Nicolas Cabrera 9, Campus de Cantoblanco de la Universidad Autónoma de Madrid, 28049 Madrid, Spain

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1 Introduction

Numerous peptides are usually found in the gastrointestinal tract (GIT). Some derive from food-protein digestion while others are released from intestinal cells. Among the food-derived peptides are biologically active peptides, commonly called bioactive peptides. These peptides have been defined as specific protein fragments that have a positive impact on body functions and may ultimately influence health (Kitts and Weiler 2003). Upon oral administration, bioactive peptides may exert an effect on the major body systems, namely the cardiovascular, digestive, immune and nervous systems.

Much effort has been devoted to evaluating in vivo effects produced by milk proteins and/or peptides (Fitzgerald et al. 2004; Jäkälä and Vapaatalo 2010; Phelan and Kerins 2011; Ramabadran and Bansinath 1989; Ricci-Cabello et al. 2012; Rutherfurd-Markwick 2012). These studies considered the ingestion of proteins and peptides and either subsequent health effects or the presence of the bioactive peptide in the blood. Few studies have focused on the presence of bioactive peptides in the digestive tract. However, to understand how food provides health benefits, it is necessary to know how food is processed throughout digestion and/or how the peptides consumed are preserved from the action of digestive enzymes. This review focuses on bioactive peptides that have been identified in the digestive tract of adult humans and animals, since digestion, the first process that ingested food undergoes, determines their formation from food proteins and their fate.

A wide range of food-derived peptides with a variety of physiological functions has been discovered over recent decades. In particular, milk represents one of the main sources of bioactive peptides. Their beneficial health effects may be classified as antimicrobial, antioxidative, antithrombotic, antihypertensive or immunomodulatory (Choi et al. 2012; Korhonen and Pihlanto 2006). In this review, milk-derived bioactive peptides present in vivo in the GIT were considered in order to provide information about their release and stability throughout digestion. The main difficulty in exhaustively collecting these data lies in compiling two kinds of information: the peptides identified in vivo in the GIT and their reported activity. We gathered peptides that are identified as bioactive in the literature and present in the GIT, even if they need to be subsequently absorbed to reach their target. We also discuss the challenge of identifying bioactive peptides within digestive effluents, which are complex mixtures of peptides and many other components.

2 Diversity of the in vivo studies

Few studies have been performed on the presence of milk-derived peptides in the GIT in vivo (Table 1). Their diversity is remarkable in terms of the milk products consumed and the segment of the GIT studied, whether in humans or in animals.

The milk products consumed ranged from single molecules to complex dairy matrices. Some examples include purified products, such as a mix of peptides of interest, i.e. phosphopeptides (Brommage et al. 1991; Hirayama et al. 1992; Kasai et al. 1995), caseinomacropeptide (Fosset et al. 2002; Ledoux et al. 1999), a single protein such as lactoferrin (Troost et al. 2001), or milk-protein fractions, i.e. casein and

Table 1 Studies of milk products present in vivo in the gastrointestinal tract

Diet	Human	Mini-pig	Calf	Rat	Site	Products	Reference
Skim milk		x			Duodenum	Peptides	Barbé et al. 2014
Casein, whey proteins	x				Jejunum	Peptides	Boutrou et al. 2013
Meal with 48% Phe-CMP				x	Stomach, small intestine	CMP	Fosset et al. 2002
Lactoferrin	x				Stomach	Lactoferrin	Troost et al. 2001
α-Lactalbumin, whey protein				x	Stomach, small intestine	α-Lactalbumin, peptides	Pantako et al. 2001
Whey protein, casein, yogurt	x				Jejunum, ileum	CMP	Ledoux et al. 1999
Milk, yogurt	x				Stomach, duodenum	Peptides	Chabance et al. 1998
CPPs, casein				x	Jejunum, ileum, cecum	CPPs	Kasai et al. 1995
Casein phosphopeptides				x	Duodenum, jejunum, ileum	CPPs	Brommage et al. 1991
Casein phosphopeptides				x	Duodenum, jejunum, ileum	CPPs	Hirayama et al. 1992
Skim milk			x		Stomach	CMP, peptides	Scanff et al. 1992
Casein, skim milk			x		Stomach	Peptides	Yvon and Pelissier 1987
Casein		x			Duodenum, distal jejunum	CPPs, β-casomorphin	Meisel and Frister 1989
Milk	x				Small intestine	β-casomorphins	Svedberg et al. 1985

CMP caseinomacropeptide, *CPPs* casein phosphopeptides

whey proteins (Boutrou et al. 2013; Meisel and Frister 1989). Some information has been gathered on skim milk, which is more complex and consists of several proteins, salts, minerals and vitamins that interact with each other (Chabance et al. 1998; Scanff et al. 1992; Yvon and Pelissier 1987). Raw whole milk, which contains lipids in addition to these components, is an emulsion of fat globules formed within a water-based fluid (Svedberg et al. 1985). Some reports also exist on in vivo digestion of yogurt, which is fermented milk whose proteins have been degraded by starter bacteria (Chabance et al. 1998; Ledoux et al. 1999). Furthermore, skim milk can be heated and processed to form a gel to evaluate the influence of matrix structure on protein digestion (Barbé et al. 2014). One must consider the impacts of the food matrix (milk versus casein meal, for example) in terms of human nutrition and biological effects (Ross et al. 2013), not just the peptides released. Barbé et al. (2014) aimed to determine the effect of dairy matrix structure on the milk peptidome generated in the gut lumen of mini-pigs after ingestion of raw and heated milk and their corresponding gels. They concluded that matrix structure strongly affects the kinetics of milk-protein digestion, with raw and heated liquid milk having approximately two and three times as many peptides identified as rennet gels, respectively. The number of bioactive peptides identified was a function of the total number of peptides. The number of biopeptides derived from β-casein in gels decreased most compared to the number in the liquid milks. The authors suggested a higher resistance of rennet gels to hydrolysis; this resistance was not observed in acid gels.

All the animal species used in digestion studies are mammals. Calves act as monogastric animals for the first 2 weeks of life, using only the abomasum to digest milk (Scanff et al. 1992; Yvon and Pelissier 1987). Rodents such as rats can be used in physiological studies of the GIT, but the results are less relevant for digestion studies because the digestive enzymes of rodents are more adapted to a cellulose-based diet (e.g. seeds and grains). In contrast, the pig represents the most relevant model to assess the human digestion process because its enzymes and the physiology of its digestive tract are the closest to those of humans (Deglaire et al. 2009). The diet to be tested can be a determining factor in the choice of the animal model. A calf needs a 5-kg test meal, i.e. milk (Yvon and Pelissier 1987), while mini-pigs need from 0.1 to 1.0 kg of liquid milk or dairy gels (Barbé et al. 2014) or 100 g of casein (Meisel and Frister 1989). However, a few g of purified protein were adequate for a rat meal (Brommage et al. 1991; Kasai et al. 1995). Around 30 g of casein (Boutrou et al. 2013) or 5 g of lactoferrin (Troost et al. 2001) was suitable to study their digestion in human.

As for the segment of the GIT studied, the literature describes studies conducted from the stomach to the cecum, involving various digestive enzymes. Pepsin is the main digestive enzyme acting on proteins in the stomach. Proteolytic pancreatic enzymes, i.e. trypsin, chymotrypsin and carboxypeptidases, are delivered to the duodenum and thus are present in the small intestine. Enzymes from gut microbiota can also hydrolyse proteins and peptides, especially in the ileum and colon.

Other factors play a key role in the digestion of milk proteins, such as the hydrolysis rate of proteins through digestion time. Boirie et al. (1997) defined casein and whey protein as slow and fast proteins, respectively.

In addition to the factors cited above, the analysis strategy and the techniques used to detect bioactive peptides within digestive effluents are important issues. The method of analysis applied in these studies has changed in recent decades. Until the 2000s, studies

mainly dealt with (i) a given bioactive peptide that was detected, and even quantified, using immunological techniques such as ELISA or (ii) a small number of peptides that were identified using liquid chromatography (LC) and Edman degradation to determine the N-terminal amino acid sequence. Since 2010, the use of LC coupled with mass spectrometry (MS) represented a breakthrough and made possible the extensive characterisation of the peptides in digestive effluents. For instance, Boutrou et al. (2013) identified more than 400 peptides from casein and whey proteins in the jejunal content of humans after ingestion of these proteins. Moreover, the use of nanoLC-MS/MS allowed more concentrated samples to be analysed in an extended separation time than conventional LC-MS/MS. Barbé et al. (2014) detected 2975 sequences in effluents from mini-pigs fed raw milk. Both studies give an overall view of the peptidic profile generated in vivo, in which bioactive peptides were particularly tracked. The application of MS-based techniques on the formation of milk peptides, especially bioactive ones, and their changes throughout digestion has been recently reviewed (Picariello et al. 2013; Sanchez-Rivera et al. 2014).

3 Bioactive peptides generated in the digestive tract through in vivo digestion of milk proteins

3.1 Bioactive peptides from β-casein

Some bioactive peptides from β-casein (38) have been observed within the GIT in adult animals and humans fed milk products (Table 2). Diverse biological activities have been attributed to these β-casein sequences. The most frequent are angiotensin-converting enzyme (ACE)-inhibitory peptides (22), which originate from different regions of the entire sequence of the protein. ACE is one of the key enzymes implicated in the regulation of blood pressure, and its inhibition represents one well-known mechanism of action for milk-derived peptides (Phelan and Kerins 2011). In addition, several peptides with other reported activities have been found in digestive effluents in vivo. For instance, ten opioid peptides originate mainly from the region 59–80, and four immunomodulatory peptides originate from the C-terminal end. Noteworthy is the similarity displayed by two of the three antioxidant peptides: β-casein(169–176) has only one more amino acid residue than β-casein(170–176). Considering the three inhibitors of proteolytic enzymes, one of the sequences, β-casein(59–67) is included in the other, β-casein(58–72). These observations question the minimal active sequence required for a peptide to exert a physiological function in vivo.

Particular attention has focused on β-casomorphins (β-CM), opioid-like peptides that behave like a μ-type opioid agonist. They consist of fragments from the β-casein sequence 60–70 (YPFPGPIPNSL), such as YPFPGPI (β-CM 7) or even shorter fragments from the C-terminal portion, i.e. β-CM 6, β-CM 5 and β-CM 4. The first in vivo demonstration of the presence of β-CM immunoreactive material in intestinal effluents of humans fed raw milk was reported by Svedberg et al. (1985). Although they detected β-CM 7 in duodenal effluents, chromatographical characterisation showed that most of the immunoreactive material was larger than β-CM 7, meaning that it might have been a precursor. This hypothesis was verified in the duodenal chyme

Table 2 Sequences of bioactive peptides derived from β-casein and that have been identified in vivo in the gastrointestinal tract of humans or animals

Sequence	Protein fragment	Activity	In vivo in GI tract
LNVPGEIVE	β-casein (6–14)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
NVPGEIVE	β-casein (7–14)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
DKIHPF	β-casein (47–52)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
SLVYPFPGPI	β-casein (57–66)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
LVYPFPGPIPNSLPQ	β-casein (58–72)	Protease/peptidase inhibitor, antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
VYPFPGPI	β-casein (59–66)	Opioid agonist, antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
VYPFPGPIP	β-casein (59–67)	Protease/peptidase inhibitor	Mini-pig (Barbé et al. 2014); human (Boutrou et al. 2013)
VYPFPGPIPN	β-casein (59–68)	Opioid agonist, antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013); mini-pig (Barbé et al. 2014)
YPFP	β-casein (60–63)	Opioid agonist	Human (Svedberg et al. 1985)
YPFPGP	β-casein (60–65)	Opioid agonist	Human (Boutrou et al. 2013; Svedberg et al. 1985)
YPFPGPI	β-casein (60–66)	Opioid agonist	Human (Boutrou et al. 2013; Svedberg et al. 1985)
YPFPGPIP	β-casein (60–67)	Opioid agonist	Human (Boutrou et al. 2013)
YPFPGPIPN	β-casein (60–68)	Opioid agonist, antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013); mini-pig (Barbé et al. 2014)
YPFPGPIPNSL	β-casein (60–70)	Opioid agonist	Mini-pig (Meisel and Frister 1989)
YPFPGPIPNSLPQNIPPLTQT	β-casein (60–80)	Opioid agonist	Mini-pig (Barbé et al. 2014)
PGPIPN	β-casein (63–68)	Immunomodulator	Human (Boutrou et al. 2013)
NIPPLTQTPV	β-casein (73–82)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
PPLTQTPV	β-casein (75–82)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
TPVVVPPFLQP	β-casein (80–90)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)

Table 2 (continued)

Sequence	Protein fragment	Activity	In vivo in GI tract
VKEAMAPK	β-casein (98–105)	Antioxidant	Mini-pig (Barbé et al. 2014)
EMPFPK	β-casein (108–113)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
YPVEPF	β-casein (114–119)	Opioid agonist	Human (Boutrou et al. 2013); mini-pig (Barbé et al. 2014)
VENLHLPLPLL	β-casein (130–140)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013); mini-pig (Barbé et al. 2014)
NLHLPLPLL	β-casein (132–140)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013); mini-pig (Barbé et al. 2014)
LHLPLP	β-casein (133–138)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
HLPLPLL	β-casein (134–140)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
SQSKVLPVPQ	β-casein (166–175)	Antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
SKVLPVPQK	β-casein (168–175)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
KVLPVPQK	β-casein (169–176)	Antioxidant	Mini-pig (Barbé et al. 2014)
VLPVPQK	β-casein (170–176)	Antioxidant	Human (Boutrou et al. 2013)
AVPYPQR	β-casein (177–183)	Antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
LLYQQPVLGPVRGPFPIIV	β-casein (191–209)	Antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
LYQEPVLQPVRGPFPIIV	β-casein (192–209)	Immunomodulator	Mini-pig (Barbé et al. 2014)
YQEPVLQPVR	β-casein (193–202)	Immunomodulator	Mini-pigs (Barbé et al. 2014)
YQEPVLGPVRGPFPI	β-casein (193–207)	Antimicrobial	Mini-pig (Barbé et al. 2014)
YQEPVLQPVRGPFPIIV	β-casein (193–209)	Immunomodulator	Calf (Yvon and Pelissier 1987); mini-pig (Barbé et al. 2014)
QEPVLQPVRGPFPIIV	β-casein (194–209)	Antihypertensive (ACE inhibitor), protease/peptidase inhibitor	Mini-pig (Barbé et al. 2014)
EPVLQPVRGPFP	β-casein (195–206)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)

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of mini-pigs fed casein, in which β-CM 11 was the main β-CM present (Meisel and Frister 1989). In the human jejunum, β-CMs were the most frequently identified bioactive peptides during 6 h of digestion after casein ingestion, while β-CM 11 was not identified using MS (Boutrou et al. 2013). Altogether, these results suggest the presence of β-CM precursors in the duodenum and that of β-CMs only in the jejunum, where they can bind opioid receptors.

3.2 Bioactive peptides from α_{s1}-, α_{s2}- and κ-caseins

Bioactive peptides from α_{s1}-, α_{s2}- and κ-caseins (18, seven and six peptides, respectively) have been identified in the GIT in vivo (Table 3). Among them, 13 antihypertensive peptides from α_{s1}-casein (nine peptides), α_{s2}-casein (two peptides) and κ-casein (two peptides) have been reviewed. In addition, seven antimicrobial peptides have been reported, including three peptides from the region 175–207 of α_{s2}-casein. The other 11 bioactive peptides have diverse activities: antioxidant, opioid, antistress, antithrombotic, protease and peptidase inhibitor, and phosphodiesterase inhibitor. We find only one immunomodulator peptide in vivo in the GIT.

3.3 Caseinomacropeptide within in vivo digests

The release of caseinomacropeptide (CMP) during milk-protein digestion in the intestine could be of nutritional and physiological significance since this peptide has been shown to have various biological activities as regulatory compounds, either directly as a neurotransmitter or indirectly by modulating the secretion of intestinal hormones (Thoma-Worringer et al. 2006). CMP in the intestine has an inhibitory effect on gastric acid secretion and stimulates pancreatic secretion through the release of cholecystokinin, although no effect on satiety could be demonstrated. CMP can modulate the cardiovascular system through its antithrombotic and antihypertensive activities. It has also been shown that CMP can exert immunomodulating and antimicrobial effects by inhibiting pathogen adhesion to cells, as well as influenza viruses.

Digestion of CMP was widely studied in the 1990s. It is a 64-amino acid peptide released from κ-casein during the first phase of digestion through the action of gastric enzymes, which rapidly cleaves the peptide bond Phe_{105}-Met_{106} and produces an N-terminal fragment (para-κ-casein, residue 1–105) and CMP (residue 106–169). CMP is a predominant breakdown product in the stomach of humans (Ledoux et al. 1999) and calves (Yvon and Pelissier 1987) after ingestion of casein, skim milk or yogurt. It is detected in stomach contents during the first few hours of digestion; thus, it resists the action of gastric enzymes. Chabance et al. (1998) also identified the antithrombotic peptide κ-casein(106–116) in human gastric samples from 20 min to 4 h after milk ingestion. In vivo studies have demonstrated that most intact CMP is rapidly released in the stomach (Fosset et al. 2002; Ledoux et al. 1999). CMP is thus present in the human duodenum and jejunum following casein and yogurt ingestion (Chabance et al. 1998; Ledoux et al. 1999). Thereafter, it undergoes partial hydrolysis by pancreatic enzymes to an extent that depends on its transit rate in the lumen (Ledoux et al. 1999). Some derived fragments are also present in the proximal and median intestine of rats fed Phe-

Table 3 Sequences of bioactive peptides derived from α_{s1}-, α_{s2}- and κ-casein and that have been identified in vivo in the gastrointestinal tract of humans or animals

Sequence	Protein fragment	Activity	In vivo GI tract
RPKHPIKHQGLPQEVLNENLLRF	α_{s1}-CN (1–23)	Antibacterial, immunomodulator	Mini-pig (Barbé et al. 2014); calf (Yvon and Pelissier 1987)
FFVAPFPEVFGK	α_{s1}-CN (23–34)	Antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
FVAPFPEV	α_{s1}-CN (24–31)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
FVAPFPEVF	α_{s1}-CN (24–32)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
FVAPFPEVFG	α_{s1}-CN (24–33)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
VAPFPEVF	α_{s1}-CN (25–32)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
HIQKEDVPSER	α_{s1}-CN (80–90)	Antioxidant	Human (Boutrou et al. 2013); mini-pig (Barbé et al. 2014)
RYLGYL	α_{s1}-CN (90–95)	Opioid	Calf (Yvon and Pelissier 1987)
YLGYLEQ	α_{s1}-CN (91–97)	Antistress	Human (Boutrou et al. 2013)
YLGYLEQL	α_{s1}-CN (91–98)	Antistress	Human (Boutrou et al. 2013)
YLGYLEQLLR	α_{s1}-CN (91–100)	Antistress	Mini-pig (Barbé et al. 2014)
LRLKKYKVPQL	α_{s1}-CN (99–109)	Antimicrobial	Mini-pig (Barbé et al. 2014)
KKYKVPQL	α_{s1}-CN (102–109)	Antihypertensive (ACE inhibitor)	Mini-pigs (Barbé et al. 2014)
YKVPQL	α_{s1}-CN (104–109)	Antihypertensive (ACE inhibitor)	Human (Boutrou et al. 2013)
AYFYPEL	α_{s1}-CN (143–149)	Antihypertensive (ACE inhibitor)	Human (Chabance et al. 1998); calf (Yvon and Pelissier 1987)
YFYPEL	α_{s1}-CN (144–149)	Antioxidant	Human (Chabance et al. 1998)
DAYPSGAW	α_{s1}-CN (157–164)	Antihypertensive (ACE inhibitor)	Calf (Yvon and Pelissier 1987)
SDIPNPIGSENSEK	α_{s1}-CN (180–193)	Antimicrobial	Mini-pig (Barbé et al. 2014)
ALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN (175–207)	Antimicrobial	Mini-pig (Barbé et al. 2014)
KTVYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN (181–207)	Antimicrobial	Mini-pig (Barbé et al. 2014)
VYQHQKAMKPWIQPKTKVIPYVRY	α_{s2}-CN (183–206)	Phosphodiesterase inhibitor, protease/ peptidase inhibitor	Mini-pig (Barbé et al. 2014)

Table 3 (continued)

Sequence	Protein fragment	Activity	In vivo GI tract
VYQHQKAMKPWIQPKTKVIPYVRYL	α_{s2}-CN (183–207)	Antimicrobial, phosphodiesterase and protease/peptidase inhibitor	Mini-pig (Barbé et al. 2014)
AMKPWIQPK	α_{s2}-CN (189–197)	Antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
AMKPWIQPKTKVIPYVRYL	α_{s2}-CN (189–207)	Phosphodiesterase inhibitor t	Mini-pig (Barbé et al. 2014)
MKPWIQPK	α_{s2}-CN (190–197)	Antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
FSDKIAK	κ-CN (18–24)	Antihypertensive (ACE inhibitor), antimicrobial	Mini-pig (Barbé et al. 2014)
ARHPHPHLSFM	κ-CN (96–106)	Antioxidant	Mini-pig (Barbé et al. 2014)
HPHPHLSF	κ-CN (98–105)	Antihypertensive (ACE inhibitor)	Mini-pig (Barbé et al. 2014)
MAIPPK	κ-CN (106–111)	Antithrombotic	Human (Boutrou et al. 2013)
MAIPPKKNQDK	κ-CN (106–116)	Antithrombotic	Human (Chabance et al. 1998; Boutrou et al. 2013); mini-pig (Barbé et al. 2014)
KNQDK	κ-CN (112–116)	Antithrombotic	Human (Boutrou et al. 2013)

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CMP, but none is bioactive; nonetheless, it is notable that two of them contain the antithrombotic sequence κ-casein(112–116) (Fosset et al. 2002). Both κ-casein(112–116) and the aforementioned κ-casein(106–116) have been identified in the human intestine (Boutrou et al. 2013).

3.4 Caseinophosphopeptides identified in the GIT in vivo

Milk's high calcium availability has been attributed to caseinophosphopeptides (CPPs) formed during digestion of casein in the small intestine. CPPs have been described as mineral carriers because of the phosphorylated serine residues in their sequence that bind minerals such as calcium, iron and zinc and thus play an important role in mineral bioavailability by forming soluble complexes that prevent, for example, calcium phosphate from precipitating (Ait-Oukhatar et al. 2002; Sato et al. 1991). Kasai et al. (1995) reported CPPs in the digestive tract of rats fed casein, from the stomach to the colon. They also estimated that the residual CPPs of rats fed casein are not distinguishable from those of rats fed a CPP diet. The large CPP α_{s1}-casein(110–142) was found in gastric effluents of calves after ingestion of skim milk (Scanff et al. 1992). In vivo formation of CPP α_{s1}-casein(66–74) was demonstrated in the distal jejunum of cannulated mini-pigs fed casein (Meisel and Frister 1989). The most abundant CPPs from α_{s1}-casein present in the ileum of rats when CPPs were ingested were the peptides α_{s1}-casein(61–74) (Hirayama et al. 1992) and α_{s1}-casein(59–79) (Brommage et al. 1991). Regarding CPPs from β-casein present in the ileum of rats fed CPPs, the following have been reported: β-casein(1–25), β-casein(1–28), β-casein(33–48) (Brommage et al. 1991) and β-casein(7–24) (Hirayama et al. 1992). Overall, in these studies, CPPs were determined according to the phosphorylation of amino acids in soluble fractions prepared from digestive effluents. In studies whose strategy was to track bioactive peptides within an entire profile of peptides identified by MS, sequences, but not always their degree of phosphorylation, was considered. For CPPs, the presence of the complete phosphorylated seryl-cluster (SpSpSpEE) in the sequence has been described as the main factor influencing their interaction with minerals, although the surrounding residues or the length of the sequence may also have an influence (Cross et al. 2005; Reynolds et al. 1994). Barbé et al. (2014) identified almost 150 CPPs in vivo at different times of digestion in duodenal effluents of mini-pigs fed a variety of dairy products. Most were either mono- or diphosphorylated; Table 4 presents the most frequently identified CPPs. It is important to note that some of the CPPs identified in vivo might be considered mineral carriers due to the presence of phosphoseryl residues in their sequences, whereas their bioactivity has not been experimentally established. Furthermore, some CPPs have been demonstrated to be active when administered individually, while others are active when present in a mixture of CPPs (Ait-Oukhatar et al. 2002). This reinforces the difficulty in identifying active CPPs within digestive effluents.

3.5 Identification of bioactive peptides by MS

It is difficult to identify all peptides in a digestive effluent with a single type MS analysis because all ranges of molecular weights (from entire proteins to dipeptides) cannot be screened (Panchaud et al. 2012). C18 reversed-phase LC is the most common

Table 4 CPPs derived from caseins that have been identified in vivo in the gastrointestinal tract of mini-pigs (data collected from Barbé et al. 2014)

Number of phosphorylated sites	Sequence	Protein fragment incriminated	Numbers of identified CPP	Some of the most frequently identified CPP
3	SpSpSpEE	β-CN (17–21)	4	β-CN (1–24), β-CN (1–29), β-CN (6–25)
		α_{s1}-CN (66–70)	0	
		α_{s2}-CN (8–12)	4	α_{s2}-CN (1–24)
		α_{s2}-CN (56–60)	0	
2	SpTSpEE	α_{s2}-CN (129–133)	7	α_{s2}-CN (124–137), α_{s2}-CN (124–140), α_{s2}-CN (124–142), α_{s2}-CN (124–146)
2	SpESpTE	α_{s1}-CN (46–50)	25	α_{s1}-CN (40–52), α_{s1}-CN (40–58), α_{s1}-CN (40–61), α_{s1}-CN (40–63), α_{s1}-CN (43–58)
1	SpEE	β-CN (35–37)	51	β-CN (29–44), β-CN (29–45), β-CN (30–52), β-CN (33–48)[a], β-CN (33–52)
1	SpTE	α_{s2}-CN (143–145)	11	α_{s2}-CN (138–149), α_{s2}-CN (138–150)
1	SpAEE	α_{s1}-CN (115–118)	45	α_{s1}-CN (109–119)[a], α_{s1}-CN (110–119), α_{s1}-CN (110–120)[a], α_{s1}-CN (110–124)

Sp=phospho-serine

[a] Also identified in humans (Boutrou et al. 2013)

method used to separate peptides before their characterisation by MS. However, small peptides containing some hydrophobic amino acids are not retained in C18 columns (Panchaud et al. 2012). In addition, peptides composed of less than six amino acids are not usually addressed using MS because it is difficult to unambiguously infer them back to one precursor protein. For very small peptides such as di- and tripeptides, separation is required prior to injection into the mass spectrometer (Panchaud et al. 2012). For example, the ACE-inhibitory peptides Ile-Pro-Pro (IPP), Leu-Pro-Pro (LPP), AW, IW, LW, VY, IY and FY were identified in plasma of humans fed a peptide-enriched milk using a LC-multiple reaction ion monitoring-MS (LC-MRM-MS) method (Foltz et al. 2007). The authors assessed the bioavailability of IPP and seven other ACE-inhibitory peptides present in a lactotripeptide-enriched yogurt beverage to determine whether meal intake affects IPP bioavailability in humans. They concluded that the tripeptide IPP selectively escapes from intestinal degradation and reaches the blood circulation undegraded. Similarly, IPP, LPP and VPP were intragastrically administered to pigs and reached blood circulation in an intact form (van der Pijl et al. 2008). Meta-analyses on the effect of lactotripeptides on blood pressure in humans also exist (Pripp 2008; Qin et al. 2013). However, to our knowledge, there is no evidence for the identification of di- and tripeptides in vivo in the GIT.

Considering the currently identified bioactive peptides from casein (Tables 2 and 3), the smallest is β-CM 4, with four amino acids; it was detected as an immunoreactive material by Svedberg et al. (1985). Sixty-seven bioactive peptides identified in vivo in

the GIT (Tables 2 and 3) contain more than five amino acids. Focusing on bioactive peptides from casein composed of more than five amino acids, 129 and 126 peptides are referenced in our research unit homemade database and in the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep), respectively. Two assumptions explain why almost half of the reported bioactive peptides are not identified in vivo in the GIT, each with completely different consequences from a nutritional viewpoint: either the region of the protein where the peptide is encrypted was almost completely digested, leading to a negligible concentration of the bioactive peptide, or the bioactive peptides were too large for efficient identification by MS in digestive effluents (Boutrou et al. 2013; Ross et al. 2013).

3.6 Bioactive peptides released from whey proteins in vivo

According to databases, a hundred or so bioactive peptides are encrypted in milk whey proteins; however, few have been identified in digestive effluents in vivo. Whey proteins are relatively resistant to peptic digestion, especially β-lactoglobulin (β-Lg), due to its particular folding (Dalgalarrondo et al. 1995). Troost et al. (2001) studied human gastric digestion of lactoferrin, an iron-binding protein that favours iron absorption and also displays antioxidative, anti-inflammatory, immunomodulatory and antimicrobial activities (Garcia-Montoya et al. 2012). They concluded that more than 65% of lactoferrin survives gastric transit. Using gel permeation chromatography, they found several lactoferrin fragments of different sizes throughout digestion. The most abundant fragments had molecular weights of 76 and 41 kDa and were able to bind the antibodies against intact lactoferrin. Pantako et al. (2001) studied the effect of digestion on dry matter recovery and peptide distribution in the gastrointestinal content of rats fed α-lactalbumin, a metalloprotein that strongly binds calcium and zinc ions and has bactericidal or antitumor activity. Their results indicated that α-lactalbumin rapidly left the stomach and was quickly digested into amino acids and absorbed in the proximal small intestine. It would be interesting to study in vivo the digestion of an α-lactalbumin/oleic acid complex, which has been reported to induce the selective death of tumour cells (Brück et al. 2014).

Recently, certain bioactive peptides from whey proteins were identified in digestive effluents using MS. Only three bioactive peptides derived from β-Lg were identified in duodenal effluents of mini-pigs fed raw milk (Barbé et al. 2014): two antihypertensive sequences, i.e. β-Lg(32–40) and β-Lg(142–148), and the antimicrobial peptide β-Lg(92–100). The latter peptide was not detected in jejunal effluents from humans fed whey proteins. Instead, various related fragments were identified, such as β-Lg(92–98), (92–99), (92–103) and (93–100) (Boutrou et al. 2013). Whether these sequences exert any biological activity deserves further investigation. Indeed, some peptides not identified as bioactive can act in situ in the GIT. Further research on the interaction of peptides with intestinal receptors would constitute a prospective strategy on this topic. Among food-derived peptides identified in the GIT, it is also important to note that some may interact in situ with receptors located in the gut. Bioactive peptides that regulate gastrointestinal function and intake have been described (Froetschel 1996; Shimizu and Ok Son 2007), and some of them were detected in digestive effluents, such as CPPs that enhance mineral absorption and opioid peptides that regulate intestinal motility (Shimizu and Ok Son 2007).

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4 Conclusion

This review collected comprehensive data that provides valuable information for (i) understanding physico-chemical parameters of protein digestion (for instance, peptides identified in vivo are important data for correlating the in vitro tools necessary to develop models that mimic protein digestion), (ii) improving protein-design or diet-structure approaches to optimise the release of bioactive peptides and (iii) shedding light on nutrient bioaccessibility (i.e. the percentage of a nutrient digested and released from the food matrix in its absorbable form).

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