Meta-Analysis for Correlating Structure of Bioactive Peptides in Foods of Animal Origin with Regard to Effect and Stability

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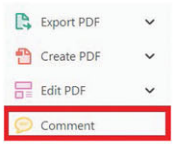

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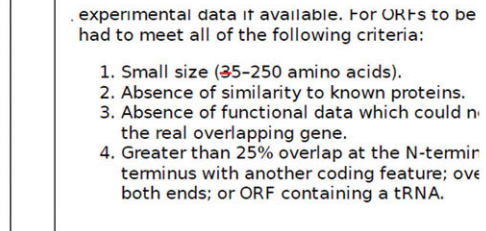

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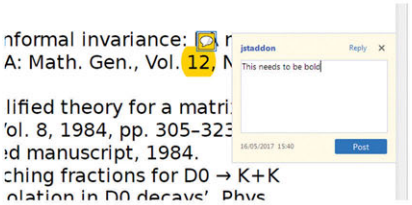

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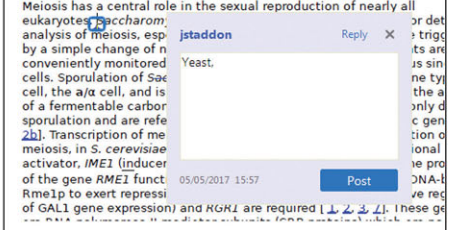

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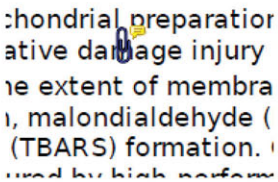

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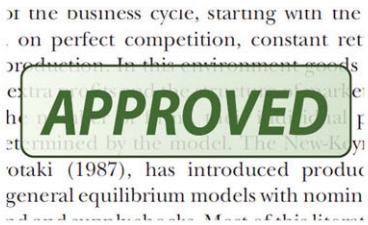

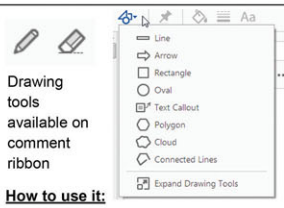

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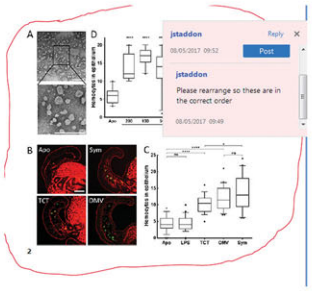

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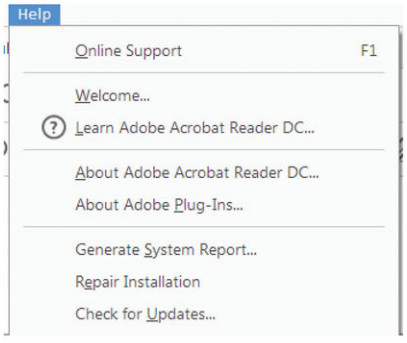

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Comprehensive REVIEWS in Food Science and Food Safety

Meta-Analysis for Correlating Structure of Bioactive Peptides in Foods of Animal Origin with Regard to Effect and Stability

Elena Maestri, Milica Pavlicevic, Michela Montorsi, and Nelson Marmiroli

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Abstract: Amino acid (AA) sequences of 807 bioactive peptides from foods of animal origin were examined in order to correlate peptide structure with activity (antihypertensive, antioxidative, immunomodulatory, antimicrobial, hypolipidemic, antithrombotic, and opioid) and stability *in vivo*. Food sources, such as milk, meat, eggs, and marine products, show different frequencies of bioactive peptides exhibiting specific effects. There is a correlation of peptide structure and effect, depending on type and position of AA. Opioid peptides contain a high percentage of aromatic AA residues, while antimicrobial peptides show an excess of positively charged AAs. AA residue position is significant, with those in the first and penultimate positions having the biggest effects on peptide activity. Peptides that have activity *in vivo* contain a high percentage (67%) of proline residues, but the positions of proline in the sequence depend on the length of the peptide. We also discuss the influence of processing on activity of these peptides, as well as methods for predicting release from the source protein and activity of peptides.

Keywords: amino acids, food processing, functional food, health effect, in vivo activity

Introduction

Although the concept of functional foods was already introduced in 1984 (Arai, 1996), consumer acceptance has been hindered by the lack of an official definition (Siró, Kápolna, Kápolna, & Lugasi, 2008). Japan's Ministry of Health and Welfare has labeled functional foods as "foods for specified health uses," for example, food that also has beneficial effects on health. Since then commissions such as FuFoSE (Functional Food Science in Europe; Diplock et al., 1998) and the Commission of the European Communities (Annunziata, Misso, & Vecchio, 2009, June) have tried to define a scientific basis for health claims, type of food modification, and nutrients added, as well as to establish the influence of production processes on the health effects of food. Claims that can be considered as "functional," for foods having beneficial effects on health, and as "reduction of disease risk claims" where daily consumption reduces the risk of particular diseases (Roberfroid, 2002) have been reviewed by the European Food Safety Authority (EFSA).

Functional foods often contain an added ingredient with confirmed health effects (Menrad, 2003; Niva, 2007). However, certain functional foods contain naturally occurring constituents with known bioactive effects (Eckert et al., 2013; López-Expósito, Quirós, Amigo, & Recio, 2007; McGregor & Poppitt, 2013; Suetsuna, 1999). During digestion or processing of these foods, proteins are degraded to peptides, which may have a positive effect on human health (Moughan, Rutherfurd, Montoya, & Dave, 2014). These are called bioactive peptides, short amino acid (AA) sequences, usually 2 to 20 residues (Sarmadi & Ismail, 2010), that are released from a source protein either during technological processing of the food or during *in vivo* digestion (Korhonen & Pihlanto, 2007).

Several review papers list various health effects of bioactive peptides from foods of animal origin (Aneiros & Garateix, 2004; Bhopale, 2016; Lafarga & Hayes, 2014; Nguyen, Johnson, Busetti, & Solah, 2015; Udenigwe & Howard, 2013; Yu, Yin, Zhao, Chen, & Liu, 2014; Zambrowicz et al., 2015) and these characterized bioactive peptides have been incorporated into databases such as BIOPEP (Table S1). However, most of these databases cover peptides of specific length, origin, and/or health effect. BIOPEP includes all bioactive peptides from foods, regardless of their health effect or size, but a literature search can reveal additional peptides not present in the list.

Interest in functional food is rapidly extending as knowledge grows about effects of bioactive peptides on human health, which has led not only to the development of new dietary supplements

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and medicines, but also to the employment of hydrolysates in food technology. For instance, fish protein hydrolysates might be used as functional ingredients due to their enhancement of protein solubility, emulsification ability, and gelling activity (Chalamaiah, Rao, Rao, & Jyothirmayi, 2010). Egg white protein is applied as a food plasticizer (Rao, Rocca-Smith, Schoenfuss, & Labuza, 2012) and as a clarifier in alcoholic drinks (Yu et al., 2014).

Bioactive peptides are also produced from waste and by-products of meat, fish, and various other seafood species (Harnedy & FitzGerald, 2012; Khiari, Ndagijimana, & Betti, 2014; Kim & Mendis, 2006; Lordan, Ross, & Stanton, 2011; Suarez-Jimenez, Burgos-Hernandez, & Ezquerra-Brauer, 2012).

Many dietary supplements containing bioactive peptides have been developed, such as BioGro, PeptiPlus, Lactium, Valtyron, Lapis Support, Calpis, Evolus, PeptACE, and Levenorm, as well as drugs like Molval, Thymosin beta-4, Pexiganan, Osteotide, C12 Peption, Bonito Peptide, Lupron, and Lantus (Fosgerau & Hoffmann, 2015; Harnedy & FitzGerald, 2012; Udenigwe & Howard, 2013; Uhlig et al., 2014). Many therapeutic peptides and proteins have been approved by the U.S. Food and Drug Administration (FDA; https://crdd.osdd.net/raghava/thpdb/index.html, accessed March 2018).

As numerous bioactive peptides are found in milk, special interest has focused on the introduction of bioactive peptides in infant formulas (Raikos & Dassios, 2014); however, due to strict regulation (Lönnerdal, 2014), any claimed effect must be well documented and verified by EFSA. For example, in 2012, EFSA rejected advertising from a Finnish company that claimed tripeptides IPP and VPP from milk have antihypertensive effects (Jauhiainen et al., 2010), in part because of the short half-lives of these peptides (Foltz, van der Pijl

Because of the complexity of isolation and purification from their natural sources, design and production of synthetic bioactive peptides have become an exciting field of research (Goodwin, Simerska, & Toth, 2012; Kolomin, Shadrina, Slominsky, Limborska, & Myasoedov, 2013; Sun, 2013). Although biological and chemical syntheses of these peptides present major challenges (Goodwin et al., 2012; Groß, Hashimoto, Sticht, & Eichler, 2016), several synthetic bioactive drugs, such as Cetapril, Acthrel, and Angiomax, are available on the market (Vlieghe, Lisowski, Martinez, & Khrestchatisky, 2010).

When evaluating the potential activity of any food, several characteristics must be taken into consideration, because the digestion process will yield different types and/or numbers of peptides from a particular food matrix. The first question is whether a source protein will release a given peptide. Furthermore, in order to be bioactive, a peptide must be stable (for example, not degraded during the gastric and intestinal phases of digestion), be transported from the gut lumen into the enterocytes, and from enterocytes into blood, and finally exhibit biological activity under physiological conditions. All these characteristics depend on the specific sequence of the peptide.

Often the activity of a whole protein hydrolysate is determined rather than the activities of its component peptides (Liaset et al., 2009; Pan, Wu, Liu, Cao, & Zeng, 2013; Sakanaka, Tachibana, Ishihara, & Raj Juneja, 2004; Shimizu et al., 2009).Thus, which sequence exhibits a given effect is unknown. Variation of conditions during preparation of hydrolysates, for example, pH, enzymes involved, and temperature, could lead to different peptide mixtures, making it harder to assign specific effects to individual peptides; also, hydrolysates can have a stabilizing effect on particular peptides. Therefore, one important question is whether a purified peptide still shows the required activity. Since peptides and/or hydrolysates are often tested *in vitro*, an additional problem is to establish a correlation between *in vitro* and *in vivo* activities. Complexities in the preparation and processing of foods, combined with purification and testing of peptides, make predictions of activity and release from a given food quite difficult.

This review attempts to correlate peptide sequences with their activity and stability, but we also discuss mechanisms related to a particular activity, factors influencing transport and stability of peptides *in vivo*, and also the effects of processing.

Animal food is rich in bioactive peptides, with considerable variation depending on the source. Figure 1 shows the process of compiling our initial dataset, together with datasets for individual analyses. We used the BioPep database, as the most complete dataset available, with a survey of the literature to identify non-listed sequences, sources of peptides, and inhibitory or effective concentration values where obtainable. Starting with 3000 known bioactive peptides, we compiled a list of 807 sequences found in food of animal origin (Table S2). Bioactive peptides derived from cyclic peptides, such as different classes of defensins (Jenssen, Hamill, & Hancock, 2006), were also included in the analysis (Table S2). Peptide hormones are usually a better source of bioactive peptides in plants (Maestri, Marmiroli, & Marmiroli, 2016; Sánchez & Vázquez, 2017; Schaller, 2001) compared to animals, where hormones, such as insulin, glucagon, and so on, are ubiquitinated and their constituent AAs are used for synthesis of other endogenous peptides and proteins (Ferro, Hyslop, & Camargo, 2004; Glickman & Ciechanover, 2002).

Peptide length varies from 2 to 40 AAs. For each peptide, we have listed its source, specific bioactive effect, AA sequence, and whether *in vitro* or *in vivo* effects have been described. We assessed effects on activity of AA type using the chi-square test, while effects of AA position were evaluated using univariate ANOVA. Additionally, for those peptides showing *in vivo* activity, we highlight which AA residues and which positions in the peptides were crucial for stability.

Proteolytic Production of Bioactive Peptides

The so-called "classic" approach to assess activity of a bioactive peptide involves proteolysis of a target source protein, fractionation and identification of peptides, activity testing, and synthesis to confirm bioactivity. Proteolysis of source proteins usually involves application of digestive enzyme preparations, although bacteria (especially lactic acid bacteria; Pessione & Cirrincione, 2016) and in some cases yeast (Rai, Kumari, Sanjukta, & Sahoo, 2016) can also be used. Choice between enzymatic digestion and microbial fermentation to obtain a protein hydrolysate depends on the food source and has a major impact on the type and activity of peptides formed (Korhonen & Pihlanto, 2003, 2006). Although enzymatic hydrolysis offers greater specificity and reproducibility, the employment of different bacterial strains and changes in conditions during fermentation can lead to a variety of peptides with different activities (Korhonen, 2009; Pessione & Cirrincione, 2016). This is relevant when using yeast as a starter culture, since carboxypeptidase and aminopeptidase exhibit synergistic action (Ferreira & Viljoen, 2003) releasing a higher number of peptides than treatment with either alone.

Fractionation and purification of extract is often performed combining filtration, usually ultrafiltration or nanofiltration, with a chromatographic technique, such as size-exclusion chromatography (Alemán, Giménez, Pérez-Santin, Gómez-Guillén, & Montero, 2011), ion-exchange chromatography (Duan et al.,

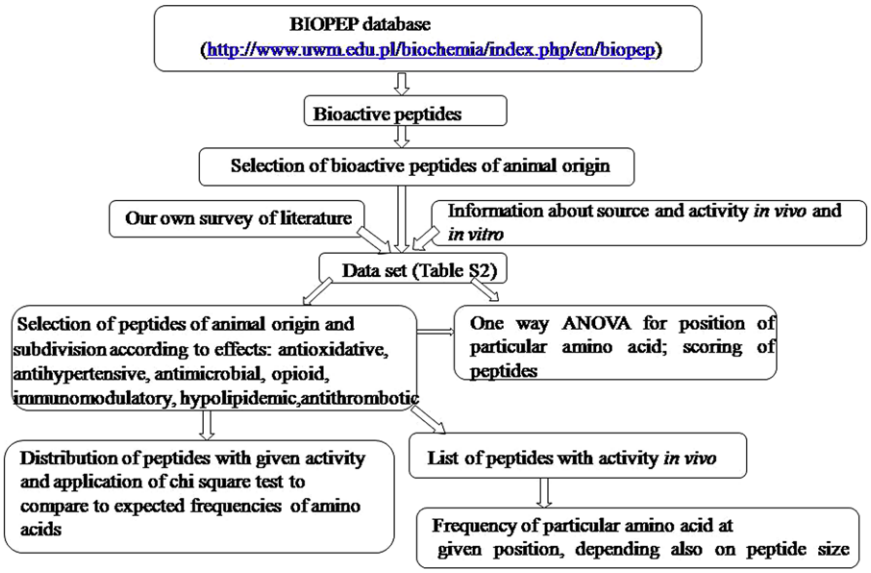


Figure 1–Flow chart followed during selection of peptides and analyses of effects of type and position of amino acid on biological activity.

2014), gel permeation chromatography (Babij et al., 2014), and RP-HPLC (reverse-phase HPLC; Hernández-Ledesma, Amigo, Ramos, & Recio, 2004).

Due to sensitivity and reproducibility (Tamvakopoulos, 2007), mass spectrometry (MS) remains the best method for the quantification of bioactive peptides. However, there are several problems with using MS for detection and quantification of bioactive peptides. Although this technique is rather sensitive and accurate, concentration of some of these peptides in food products may be below its detection limit. Another problem arises when digestion process (prior to MS) generates nonactive peptides from endogenous proteins. Since these proteins are abundant in hydrolysate, they can interfere with signal coming from bioactive peptides and prevent correct determination of their concentration (Dallas et al., 2015; Tsakelidou et al., 2017). Additionally, identifying peptides from the plethora of MS data could be challenging.

Health Claims Related to Bioactive Peptides

Literature reports present different classifications of bioactive peptides (Table 1): it is important to note that classification is not uniform and definitions of effects also show variations.

Antioxidative peptides minimize damage to lipids, nucleic acids, and proteins caused by free radicals. Chalamaiah, Dinesh Kumar, Hemalatha, and Jyothirmayi (2012) and Najafian and Babji (2012) define an antioxidant broadly "as any substance that considerably delays or inhibits the oxidation of a substance." However, since one of the main causes in food deterioration is the oxidation of lipids, along with production of peroxides, aldehydes, and short-chain fatty acids, definitions of antioxidant bioactive peptides focus on avoidance of lipid oxidation (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011; Yu et al., 2014). Antioxidant peptides are also involved in the protection of developing diseases, such as artherosclerosis, that are partially caused by oxidation of lipoproteins (Salvayre, Negre-Salvayre, & Camaré, 2016). Antihypertensive peptides are involved in regulating blood pressure. Since angiotensin-converting enzyme (ACE) plays a major role in the regulation of blood pressure by converting angiotensin I to angiotensin II, which acts as a vasoconstrictor, these peptides are sometimes referred as ACE inhibitors (Smacchi & Gobbetti, 2000; Wada & Lönnerdal, 2014).

Strictly, antimicrobial peptides act like antibiotics, for example, by providing protection during bacterial infection. However, peptides reported as antimicrobial in the literature also include antifungal peptides, antiviral peptides, and peptides active against protozoa (Bhopale, 2016; Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011; Kim & Wijesekara, 2010; Najafian & Babji, 2012). Thus, antibacterial peptides may exhibit antiviral and antifungal activity (Avrahami & Shai, 2004; Boman, 2003; Gordon, Romanowski, & McDermott, 2005; Makovitzki, Avrahami, & Shai, 2006; Skerlavaj, Benincasa, Risso, Zanetti, & Gennaro, 1999), and all 3 types of peptide share a similar mechanism (Brogden, Ackermann, McCray, & Tack, 2003; Hancock & Sahl, 2006; Klotman & Chang, 2006; Mulder, Lima, Miranda, Dias, & Franco, 2013; Théolier, Fliss, Jean, & Hammami, 2014). Antimicrobial peptides exhibit their effect primarily through activation of innate immunity (Epand & Vogel, 1999; Shai, 1999), but it has been shown that antimicrobial peptides also activate the adaptive immune response through modulation of cytokine release, proliferation of different subgroups of leukocytes, chemotaxis, and activation of proteases (Bals, 2000; Ho, Sung, & Chen, 2012; Tu, Ho, Chuang, Chen, & Chen, 2011). Therefore, antimicrobial peptides might also be considered as a subclass of immunomodulatory peptides.

Antithrombotic peptides prevent aggregation of platelets and formation of blood clots by inhibiting fibrinogen binding to receptor GP IIb-IIIa on the platelet surface. Due to structural similarities among fibrinogen, fragments of κ-casein, and fragments of lactotransferrin (Clare & Swaisgood, 2000; Jollès et al., 1986; Meisel, 1997; Nagpal et al., 2011; Rutherfurd & Gill, 2000), milk is considered to be a rich source of antithrombotic peptides.

Immunomodulating peptides are difficult to define, due to their variety of effects and often overlapping mechanisms of action. They are peptides that affect the immune response of an organism in the presence of an antigen. This effect could be viewed as an activation/deactivation of innate (nonspecific) or adaptive (acquired) immunity. However, to distinguish effects on innate

Table 1–A list of the different classifications of bioactive peptides as reported in papers and reviews (updated March 2018).

Main bioactivities (always presented in reviews)	Common bioactivities (often presented, but not always)	Less frequent bioactivities (referring to specific actions)
• ACE inhibitory (antihypertensive) • Antioxidant • Antimicrobial (FitzGerald & Murray, 2006; Gomez-Guillen et al., 2011; Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Korhonen, 2009; Madureira, Tavares, Gomes, Pintado, & Malcata, 2010; Meisel, 1998; Nagpal et al., 2011; Najafian & Babji, 2012; Silva & Malcata, 2005)	• Hypolipidemic • Mineral binding (osteoprotective) • Anticancer • Antithrombotic • Anti-inflammatory • Immunomodulatory • Opioid (Aimutis, 2004; Baldi et al., 2005; Baltić et al., 2014; Kim & Wijesekara, 2010; Mohanty & Mohapatra, 2016; Pepe, Tenore, Mastrocinque, Stusio, & Campiglia, 2013; Udenigwe & Howard, 2013; Wang et al., 2010; Yu et al., 2014)	• Antidiabetic (Hernández-Ledesma et al., 2014; López-Expósito et al., 2012; Ryan et al., 2011) • Antisclerotic (Lafarga & Hayes, 2014) • Antiviral (Lordan et al., 2011; Schanbacher et al., 1998) • Anti-HIV (Ngo, Vo, Ngo, Wijesekara, & Kim, 2012; Smacchi & Gobbetti, 2000) • Antiappetizing (Park & Nam, 2015) • Gut mucosa (often defined as part of immunomodulatory; Hernández-Ledesma et al., 2014; Hsieh et al., 2015) • Antinociceptive (Hernández-Ledesma et al., 2014) • Anti-alopecia (Yoshikawa, 2015)

and/or acquired immunity is difficult, because innate immunity also regulates adaptive immunity through cell-to-cell interaction and response to cytokine profiles (Iwasaki & Medzhitov, 2010, 2015; Parish & O'Neill, 1997). Furthermore, both innate and acquired immunities are involved in the development of cancer and in controlling apoptosis (Gajewski, Schreiber, & Fu, 2013; Mantovani & Sica, 2010; Peng et al., 2007). Udenigwe and Aluko (2012) define immunomodulatory peptides as those that "act by enhancing the functions of the immune system, including regulation of cytokine expression, antibody production, and ROS-induced immune functions." Meisel (1997) instead considers immunomodulatory peptides those that "stimulate proliferation of human lymphocytes and phagocytic activities of macrophages." Schanbacher, Talhouk, Murray, Gherman, and Willett (1998) also include antiproliferative peptides as immunomodulatory, although in some reviews they are presented as a separate category (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; López-Expósito, Amigo, & Recio, 2012; Ryan et al., 2011). However, the immune system has a crucial role in preventing and controlling proliferation of neoplasia (Chalamaiah et al., 2014), and many papers confirm a relation between immunosuppression and development of cancer (Cata, Wang, Gottumukkala, Reuben, & Sessler, 2013; Godbout & Glaser, 2006; Sephton & Spiegel, 2003; Seruga, Zhang, Bernstein, & Tannock, 2008; Terme et al., 2011; Whiteside, 2006). This relationship originates from changes in concentration and/or activity of some key components of humoral and cellular immunity, including interleukins and growth factors (de Jong, van Dienst, van der Valk, & Baak, 1998; Goustin, Leof, Shipley, & Moses, 1986; Seruga et al., 2008; Waugh & Wilson, 2008; Whiteside, 2006). Similarities between inflammation and carcinogenesis and higher concentrations of proinflammatory cytokines present during the initial stages of tumor formation (Bernstein, Blanchard, Kliewer, & Wajda, 2001; Grivennikov & Karin, 2011; Seruga et al., 2008; Sporn & Roberts, 1986) have led to the conclusion that anti-inflammatory peptides should also be considered as immunomodulatory. This is supported by the anti-inflammatory peptides, which inhibit neutrophil aggregation and regulate expression of adhesion molecules (such asselectins and integrins) on the surface of leukocytes (Camussi, Tetta, & Baglioni, 1990; Zouki, Ouellet, & Filep, 2000).

Opioid peptides bind to opioid receptors present in both the central and peripheral nervous systems; they are involved in the regulations of pain (Labuz, Celik, Zimmer, & Machelska, 2016; Madden, Akil, Patrick, & Barchas, 1977), a person's mood through their effect on dopamine release (Spanagel, Herz, & Shippenberg, 1990), drinking and feeding (Duraffourd et al., 2012; Kaneko, Yoshikawa, & Ohinata, 2012; Reid, 1985), sleep (Wang & Teichtahl, 2007), body temperature (Clark, 1979), stress responses (Madden et al., 1977; Shavit, Lewis, Terman, Gale, & Liebeskind, 1984), sexual maturation by modulating secretion of luteinizing hormone (Blank, Panerai, & Friesen, 1979), taste preferences (Drenowski, Krahn, Demitrack, Nairn, & Gosnell, 1992), and in the development of the nervous system (Zagon & McLaughlin, 1991). They are usually referred to as opioid receptor ligands that exhibit morphine-like effects, which can be inhibited by naloxone (Meisel, 1998; Park & Nam, 2015; Silva & Malcata, 2005; Wada & Lönnerdal, 2014).

Hypolipidemic peptides lower the levels of cholesterol and other lipids in blood. Udenigwe and Howard (2013) defined the hypolipidemic effect as changes in the rates of anabolism and catabolism of cholesterol and lipids.

However, a single peptide frequently exhibits more than one effect. This is due to interlinking metabolic pathways controlling specific functions and to the fact that bioactive peptides might serve as signaling molecules, thus functioning at the systemic level (Boonen, Creemers, & Schoofs, 2009). For example, the tripeptide VPP is classified as both antioxidative and antihypertensive (Hernández-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005). The link between antioxidative and antihypertensive activities could be explained by the involvement of reactive oxygen species in renal injuries that can affect both renin–angiotensin and kallikrein–kinin systems, thus leading to hypertension (Shou, Wang, Suzuki, Fukui, & Tomino, 1997). Since the ACE products angiotensin II and angiotensin III are involved in chemotaxis and adhesion of monocytes and macrophages (Godsel, Leon, & Engman, 2003) and in the activation of transcription factors NF-kB and AP-1 (Ruiz-Ortega, Lorenzo, & Egido, 2000), antihypertensive peptides could have an immunomodulatory effect. For example, milk-derived peptide TTMPLW shows both ACE-inhibitory and cytomodulatory activity (Udenigwe & Aluko, 2012) and VPP, besides having antioxidative and antihypertensive effects, also modulates adhesion of monocytes to vascular endothelium, which is an immunomodulatory activity (Aihara, Ishii, & Yoshida, 2009). Bali, Randhawa, and Jagg (2014) state that opioids activate components of the renin angiotensin system, thus influencing ACE inhibition: the milk-derived peptide YLLF shows both antihypertensive and opioid-like activity (Antila et al., 1991). Also, antimicrobial peptides, through their interaction with components of adaptive immunity and interaction with multifunctional enzymes like Ca^{2+}-dependent phosphoesterase (Table 2), exhibit immunological effects (Izadpanah & Gallo, 2005; Niyonsaba, Nagaoka, Ogawa, & Okumura, 2009; Wang, 2014; You,

Table 2–Mechanisms and modes of action of peptides belonging to different classes of bioactivity (updated March 2018).

	Mode of action			
Bioactive effect	Direct interaction with metabolite or organelle	Effect on protein activity	Effect on gene expression	Other
Antihypertensive	Production of NO through modulation of bradykinin-mediated vasorelaxant pathways (Hirota et al., 2011; Udenigwe & Aluko, 2012)	Inhibition of angiotensin-converting enzyme (ACE; Martínez-Maqueda et al., 2012). Inhibition of renin and endothelin-converting enzyme (Udenigwe, 2014)	Decreased expression of renal AT-II receptor mRNA (Yu et al., 2014)	
Antimicrobial			Modulation of expression for different genes in monocytes, epithelial cells, and other immune cells involved in chemo-attraction, induction of chemokines, and differentiation responses through signal transduction, promotion of angiogenesis (Hancock & Sahl, 2006)	Cell membrane perforation, for example, α-casein peptide fragment (183 to 207; Ibrahim, Sugimoto, & Aoki, 2000; López-Expósito, Amigo, & Recio, 2008) and/or disruption of pathways or organelles in parasite
Antioxidative	Radical scavenging or metal chelation, for example, carnosine in meat (Sarmadi & Ismail, 2010); inhibition of lipid peroxidation through enhanced interaction with fatty acids (Byun, Lee, Park, Jeon, & Kim, 2009); sequestration of metal ions, for example, ferrous ions with casein phosphopeptides (Díaz & Decker, 2004)			
Antithrombotic		Inhibition of platelet aggregation, possibly due to structural similarities between κ-casein and fibrinogen (κ-casein could act as a competitive inhibitor of fibrinogen for binding to activated platelet; Rutherfurd & Gill, 2000)		
Hypolipidemic	Removal of bile acids from enterohepatic circulation through formation of complexes with peptides, mainly via hydrophobic forces (Udenigwe & Howard, 2013)	Inhibition of lipase (Kagawa et al., 1996) Inhibition of acylCoA: cholesterol acyltransferase (Wergedahl et al., 2004) Inhibition of glucose-6-phosphate dehydrogenase, fatty acid synthase and carnitine palmitoyltransferase (Shimizu et al., 2006) Changes in signal transduction, for example, lactoferrin activation of CYP7A1 (Wakasa et al., 2011)	Elevated expression of mRNA of proteins involved in process of fatty acid oxidation (PGC-1a, UCP-1, CPT1B, CPT2, MCAD, LCAD, and so on; Liaset et al., 2009)	Inhibition of cholesterol micellar solubility through formation of complexes with bile acids (Wakasa et al., 2011)
Immunomodulatory	Anticancer effect due to inhibition of cell proliferation, through conformational changes in tubulin molecule as result of formation of complex with peptide (Panda et al., 2000)	Cytotoxicity due to inhibition of protein synthesis, through binding toribosome-EF-1a complex (Mayer & Gustafson, 2003) Downregulation of signaling cascades (Thell, Hellinger, Schabbauer, & Gruber, 2014) Inhibition of calmodulin through association with cyclosporine A–cyclophilin complex and modulation of activity of its substrate (Matsuda & Koyasu, 2000)	Elevated expression of genes coding Caspase 3 and Caspase 8 in tumor cells (Su et al., 2014) Downregulation of genes regulated by NFAT (nuclear factor of activated T-cells; Hogan, Chen, Nardone, & Rao, 2003; Thell et al., 2014)	Anti-inflammatory effect due to changes of adhesion of monocytes to endothelia, through modulation of proinflammatory c-Jun N-terminal kinase pathway (Aihara et al., 2009) Antinociceptive effect due to inhibition of prostaglandin synthesis (Tavares et al., 2013)
Opioid		Binding to opioid receptors, agonist (for example, α_{s1}, β casein) or antagonist (κ casein; Pihlanto-Leppälä, 2000)		

Udenigwe, Aluko, & Wu, 2010). As one example, the peptide GFHI isolated from beef sarcoplasmic protein hydrolysates inhibited growth of both *Escherichia coli* and *Pseudomonas aeruginosa*, increased macrophage activity, and showed cytotoxic effects (Jang, Jo, Kang, & Lee, 2008). Since angiotensin II promotes thrombosis by increasing secretion of plasminogen activator inhibitor type 1 (Kucharewicz, Pawlak, Matys, Pawlak, & Buczko, 2002), a correlation may exist between antihypertensive and antithrombotic activity of bioactive peptides. Several peptides, including κ-CN f (152-160) and f (155-160; Gobbetti, Minervini, & Rizzello, 2004), MAIPPKK (Marques et al., 2012), and YQEPVLGPVRGPFPIIV (Rojas-Ronquillo et al., 2012), have both antihypertensive and antithrombotic effect. Since inflammation-inducing tumor necrosis factor and interleukin 1-α also play roles in tissue factor expression, and thus in initiation of thrombosis (Esmon, 2003), many anti-inflammatory peptides also exhibit antithrombotic effects, including egg-derived peptides IQW and LKP (Majumder et al., 2015). Due to the link between innate and acquired immunity, immunomodulatory peptides also show antimicrobial activity. Dolastatin 10, isolated from marine sources, has both antitumor (Beckwith, Urba, & Longo, 1993) and antimicrobial activities (Pettit, Pettit, & Hazen, 1998), while the peptide RRWQWR derived from lactoferricin has both antimicrobial and anticancer effects (Richardson, de Antueno, Duncan, & Hoskin, 2009). Certain peptides, such as GFHI, DFHING, FHG, and GLSDGEWQ (isolated from beef muscle), are multipotent, showing immunomodulatory, antimicrobial, and antihypertensive activities (Jang et al., 2008). Results suggest that opioids can act as cytokines (Peterson, Molitor, & Chao, 1998), and it is not uncommon for opioid peptides to show immunomodulatory activity. Moreover, casomorphine (FFVAPFPEVFGK) also stimulates phagocytosis by murine peritoneal macrophages *in vitro* (Gill, Doull, Rutherfurd, & Cross, 2000). Due to the involvement of lipids in the formation of atherosclerotic plaques, hypolipidemic peptides often show antihypertensive effect. Turpeinen et al. (2009, 2012) showed that IPP and VPP peptides possess both antihypertensive and cholesterol-lowering activities.

We have listed multi-active peptides in all categories for which they show activity. Thus, effects of type and position of particular AAs in multi-active peptides were assessed individually in different categories, which allowed for a comparison of influence of AA sequence on the different activities.

Lately, functional genomics and chemical biology have been utilized to elucidate mechanisms of action of bioactive compounds (Azad & Wright, 2012; Ho et al., 2011), which has led to increased interest in the field of nutridynamics (Serrano, Jove, Gonzalo, Pamplona, & Portero-Otin, 2015). Globally, bioactive peptides can exhibit their activity in many ways: via direct interaction with metabolites, by altering the activity of an enzyme, and/or gene expression. Other mechanisms include changes in solubility or facilitating perforation of microbial cell membranes. Direct interaction with metabolites involves removal of pathway intermediates or products through sequestration, chelation, scavenging or complexation, or production of signaling molecules like NO. Altering activity or structure of key enzymes may change metabolic flux through particular pathways and thus cause redistribution of compounds into pathways with shared intermediates, or modulate signal transduction pathways. Regulation of gene expression may involve direct interaction with the promoter, silencing (for example, methylation) or interference with RNA processing. Some peptides, such as opioids and antithrombotics, have a preferred mechanism; others utilize several mechanisms more or less simultaneously. Table 2 lists mechanisms of action for peptides with different biological activities. Immunologically active peptides show the most variation, that is, they interact via the greatest number of mechanisms; this complexity is partially due to interlinking between pathways involved in the immunological response.

The type of activity likely to be displayed by a bioactive peptide is dependent on its source. Milk and milk products are characterized by a high frequency of ACE inhibitory peptides (Figure 2A), while eggs, fish, and various other seafood have been found to be good sources of antioxidative peptides (Figure 2B and 2C). Meat and meat products have a high content of antimicrobial and antithrombotic peptides (Figure 2D).

Measuring the Effects of Bioactive Peptides

Individual effects of bioactive peptides can be determined with *in vivo* and *in vitro* assays. However, each assays measures only one aspect of bioactive peptide activity, and each has its own limits in terms of sensitivity, reproducibility, and presence of interfering compounds. Moreover, there is also a problem of correlation between *in vitro* and *in vivo* results. Thus, several tests are usually performed simultaneously and the majority of methods are modified for particular food matrixes.

Antihypertensive activity

In vitro tests of antihypertensive activity usually involve measurement of ACE inhibition, expressed either as percentage of inhibition or as by IC_{50} values—the concentration of inhibitor that causes 50% loss of enzyme activity. Mechanisms of action of the inhibiting peptide are deduced by assessing *in vitro* inhibition patterns (Balti et al., 2015; Duan et al., 2014), by *in silico* location of the binding site using software such as Q-SiteFinder (Hayes, Stanton, Fitzgerald, & Ross, 2007), or by molecular docking with alignment of peptides and assessment of bond formation between peptides and enzyme (Duan et al., 2014). *In vivo* tests for measuring antihypertensive activity monitor the changes in systolic and diastolic blood pressures in spontaneously hypertensive rats (SHR; Babij et al., 2014; Contreras, Carrón, Montero, Ramos, & Recio, 2009; Lee, Qian, & Kim, 2010). Usually the effect of antihypertensive peptides on blood pressure is compared to antihypertensive drugs, such as captopril. *In vitro* assays use different substrates: hippuryl-histidine-leucine, furanacroloyl-phenylalanine-glycine-glycine, or *o*-aminobenzoylglycyl-*p*-nitro-L-phenylalanyl-L-proline (Alemán et al., 2011; Castellano, Aristoy, Sentandreu, Vignolo, & Toldrá, 2013; Phelan, Khaldi, Shields, & Kerins, 2014), and consequently they use different methods for enzyme product detection: RP-HPLC, spectrophotometry, and capillary electrophoresis (Byun & Kim, 2002; Garcia-Tejedor et al., 2014; Lee, Cheng, Enomoto, & Nakano, 2006). When correlating *in vitro* and *in vivo* antihypertensive tests on peptides, their stability has to be considered (Boutrou, Henry, & Sanchez-Rivera, 2015; Vermeirssen, Van Camp, & Verstraete, 2004). For example, García-Tejedor et al. (2014) found that longer peptides liberated during milk fermentation (such as DPYKLRP), which showed significant ACE-inhibitory activity *in vitro* ($IC_{50} = 30.5\ \mu M$), were almost completely hydrolyzed during simulated gastric digestion. However, gastric digestion can liberate more active, but smaller, peptides from longer sequences. For example, Miguel, Aleixandre, Ramos, and Lopez-Fandiño (2006) showed that 2 peptides from ovalbumin hydrolysate, YAEERYPIL and RADHPFL, showed relatively small ACE-inhibitory activities ($IC_{50} = 446$ and $521\ \mu M$, respectively), but *in vitro* after simulated gastric digestion, with liberation of products YPI and RADHP,

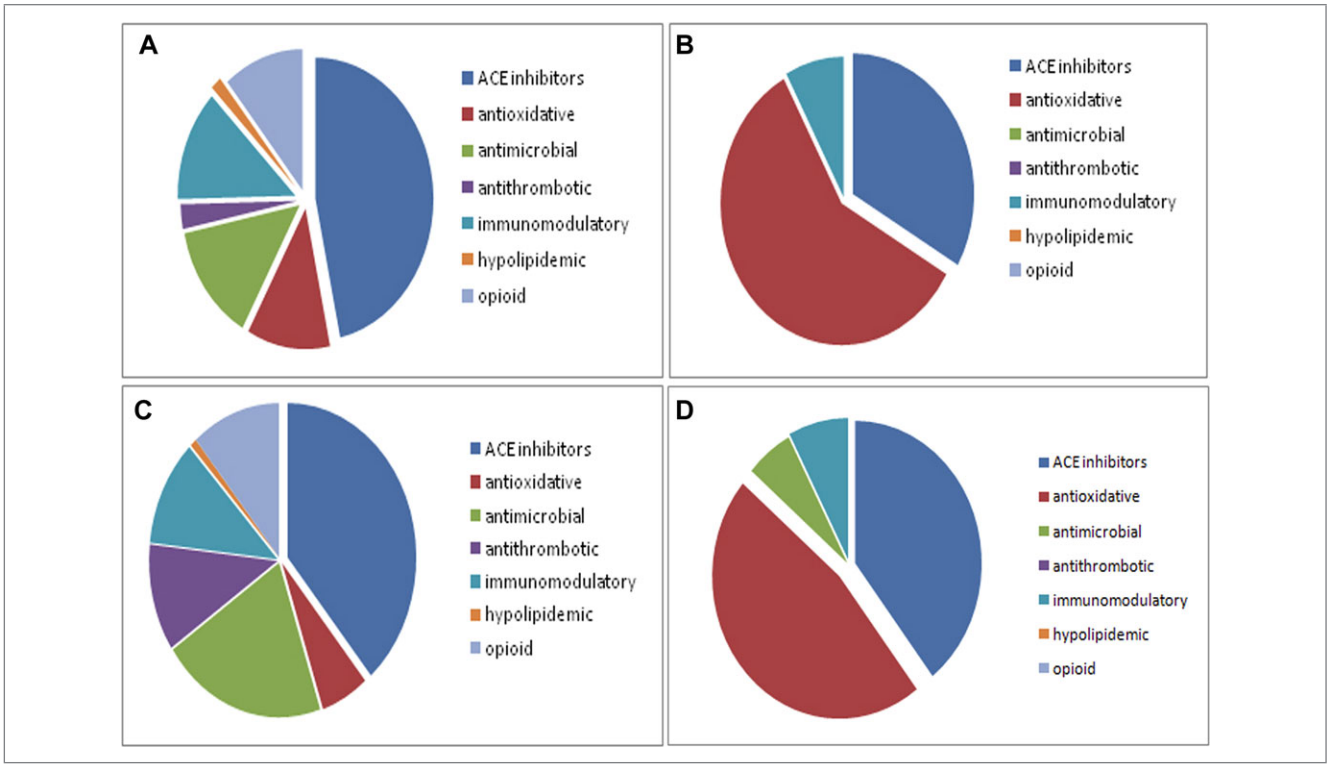


Figure 2–Frequency of different categories of bioactive peptides in particular food sources: (A) milk and dairy products, (B) meat, (C) eggs, and (D) marine products. Categories of bioactive effects are reported in the legend.

a significant decrease in systolic blood pressure was obtained (21.7 mm Hg and 32.13 mm Hg after 4 hours). But, even if a peptide is stable during simulated gastric digestion, activity can differ between *in vitro* and *in vivo* tests. Ren et al. (2011) reported that, although VSPVW from hydrolysate of porcine hemoglobin shows high ACE-inhibitory activity *in vitro* ($IC_{50} = 0.254$ mg/mL) and is stable under simulated gastric digestion, its administration in SHR led to only a small decrease of systolic pressure (only 4.9 mm Hg after 15 hours). This indicates that there is more than one mechanism by which antihypertensive peptides can exert their effect (Table 2; Majumder & Wu, 2015; Marques et al., 2012; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Moreover, the effects of antihypertensive peptides strongly depend on race, age, and gender of examined subjects (Cicero, Gerocarni, Laghi, & Borghi, 2011).

Antioxidative activity

In vitro antioxidative tests are generally divided into 2 categories based on their mechanisms: involving either hydrogen atom transfer (HAT) or electron transfer (ET) (Di Bernardini et al., 2011; Sarmadi & Ismail, 2010). Oxygen radical absorbance capacity is among the most popular HAT methods. The most-used ET tests are the 1,1-diphenyl-2-picrylhydrazyl assay, the 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid assay, and measurement of ferric ion-reducing antioxidant power (Alemán et al., 2011; Bougatef et al., 2009; Cheung, Cheung, Tan, & Li-chan, 2012; You et al., 2010). These tests are measuring very different parameters and consequently express antioxidative activity very differently. Therefore, correlation between results is hindered. Antioxidant activity can also be measured using lipid oxidation and peroxidation with the β-carotene bleaching assay and the thiobarbituric acid-reactive substances assay (Bougatef et al., 2009; Meister Meira et al., 2012). Unfortunately, antioxidative activities of peptides have rarely been tested *in vivo* and this is a major issue, because it has been shown that antioxidative activities of duck egg hydrolysate diminished after simulated gastric digestion (Ren, Wu, Li, Lai, & Xiao, 2014). Few *in vivo* tests have been performed that show activities of the enzyme involved in response to oxidative stress, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione transferase, or the concentration of malondialdehyde, the final product of lipid peroxidation (Ding et al., 2011; Nazeer, Kumar, & Ganesh, 2012).

Antimicrobial activity

Like antioxidative activity, antimicrobial activity is rarely tested *in vivo*. For *in vivo* testing, antimicrobial peptides are usually added to food or a food matrix, and in these conditions, the presence of compounds such as verbascoside and glycerophosphoinositol lysine changes the stability of peptides and also exerts synergistic effects (Tang et al., 2011; Vercelli et al., 2015). However, Ding, Liu, Bu, Li, and Zhang (2012) showed that Pt 5 peptide (phosvitin-derived peptide, which consists of C-terminal 55 residues of phosvitin) exerts an antibacterial effect against *Aeromonas hydrophila* in zebra fish. Bateman et al. (1996) found in an *ex vivo* experiment that MPCSCKKYCDPWEVIDGSCGLFNSKYICCREK peptide isolated from rat kidney not only affected the volume of villi cells in the jejunum of guinea pigs, but also stimulated synthesis of corticosterone (Table 2). Activity against microorganisms may be measured in a variety of ways, which again makes comparing results difficult.

Immunomodulatory activity

Due to the variety of mechanisms of activity of immunomodulatory peptides (Table 2), different assays target different aspects

of their antiallergenic, cytotoxic, antiproliferative/anticancer, or anti-inflammatory activities. The innate (nonspecific) immune response can be assessed through activation of complement, phagocytosis, activity of natural killer (NK) cells, mast cells, dendritic cells, and neutrophils (Beutler, 2004; Galli, Borregaard, & Wynn, 2011; Medzhitov & Janeway, 1997). Adaptive immunity response may be evaluated determining lymphocyte proliferation and concentration of subpopulation of T and B cells (Pancer & Cooper, 2006; Steinman & Hemmi, 2006). However, responses of innate and adaptive immunities are often linked through signaling by cytokines (Luster, 2002) or cell-to-cell interactions, for example, cooperation of dendritic cells and T lymphocytes in antigen presentation (Liu, 2001; Steinman & Hemmi, 2006) or involvement of certain cell types or groups of cells, such as NK cells (Moretta, Marcenaro, Parolini, Ferlazzo, & Moretta, 2008; Vivier, Raulet, Moretta, & Caligiuri, 2011), complement (Carroll, 2004), or neutrophils (Rosales, Demaurex, Lowell, & Uribe-Querol, 2016).

Due to the complexity of mechanisms and to variability of *in vivo* responses (Leroux-Roels et al., 1994; Table 2), focusing on specific changes in either innate or adaptive immunity leads to incorrect conclusions about peptide activities, which can be corrected by measuring changes in both adaptive and innate immunities (Maestri et al., 2016). Anti-inflammatory activity can be determined *in vivo* by the paw edema test, measuring swelling (as change in volume) after injection of inflammatory substance (Tavares et al., 2013), or *in vitro* by monitoring macrophage activity using chemiluminescence assays (changes in respiratory burst; Tompa et al., 2011), cytokine secretion (usually by ELISA; Tompa et al., 2011), or by monitoring monocyte adhesion to vascular endothelium (Aihara et al., 2009). Antiallergenic properties are measured *in vitro* determining IgE content by the reverse enzyme allergosorbent, ELISA, or enzyme allergosorbent tests (Falagiani et al., 1999; Wigotzki, Steinhart, & Paschke, 2001), or *in vivo* by measuring indexes of spleen and thymus status, such as changes in weight, hemolysin content, changes in optical density as a result of macrophage activity, and release of histamine from mast cells (Pan et al., 2013). Q8 Cytotoxic activity and apoptosis have been assessed measuring cell viability and proliferation by staining, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, acid phosphatase assay, neutral red uptake assay, lactate dehydrogenase assay, incorporation of [^{3}H] thymine, NK assay, or JAM test (Bateman et al., 1996; Chalamaiah et al., 2014; Hagiwara, Shinoda, Fukuwatari, & Shimamura, 1995; Hou et al., 2014; Repetto, del Peso, & Zurita, 2008; Richardson et al., 2009; Rodríguez Saint-Jean et al., 2013; Yang, Sinai, & Kain, 1996), apoptosis by the annexin V test and caspase assay (Green & Steinmetz, 2002; Hartmann, Wal, Bernard, & Pentzien, 2007), mitochondrial transmembrane potential (Hartmann et al., 2007), or DNA synthesis and damage (Hsieh, Ho, Lai, & Yen, 2003).

Correlation between *in vitro* and *in vivo* tests varies depending on the aspect of immunomodulatory activity, which is monitored (Clay, Hobeika, Mosca, Lyerly, & Morse, 2001). Thorpe et al. (2013) concluded that different cytokine subpopulations had different comparability with *in vivo* results. Leroux-Roels et al. (1994) reported that lymphocyte proliferation measured using the hepatitis B antigen-specific lymphoproliferative assay showed a high correlation (>95%) between results *in vitro* and *in vivo*. Development of a CFSE method (carboxyfluorescein diacetate succinimidyl ester) to measure lymphocyte proliferation (Quah, Warren, & Parish, 2007) allowed for a better correlation, whereas measurement of cytokine release and direct cytotoxicity showed a lower correlation with *in vivo* findings (Clay et al., 2001). These poor correlations with *in vivo* results are attributed to inaccurate evaluation of cytokine profiles in tumor cells and to relative insensitivity of cytotoxic assays (Clay et al., 2001). Experiments were performed either *ex vivo* or *in vivo* in mice and rats, but due to differences in the immunological response between mice and humans (Mestas & Hughes, 2004; Zschaler, Schlorke, & Arnhold, 2014), correlating responses between these species remain uncertain.

Hypolipidemic activity

Similarly as for antioxidative and immunomodulatory peptides, hypolipidemic peptides can exert their effect through different mechanisms (Table 2). For several of those mechanisms, such as removal of bile acids or inhibition of cholesterol solubilization, concentration of present peptides is of crucial importance. Tests for hypolipidemic activity measure different metabolic stages in blood, liver, and feces, or measure metabolite concentration and/or enzyme activity or changes in functionality. Functionality tests by monitoring, for example, arterial stiffness via pulse wave analysis (Turpeinen et al., 2009), pancreatic exocrine activity (Kagawa, Matsutaka, Fukuhama, Watanabe, & Fujino, 1996), taurocholate capacity, micellar solubility and absorption of cholesterol (Nagaoka et al., 2001), or speed of β-oxidation in liver mitochondria (Liaset et al., 2009), may help to elucidate the actions of particular peptides or to evaluate the risk of developing disease, such as atherosclerosis. Changes in metabolite concentrations as the final effect of bioactive peptides can be assessed, whereas measuring changes in activity of key enzymes allows identification of metabolic pathways, which are affected by particular peptides. Measurement of concentration of triglycerides, phospholipids, and total cholesterol in blood and liver may be done either by chromatography or by enzymatic kits (Liaset et al., 2009; Shimizu et al., 2006), while profiling of fatty acids in liver is done by HPLC or GC methods (Liaset et al., 2009). Changes in activity of enzymes involved in synthesis of fatty acids or their transport can highlight changes in metabolic flux and elevated or decreased risk of development of hyperlipidemia (Liaset et al., 2009; Wergedahl et al., 2004).

Opioid activity

Opioid activity is measured *in vitro* by the dissociation constant of opioid to opioid receptor complexes or by maximal binding capacity of peptide to opioid receptor (Antila et al., 1991; Meisel, 1986). Since adenylate cyclase is sensitive to opioid concentration, due to opioid-induced changes in the MAPK signaling pathway (Al-Hasani & Bruchas, 2011), opioid activity can also be measured by inhibition (or stimulation) of adenylate cyclase activity (Zioudrou, Streaty, & Klee, 1979). Another method is testing the antagonistic effect of opioid peptides on inhibition of muscular contraction (Chiba, Tani, & Yoshikawa, 1989; Zioudrou et al., 1979). Opioid activity is rarely measured *in vivo*; in some cases, observation of behavioral patterns has been used (Belyaeva, Dubynin, Stovolosov, & Kamensky, 2008). Q9

Antithrombotic activity

There are generally 2 types of tests for determining antithrombotic (anticlotting) activity depending on whether the effect is measured *in vivo* or *in vitro*. For *in vivo* measurements, percentage inhibition of thrombosis is measured after intravenous administration of peptide in lesions formed in arterial endothelium using lasers (Bal dit Sollier et al., 1996; Rojas-Ronquillo et al., 2012; Rutherfurd & Gill, 2000; Shimizu et al., 2009). *In vitro* tests measure effects of peptides on different stages of the coagulation

process. The activated partial thromboplastin time test is used to evaluate activity of coagulation factors in the presence or absence of anticoagulants, but it is known to have a nonlinear response (Gribkova et al., 2016). The influence of anticoagulants inactivating coagulation factor can be measured via specific factor activity tests, the intrinsic factor tenase (FXase) assay, or by assay of prothrombinase (Jung & Kim, 2009; Rajapakse, Jung, Mendis, Moon, & Kim, 2005). Inhibition at later stages of thrombosis can be monitored by platelet activation and adhesion tests, as well as by the fibrinogen binding assay (Jollès et al., 1986; Mazoyer et al., 1990; Rajapakse et al., 2005). Hemostatometry (shear-induced platelet function) gives results comparable with *in vivo* tests (Yamamoto et al., 2015). With the fibrin plate method, activity of fibrinolytic enzymes can be measured (Jung, Je, Kim, & Kim, 2002). But in terms of the mechanism of action of a given bioactive peptide, the most instructive data are obtained by surface plasmon resonance measuring the binding activity of peptide to coagulation factors (Jung & Kim, 2009).

Correlating Peptide Sequence with Bioactive Effect

Difference in types and number of AA residues can affect stability and activity of peptides (Li & Yu, 2015). To test how the frequency of particular classes of AA differed in bioactive peptides exhibiting different medicinal effects, a chi-square test was performed (Table 3). Taking data from Table S2, 807 peptide sequences were classified according to their activity and observed frequency of particular types of AA. We classified AA residues as aliphatic (glycine [G], alanine [A], leucine [L], isoleucine [I], proline [P], and valine [V]), aromatic (tryptophan [W], phenylalanine [F], and tyrosine [Y]), polar noncharged (asparagine [N], glutamine [Q], methionine [M], cytosine [C], serine [S], and threonine [T]), positively charged (histidine [H], arginine [R], and lysine [K]), and negatively charged (aspartic acid [D] and glutamic acid [E]; Table 3). The expected frequency of a particular type of AA residues was calculated assuming a normal distribution using the formula:

$$\text{Expected frequency} = \frac{(\text{number of amino acids in given group} \times \text{total number of peptides with particular effect})}{20}.$$

As seen from Table 3, for all types of AA, the chi-square test showed significant differences from expected frequencies: $\chi^2(1, 7) = 468.01; 82.69; 96.72; 60.19; 105.51$ for aliphatic, aromatic, polar, and positively and negatively charged AAs, respectively.

High percentages of aromatic and aliphatic AA residues (Table 3) in antihypertensive peptides are necessary for the formation of a complex with ACE (Yousr & Howell, 2015). Similarly, for the formation of stable complexes with opioid receptors, higher frequencies of aromatic AA residues are required (Table 3). The frequencies of both polar noncharged and aromatic AA are relatively high in antioxidative peptides (Table 3); H, P, C, Y, W, F, and M are all involved in the prevention of lipid peroxidation (Li & Yu, 2015) and involved in electron and proton transfer (Chi et al., 2015; Udenigwe & Aluko, 2011). Additionally, a high percentage of hydrophobic AAs helps solubilization in a lipid environment and allows for better access to hydrophobic targets (Yousr & Howell, 2015). In case of hypolipidemic peptides, L and K are crucial for interaction with bile acids (Howard & Udenigwe, 2013). Their complex formation involves hydrophobic bonds, which might explain the relatively high frequency of aliphatic and positively charged AA residues in hypolipidemic peptides (Table 3). Antithrombotic peptides act as competitive inhibitors for fibrinogen (Table 2), and a higher frequency of charged AAs in antithrombotic peptides (Table 3) can be explained with the similarities between AA composition of peptides and fibrinogen (Chabance et al., 1995). But a single explanation for the different frequencies of particular AA types for antimicrobial, and particularly for immunological peptides, is difficult because of the different modes of action (Table 2).

However, not only the type of AA, but also its position in the peptide can influence its activity. To correlate differences in positions and proportions of individual AAs in the peptides, we analyzed sequences using univariate ANOVA. The statistical significance was decided assigning to each AA a numerical value for unequivocal representation. The choice of values was made by referring to tabulated values or scores taken from the literature. The database AA index (www.genome.jp/aaindex/AA index/list of indexes, accessed in August 2018) and the accompanying paper by Tomii and Kanehisa (1996) list over 544 indexes and scores that have been used to classify AAs on the basis of their biochemical and physicochemical properties. Out of 46 indexes representing the physical and chemical properties of each AA, we focused on the scoring systems illustrated in Figure 5 [illegible]ion P of Tomii and Kanehisa (1996), choosing the index that showed the highest number of significant correlations to other indexes in the database. The rationale was to choose an index that could represent different properties of the AA. The index defined as "normalized van der Waals volume" developed by Fauchère, Charton, Kier, Verloop, and Pliska (1988) was considered the best for this purpose, because it correlates to 28 other indexes and assigns different numerical values to each AA, except for I and L, which have the same value. The AA values assigned by this index ranged from 0 for G to 8.08 for W; a complete list of the values is reported in Table S3. A subset of 649 peptides, of 2 to 10 AAs, was further analyzed by assigning to each AA the value given by Fauchère index. Since peptides longer than 10 AAs were represented by only a few examples, they were not amenable to statistical analysis. For each sequence, the values of Fauchère index corresponding to each AA were computed and subjected to statistical analyses (by SPSS v.21), taking into account the position of the AA in the sequence. As suggested by Udenigwe (2014), position of AA in peptide was labeled depended from its distance from C-terminus. Thus, position of AA next to C-terminus was labeled as –1, that on the second position from C terminus as –2, and so on. Figure S1 shows the distribution of the scores for each AA position in the subset of 649 peptides. The highest values ($P < 0.001$) corresponded to the AA residues at the C-terminus and at the N-terminus. AA residues position at the C-terminus had an average Fauchère index of 4.0, whereas AAs at the N-terminus had an average index of 3.9. To compare peptides with different biological effects, we averaged the scores for all sequences belonging to specific categories, calculating all AAs at all positions (ANOVA, $P < 0.0001$; Figure S2). Opioid peptides have a significantly higher average score, whereas antithrombotic peptides have the lowest value. Thus, with univariate ANOVA (Table 4), we showed that between categories of peptides, the only significant position was the N-terminus AA, and to a lesser extent, the AA next to the C-terminal residue. These

Table 3–Distribution and frequencies of aliphatic, aromatic, polar non-charged, positive charged and negative charged amino acids in peptides with different biological activities.

Amino acid type Biological activity	Aliphatic	Aromatic	Polar, noncharged	Positively charged	Negatively charged
Antihypertensive	49.93	14.49	17.58	12.85	5.14
Antimicrobial	46.78	11.76	25.04	21.68	5.71
Antioxidative	43.94	13.80	17.70	14.01	10.54
Antithrombotic	46.78	3.51	20.46	18.72	10.52
Hypolipidemic	60.00	4.00	8.00	14.00	14.00
	49.63	14.46	16.99	14.16	4.77
Immunomodulatory					
Opioid	46.92	28.85	17.84	4.41	1.99
χ^2 value	486.01	82.69	96.72	60.19	105.51
Degrees of freedom	1,7	1,7	1,7	1,7	1,7
Significance	***	***	***	***	***

***$P < 0.001$

results are in agreement with results obtained using c itative structure-activity relationship modeling (Chou, 2011).

Univariate ANOVA showed that the opioid peptides had a significantly higher value, 5.4, for AA at the N-terminus with respect to other classes of peptides (Table 4), which is consistent with the finding that opioid peptides are characterized by high percentages of aromatic AAs, especially for those located at the N-terminus (Kostyra, Sienkiewicz-szapka, Jarmoowska, Krawczuk, & Kostyra, 2004; Meisel, 1998; Silva & Malcata, 2005). Aromatic AAs (Y, P, and W) have the highest value of the Fauchère index (Table S3).

The analysis was carried out independently for each class of peptide length; nonparametric tests were applied due to deviations from the normal distribution (Table S4). The N-terminal AA shows significant differences in distribution in peptides of 4, 5, 7, and 8 residues, with opioid peptides showing consistently higher values, as reported in Table 4. The C-terminal AA is significant in peptides of 2 and 5 AAs. Within dipeptides, the antioxidants show significantly higher values in the 2nd AA. In 9 AA peptides, 2 positions are significant: opioid peptides have higher values for AA minus 6, whereas antimicrobial peptides show higher values in AA minus 7.

Additionally, we used the WebLogo tool (Crooks, Hon, Chandonia, & Brenner, 2004) to test frequencies of individual AA residues in peptides of 2 to 10 AA residues. Distribution of AAs at different positions was skewed; for instance, Y was prevalent in the first position and P at intermediate positions (Figure S3).

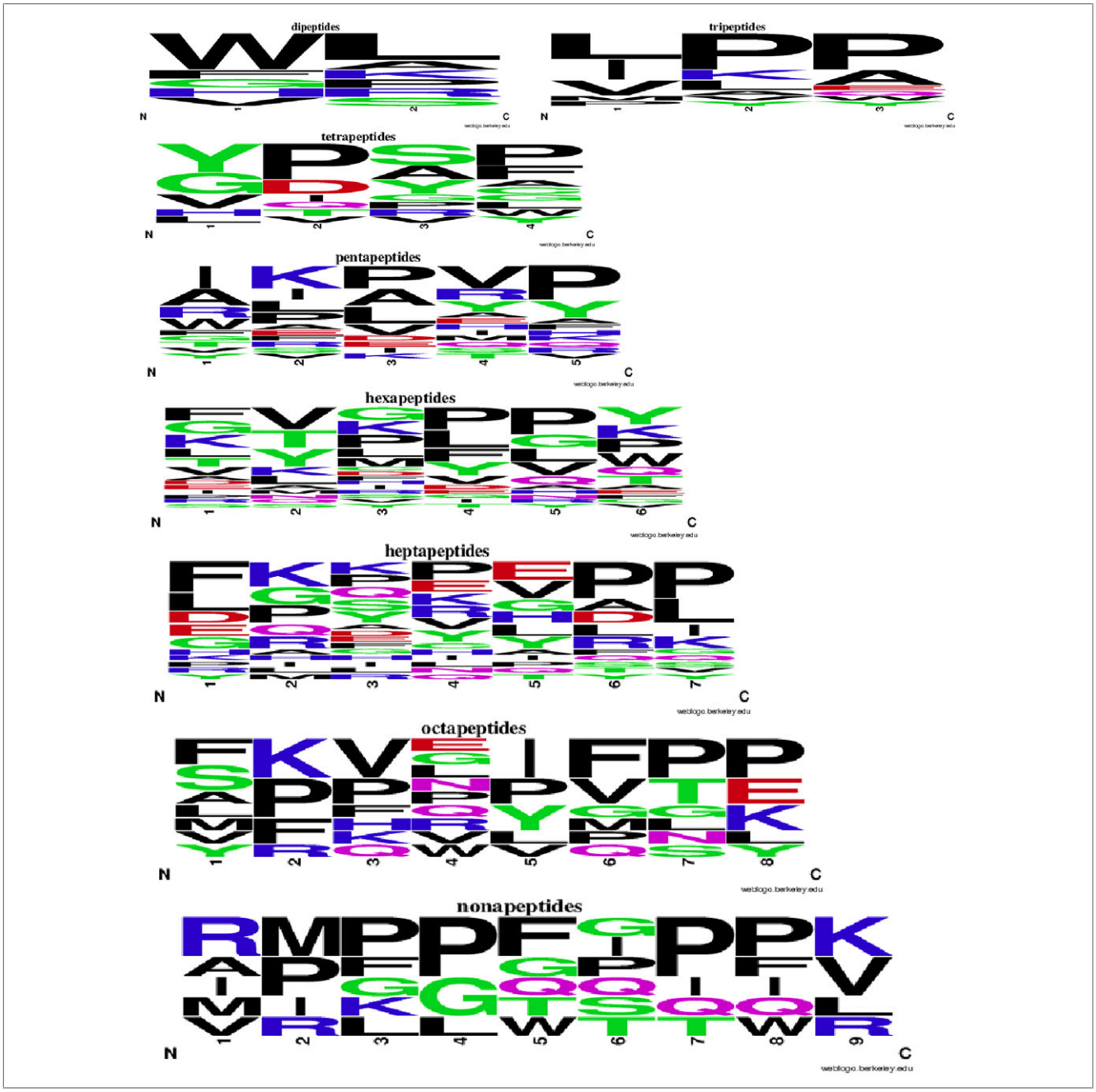
The significance of some types of AAs at particular positions is well documented in the literature. For example, antioxidative peptides containing the LG sequence or with H close to the N-terminus exhibit higher activity compared to antioxidative peptides with the same sequences elsewhere in the chain (Li & Yu, 2015; Zou, He, Li, Tang, & Xia, 2016). Similarly, peptides with pronounced antihypertensive effect often have branched AAs at the N-terminus and aromatic AAs at the C-terminus (Li, Le, Shi, & Shrestha, 2004). Opioid peptides with marked activity possess Y as the N-terminal AA because the phenolic hydroxyl enhances opioid effects (Meisel, 1998). If secondary structure is present, as for example with anticancer cationic amphiphilic peptides, which are characterized by the presence of an α-helix, selectivity at individual residues on the basis of AA structure could both enhance activity and provide a stabilizing effect (Schweizer, 2009).

Behavior of Bioactive Peptides in Humans

An interesting question when preparing functional food is: should bioactive peptides, pre-prepared by enzymatic hydrolysis or fermentation, be added directly into food products? The answer depends mainly on absorption and stability of bioactive peptides (Brandsch, Knütter, & Bosse-Doenecke, 2008; Foltz et al., 2010; Gao, Sudoh, Aubé, & Borchardt, 2001; Jahan-Mihan, Luhovyy, El Khoury, & Anderson, 2011; Renukuntla, Vadlapudi, Patel, Boddu, & Mitra, 2013; Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). Factors influencing peptide resistance to protease degradation and their absorbance from enterocytes into blood include:

Number and type of AA residues

There are 3 ways in which protein can enter the cell: paracellular, transcellular, and via specific transporters. Paracellular transport is involved in transport of small molecules, such as glucose through gaps between cells. The paracellular route is usually inaccessible to peptides due to the existence of the tight junctions between enterocytes (Assimakopoulos, Papageorgiou, & Charonis, 2011; Stevenson & Keon, 1998). However, permeability of this tight junction is dependent on Ca^{2+} levels and is different for different epithelia; thus, permeability of tight junctions can be altered temporarily to allow flow of water-soluble compounds (Arhewoh, Ahonkhai, & Okhamafe, 2005).The preferred route is dependent on the number of AA residues in the bioactive peptide. Peptides with 2 or 3 AA residues might be absorbed into enterocytes utilizing the PepT1 transporter (Brodin, Nielsen, Steffansen, & Frokjaer, 2002) or in renal epithelia via either PepT1 or the additional PepT2 transporter (Yang, Dantzig, & Pidgeon, 1999). PepT1 and PepT2 belong to a family of proton-dependent oligopeptide transporters, which use gradients created by Na^+/H^+ exchange to cotransport peptides with H^+ ions (Newstead et al., 2011). Son, Satsu, Kiso, and Shimizu (2004) showed in Caco-2 cells that the peptide carnosine is transported via PepT1. The major difference between PepT1 and PepT2 is that PepT2 is a high-affinity transporter, while PepT1 is low-affinity. This difference in affinity stems from the different physiologies of intestines and kidneys. But not all di- and tripeptides enter via Pep transporters. Again in Caco-2 cells and for the tripeptide VPP, the paracellular route was preferred for transport; inhibition of PepT1 did not interfere with transport of peptide across the Caco-2 monolayer (Satake et al., 2002). Peptides of greater than 3 or 4 residues are adsorbed via the transcellular route, using receptor- or nonreceptor-mediated endocytosis (Segura-Campos et al., 2011). Peptides transported via the transcellular route include bradykinin (RPPGFSPFR), FRADHPFL, YPFPG, PFGK, and GGYR (Shimizu, Tsunogai, & Arai, 1997).



COLOUR ONLINE, B&W IN PRINT

Figure 3–WebLogo representation of the distribution of individual amino acid residues in peptides showing activity *in vivo*. Color code: black—hydrophobic AA (A, V, L, I, P, W, F, and M); red—acidic (D and E); blue—basic (K, R, and H); purple—neutral (Q and N); green—polar (G, S, T, Y, and C). Height of the letter represents the frequency of amino acids at the given position.

Type of AA

Stability of peptides to proteolysis depends on the specific AA residues present. It has been shown that peptides containing proline and hydroxyproline residues are less prone to proteolysis in the intestines: dipeptides and oligopeptides isolated from casein and gelatine contain proline residues in the C-terminal region and are therefore resistant to peptidases specific for this AA (FitzGerald & Meisel, 2000); these results were confirmed in experiments by van der Pijl, Kies, Ten Have, Duchateau, and Deutz (2008) who showed that peptides rich in proline reach circulation intact. They also found that the half time for elimination was at maximum 15 minute, suggesting this peptide could have an acute effect. To test whether P was more frequent as compared to other AA residues in bioactive peptides, we extracted 120 bioactive peptides from Table S2 whose activity was tested *in vivo*. Percentages of individual AAs in peptides of 2 to 9 residues were calculated and their frequency was represented with the tool WebLogo (Crooks et al., 2004; Figure 3). P was present in 67% of the sequences, and 6.7% of these had PP doublets, which confer stability against serum proteases (Foltz et al., 2007; Jambunathan & Galande, 2014). This high percentage of P residues in stable peptides might be the consequence of conformational changes, which reduce affinity of proteases to such peptides (Apostolovic et al., 2016). However, the position of P depended on peptide length (Figure 3).

Table 4–Scores for normalized van der Waals volume of amino acids at the different positions of peptides (up to 10 amino acids), classified by category of biological activity.

AA position (nr of cases) Biological activity[$]	First AA (649)	Minus 8 (31)	Minus 7 (81)	Minus 6 (135)	Minus 5 (202)	Minus 4 (301)	Minus 3 (396)	Minus 2 (478)	Minus 1 (578)	Last AA (649)
Antihypertensive	3.68^{bc}	3.15^{a}	3.48^{a}	3.18^{ac}	3.40^{a}	3.76^{a}	3.69^{a}	3.28^{a}	3.49^{ab}	3.93^{ab}
Antimicrobial	3.40^{bc}	//	3.85^{a}	4.39^{b}	3.22^{a}	3.18^{ac}	3.33^{a}	3.29^{a}	4.27^{a}	4.27^{a}
Antioxidative	4.05^{b}	3.30^{a}	2.62^{b}	3.72^{a}	3.32^{a}	3.36^{ac}	3.47^{a}	3.33^{a}	3.42^{ab}	4.24^{a}
Antithrombotic	2.82^{c}	1.36^{c}	1.45^{c}	3.69^{a}	2.61^{b}	2.48	2.98^{b}	2.93^{a}	3.36^{ab}	3.00^{b}
Hypolipidemic	2.78^{c}	//	//	//	//	4.00^{ab}	3.59^{a}	2.93^{a}	3.96^{ab}	3.94^{ab}
Immunomodulatory	4.24^{b}	3.38^{a}	2.88^{b}	3.52^{a}	3.38^{a}	3.40^{a}	3.45^{a}	3.70^{a}	3.05^{b}	4.00^{ab}
Opioid	5.49^{a}	4.23^{b}	3.46^{a}	4.34^{b}	3.22^{a}	3.99^{ab}	3.12^{a}	3.70^{a}	3.94^{ab}	4.11^{ab}
Total sample	3.93^{bc}	3.30^{a}	3.18^{ab}	3.56^{a}	3.33^{a}	3.60^{a}	3.51^{a}	3.36^{a}	3.52^{ab}	4.01^{ab}
Degrees of freedom	6	4	5	5	5	5	6	6	6	6
F	9.17	1.85	2.34	1.60	0.28	1.27	0.95	0.80	2.21	1.77
Significance	***	ns	*	ns	ns	ns	ns	ns	*	ns

[$] For each amino acid position, the average values are reported for each category, without standard error. Different letters in columns represent significant differences among categories according to Duncan post hoc test.
* $P < 0.05$
*** $P < 0.001$
ns, not significant.

Although P was located at the C-terminus in the majority of peptides, in smaller peptides (tripeptides to pentapeptides), additional P residues occurred at position minus 2; in larger peptides (6 to 9 AA residues), additional P residues are located at position minus 1 and at the position adjacent to the N-terminus. From WebLogo results (Figure 3), it can be seen that PP sequences were more frequent in heptapeptides to nonapeptides. However, this analysis did not include a sufficiently large number of antioxidant and antimicrobial peptides with an activity tested *in vivo*. Moreover, peptides larger than 9 AA residues are rare, and therefore excluded from the analysis.

Although the presence of P enhances stability toward proteases, it also interferes with transport inside the cell; P-containing sequences (for example PA, PD, PS, PE, and PG) showed a low affinity toward PepT1 (Vig et al., 2006). In addition, Chen, Pan, Wong, and Webb (2005) discovered that, although tripeptide WWW displays high affinity to PepT1, it is not transported into the cell. The factors that limit transport via PepT1 and PepT2 are not clear. Moreover, the individual residues present at the C- or N-terminus can affect stability of peptides. In Caco-2 cells, it was shown that peptides of different sizes, in which the C- and N- terminals were blocked, pass through the intestinal epithelium (Segura-Campos et al., 2011). In addition, even a single AA residue can change enzyme activity; Quirós et al. (2009) showed that when penultimate L in LHLPLP was substituted with G (giving peptide LPLPGP), ACE-inhibitory activity increased 2-fold.

Overall charge of peptide

Studies in Caco-2 monolayers (Pauletti, Okumu, & Borchardt, 1997; Satake et al., 2002) showed that charge influences paracellular transport of peptides. In the case of larger peptides (for example, hexapeptides), size is the limiting factor, while for tri- or tetrapeptides, transport is charge-dependent in the order negatively charged > positively charged > neutral. Yang et al. (1999) showed that for dipeptides to be transferred into a cell, the overall positive charge must be less than 2 units. This is consistent with the transporter proton-coupling discussed below.

Concentration of bioactive peptides

As for all protein carriers, PepT1 and PepT2 show typical saturation kinetics (Chen, Zhu, Smith, & Hediger, 1999; Sala-Rabanal, Loo, Hirayama, Turk, & Wright, 2006; Sala-Rabanal, Loo, Hirayama, & Wright, 2008); Chen et al. (1999) found that PepT2 couples transport of 1 peptide molecule to 2 H^+, while PepT1 transports only 1 peptide per H^+. These findings were confirmed by experiments showing that the time of gastrointestinal absorption of casomorphins was dose-dependent (Jahan-Mihan et al., 2011). However, Chen et al. (1999) monitored transport of radioactive labeled (D)F-(L)A and (D)F-(L)E and found that transport of neutral and anionic dipeptides had different stoichiometries for anionic peptides, substrate was cotransported with 3 proton ions, while for neutral peptides, 2 protons were transported per 1 molecule of peptide.

pH

Energy for transport of bioactive peptides is derived from the pH gradient across cell membranes and changes in pH can affect their transport. Brandsch et al. (1997), in experiments with Caco-2 cells, showed that treatments with both H^+ and diethylpyrocarbonate modulate the speed of transport. Since diethylpyrocarbonate blocks the acceptor/donor function of

histidine residues, these results lead to the conclusion that H plays a crucial role in activity of PepT1 and PepT2.

Age of the studied organism

Changes in concentration and activity of digestive enzymes during aging are well documented (Bauer et al., 2013; Greenberg & Holt, 1986; Lindemann, Cornelius, el Kandelgy, Moser, & Pettigrew, 1986); this can affect the kinetics of peptide degradation (Barbé et al., 2014; Mandalari et al., 2009; Roufik, Gauthier, & Turgeon, 2006). *In vivo* experiments with ^{15}N-labeled casein and lactoferrin (Drescher et al., 1999) showed that in suckling pigs, but not in adults, lactoferrin is digested more slowly than casein. One possible explanation for this result is that the gastrointestinal tract of young pigs is not completely developed and therefore digestion is slower. Similarly, in human infants, digestibility of lactoferrin is slower compared to casein (Lönnerdal, 2014).

Nutrition

A high-protein diet results in a greater number of PepT1 molecules (Chen et al., 2005) and, therefore, in a higher number of circulating peptides; there was an increase in PepT1 mRNA when chickens were fed with 18% and 24% crude protein, while feeding chickens with 12% crude protein resulted in a decrease in PepT1 mRNA. Receptor-mediated transport was affected in both cases. One possible explanation for this dependency was given by Gilbert, Wong, and Webb (2008), who suggested that a possible mechanism for regulation of the number of peptide transporters might occur in response to direct interaction with substrate.

Moreover, for certain peptides although their effect can be observed at the systemic level, there are tissues that preferentially exhibit the effect. Matsui et al. (2004) found that the antihypertensive peptide VY shows the highest degree of ACE-inhibitory activity in abdominal aorta and kidneys.

Some naturally occurring modifications can also have a positive effect on protein stability. Picariello et al. (2013) found that monophosphorylated derivatives of lactoglobulins showed stability toward degradation in the gut; according to the authors' interpretation, these modifications increase the size of a peptide, which in turns make the paracellular transport more plausible, even though paracellular transport usually concerns smaller molecules. Ulluwisheva et al. (2011) suggested that such change in permeability might be due to interaction with intestinal bacteria. It should be pointed out that higher stability of bioactive peptides may lead to toxicity, due to their slower degradation (Hartmann & Meisel, 2007).

Impact of Food Processing Operations

Table 6 shows the main factors that influence bioactive peptides during processing: temperature, pressure, hydrolysis, and fermentation; these treatments change the structures of source proteins, influencing the release of bioactive peptides and their stability and adsorption in the gut lumen. Since stability toward gastrointestinal proteases, adsorption into enterocytes, and transport from enterocytes into the circulation are necessary requirements for peptides to exhibit their effects, changes in these parameters can result in alteration of food properties, which is of crucial importance in claims about food functionality.

Effect of thermal processing

Heat treatments are used in food industry to increase the safety of products and to prolong shelf-life. Effects of heat processing are greatly dependent on temperature applied and time of processing. Generally, heat treatment results in the denaturing of proteins and in subsequent aggregation of peptides due to formation of covalent and/or noncovalent bonds (Rombouts et al., 2015). Such intra- and intermolecular bonding can influence both digestibility and absorption of the produced bioactive peptides (Wada & Lönnerdal, 2014). Additional factors that reduce digestibility, and thus lead to a higher adsorption of bioactive peptides, are the possibilities of racemization of L-AAs into D-isomers during heat treatment (Hajirostamloo, 2010). Reactions enhanced by heat treatment, such as the Maillard reaction, can have a detrimental effect on nutritive value (Tamanna & Mahmood, 2015) but can also enhance the availability (Jiang, Rai, O'Connor, & Brodkorb, 2013) and the physiological effect of bioactive peptides. For example, conjugation of fragments from β-lactoglobulin with sugar residues during the Maillard reaction can reduce allergenicity of cow milk (Bu, Luo, Chen, Liu, & Zhu, 2013), a result that can be explained by the destruction of epitope(s) (Thomas et al., 2007). Pretreatment heat can enhance existing health effects of bioactive peptides: Adjonu, Doran, Torley, and Agboola (2013) found that pretreatment at temperatures higher than 90 °C enhanced ACE-inhibiting activity of a β-lactoglobulin fragment. Results of Jayatilake et al. (2014) imply that treatment at low temperatures could also enhance the anti-inflammatory action of whey proteins, due to higher retention of immunoactive proteins and factors, such as immunoglobulins, lactoferrin, and growth factors (Nguyen et al., 2015). Other factors that could be important include the type of heat source: Gomaa (2010) found that microwave heating not only enhanced antioxidative activity of β-lactoglobulin hydrolysate, but the same hydrolysate also presented unique proteins, an indication that microwave treatment may have exposed new cleavage sites within the protein. Since geometry of proteins in skim milk was also changed during microwave heating (Tu et al., 2014), microwave treatment can also enhance digestibility of milk.

Influence of enzymatic hydrolysis

The effect of enzymatic hydrolysis on activity of peptides obtained from different food sources has been analyzed (Huang et al., 2010; Lahart et al., 2011; Liaset et al., 2009; Liu, Baggerman, Schoofs, & Wets, 2008; Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008; Morimatsu et al., 1996; Rizzello et al., 2005; Stuknyte, De Noni, Guglielmetti, Minuzzo, & Mora, 2011). Does enzymatic proteolysis result in peptides with improved effect when compared to the activity of the whole protein? Table 5 lists examples of activity in protein hydrolysates as compared to the whole protein in different types of food products. It is evident that in all cases, with the 1 exception of antioxidative activity of eggs, protein hydrolysates had higher activity. It is also evident that comparison of hydrolysate activities with those of source protein(s) is limited to only a few food sources, and that most of these experiments were performed *in vitro* rather than *in vivo*.

The number and the structure of peptides released during hydrolysis depend on type of enzyme(s) used and on reaction conditions. For example, yak milk casein hydrolysate showed a higher anti-inflammatory activity when prepared with Alcalase and trypsin rather than when prepared with pepsin or papain (Mao, Cheng, Wang, & Wu, 2011). Roufik et al. (2006) found that β-lactoglobulin fragment 142 to 148 remains intact after incubation with pepsin, but is strongly hydrolyzed after incubation with chymotrypsin. When the antioxidative activities were compared of different hydrolysates, obtained from tuna backbone protein, it was found that the peptic hydrolysate exhibited the highest activity as compared to those obtained with Alcalase, chymotrypsin,

Table 5–Differential effects of bioactive peptides in food subjected or not subjected to enzymatic pretreatment.

Effect	Source of bioactive proteins	Bioactive peptides present in hydrolysate	Whole protein product
Antihypertensive	Meat and meat products	Hydrolysate of myosin (Nakashima et al., 2002)	
	Milk and milk products	Fermented milk (Chen et al., 2007; Quirós et al., 2007; Tsai et al., 2008) Extract from cheeses (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000) Whey protein (Guo et al., 2009)	Low ACE inhibition of casein compared to hydrolysates (Mao, Ni, Sun, Hao, & Fan, 2007; Mullally, Meisel, & FitzGerald, 1997)
	Fish and fish products	Hydrolysates of sardinella (Bougatef et al., 2008)	Protein extract showed lower activity compared to hydrolysate (Bougatef et al., 2008)
	Eggs	Hydrolysate of egg white protein (Liu et al., 2010)	Ovotransferrin had lower ACE inhibitory activity compared to hydrolysates (Liu et al., 2010)
Antimicrobial	Meat and meat products	Hydrolysate of bovine hemoglobin (Froidevaux et al., 2001)	
	Milk and milk products	Hydrolysates of casein (McCann et al., 2006; McClean, Beggs, & Welch, 2014)	Extract of several cheeses showed no antimicrobial activity (Rizzello et al., 2005)
	Fish and fish products	Hydrolysate of phosvitin (Ding et al., 2012)	
	Eggs	hen egg lysozyme (Abdou, Higashiguchi, Aboueleinin, Kim, & Ibrahim, 2007)	
Antioxidative	Meat and meat products	Porcine protein hydrolysate (Yang et al., 2013)	
	Milk and milk products	Caseinophosphopeptide (Kim et al., 2007)	Lower effect than for casein hydrolysates (Mao et al., 2011)
	Fish and fish products	Tuna backbone protein (Je et al., 2007)	
	Eggs	Hydrolysis of crude egg white (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004)	Hydrolysate showed higher antioxidative activity than ovotransferrin (Huang, Majumder, & Wu, 2010)
Antithrombotic	Meat and meat products	Defatted pork meat (Shimizu et al., 2009)	
	Milk and milk products	Hydrolysates of casein (Chabance et al., 1995; Jollès et al., 1986; Rojas-Ronquillo et al., 2012)	
	Fish and fish products	n.a.	n.a.
	Eggs	Egg white protein hydrolysate (Yang, Wang, & Xu, 2007)	
Hypolipidemic	Meat and meat products	Pork liver hydrolysate (Shimizu et al., 2006)	Lower hypocholesterolaemic effect of untreated pork meat compared to papain hydrolysate (Morimatsu et al., 1996)
	Milk and milk products	Hydrolysate of β-lactoglobulin (Nagaoka et al., 2001)	Lower effect of casein compared to either casein hydrolysate or fish protein hydrolysate (Liaset et al., 2009; Wergedahl et al., 2004)
	Fish and fish products	Fish protein hydrolysate (Liaset et al., 2009; Wergedahl et al., 2004)	
	Eggs	n.a.	n.a.
Immunomodulatory	Meat and meat products	Beef sarcoplasmic protein hydrolysates (Jang et al., 2008)	
	Milk and milk products	Casein hydrolysates (Lahart et al., 2011; Stuknyte et al., 2011)	Lower effect compared to hydrolysate (Lahart et al., 2011; Stuknyte et al., 2011)
	Fish and fish products	Fish protein concentrate (Duarte, Vinderola, Ritz, Perdigón, & Matar, 2006)	Lower effect compared to hydrolysate
	Eggs	IRW peptide from egg protein (Huang et al., 2010)	
Opioid	Meat and meat products	n.a.	n.a.
	Milk and milk products	α_{s1}-, β- and κ-caseins from bovine milk (Antila et al., 1991; Pihlanto-Leppälä, Antila, Mäntsälä, & Hellman, 1994)	
	Fish and fish products	n.a.	n.a.
	Eggs	n.a.	n.a.

n.a., information not available.

Neutrase, papain, and trypsin (Je, Qian, Byun, & Kim, 2007). In addition, activity of a hydrolysate might be dependent on the pH at which it was obtained. Kim, Jang, and Kim (2007) found that antioxidative activity of caseinophosphopeptides obtained by hydrolysis with Alcalase at pH 8 was lower when compared to hydrolysates prepared under acidic conditions (pH 3 to 5). These differences were due to changes in the contents of particular AA residues. Interestingly, no direct correlation was found between degree of hydrolysis and immunological effect of casein hydrolysate (Lahart et al., 2011). Extent of hydrolysis will also affect the activity of peptides. For example, Miguel et al. (2006) found that when LHLPLPL was hydrolyzed to LHLPLP, its ACE-inhibitory activity increased, because LHLPLP is the true inhibitor of the enzyme. The same observation was made when TQPKTNAIPY was hydrolyzed to TNAIPY (Ruiz, Ramos, & Recio, 2004).

Table 6–Effect of temperature, pressure, enzymatic hydrolysis, and fermentation on stability and activity of bioactive peptides during food processing.

Bioactive peptides			
Enzymatic hydrolysis	**Fermentation**	**Thermal processing**	**Pressure**
- Type of enzyme used in hydrolysis influences degree of bioactive effect (for example, pepsin hydrolysate of casein shows higher antimicrobial activity compared to hydrolysate of trypsin and chymotrypsin; McCann et al., 2006). - Extent of hydrolysis has a variable influence on activity (Lahart et al., 2011), but hydrolysate is more active (Mao et al., 2011).	- Effect of fermentation is highly dependent on type of microorganisms used. (Marshall & Tamime, 1997) - Often used with proteases to enhance production of bioactive peptides (for example, ACE-inhibitory peptides; Chen et al., 2007). - Proteinases from different strain of *Lactobacillus* are unable to produce bioactive peptides themselves, but produce oligopeptides that can generate bioactive peptides after digestion by pepsin and trypsin (Gobbetti et al., 2002). - Fermented products contain high number of bioactive peptides, relatively resistant to proteolysis in simulated condition of gastrointestinal digestion (Kopf-Bolanz et al., 2014).	- Possibility of Maillard reaction between the lysine residues and carbohydrates (Korhonen, Pihlanto-Leppäla, Rantamäki, & Tupasela, 1998)—this might lead to changes not only in nutritional value, but also in biological effect. - Milk is especially prone to undergo Maillard reaction (high content of Lys residues and lactose). - Treatment with Alcalase and increased temperature increases content of sulfhydryls groups (therefore, enhances antioxidative effect) in porcine plasma hydrolysate (Yang et al., 2013). - Heat treatment also enhances antioxidative, ACE-inhibitory and opioid properties of bioactive peptides (Adjonu et al., 2013). - Use of slightly higher temperature during ripening enhances ACE-inhibitory activity in cheese (Sahingil et al., 2014).	- Low pressures usually induce reversible changes such as dissociation of protein-protein complexes, the binding of ligands and conformational changes (Korhonen et al., 1998). - High pressure treatment induces irreversible denaturation and reduces antigenicity of product (Cheftel & Culioli, 1997; Messens et al., 1997). - High pressure treatment of casein results in increase in both hydrolysis by pepsin and digestibility of β-lactoglobulin (Zeece et al., 2008).

Influence of pressure

Pressure affects the functionality of food and has been tested as a possible replacement for heat treatment to prolong shelf-life and/or to enhance digestibility of allergenic proteins. During high-pressure treatment, hydrogen and ionic bonds in protein molecules are broken (Messens, Van Camp, & Huyghebaert, 1997), which in turn, facilitates digestibility of proteins (Zeece, Huppertz, & Kelly, 2008) even more efficiently than heat treatment (Hoppe, Jung, Patnaik, & Zeece, 2013). This may be desirable for allergenic proteins, like ß-lactoglobulin or egg white protein. Although the effects on bioactivity depend mainly on the applied conditions, the structure of the whole protein may also be important: Castellani, Guérin-Dubiard, David-Briand, and Anton (2004) found that the irregular structure of phosvitin makes it very resistant to high pressure and does not change its iron-binding capacity, whereas Piccolomini, Iskandar, Lands, and Kubow (2012) demonstrated that application of high pressure for pretreatment of hydrolysates increases their antioxidant and anti-inflammatory effects.

Influence of fermentation

The effect of fermentation on production of bioactive peptides depends on several factors, the most significant being the structure of the food matrix (FitzGerald & Murray, 2006; Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002), the important bacterial strains, species, or genera, in both starter and adjunct culture (Marshall & Tamime, 1997; Sahingil, Hayaloglu, Kirmaci, Ozer, & Simsek, 2014), and the fermentation conditions including duration, temperature, and pH (Chen, Tsai, & Sun Pan, 2007; Guo, Pan, & Tanokura, 2009; Tsai, Chen, Pan, Gong, & Chung, 2008). *Lactobacillus* species or specific strains thereof are commonly used for making fermented milk products, and even the type of strain can influence not only the number of released bioactive peptides, but also their activity. These differences are due to the presence of different isomers of cell wall proteases in different bacteria (Hafeez et al., 2014), while activity of cell wall proteases may differ across strains (Galia, Perrin, Genay, & Dary, 2009; Stuknyte et al., 2011); however, it is not known whether peptides formed in this way can remain intact long enough to exert their activity. Some peptides are stable during homogenization and pasteurization, including antihypertensive peptides RYLGY and AYFYPEL (Contreras et al., 2009). Similarly, after ingestion of yogurt, bioactive peptides with antioxidative activity obtained by digestion of α and γ casein have been found in human blood (Chabance et al., 1995). On the other hand, peptides like isracidin and casocidin are degraded during fermentation and can only be added at the very end of production, or to the final product (Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014).

Prediction of Peptide Release, Stability, and Activity

The greatest challenges in formulating a novel bioactive food are to predict which peptides will be released from a particular protein, if they will be resistant to degradation by proteases, and what their biological activity will be. Antunes, Andrade, Ferreira, Nielsen, and Sarmento (2013) have discussed the application of models to predict intestinal absorption of therapeutic peptides.

Experiments assessing activity of peptides are usually performed *in vitro*; liberation of bioactive peptides from source protein is determined with digestion models intended to simulate the condition present in the gastrointestinal tract. Two types of digestion models have been employed: static and dynamic. Static, or biochemical, models imply that digestion phases (oral, gastric, and intestinal) are performed in a single bioreactor without mimicking mixing of food in the stomach or peristalsis in the gastrointestinal tract. Static models differ in several important aspects: type and concentration of enzyme used, number of steps and time of particular phase of digestion, rate of stirring, and other parameters (Hur, Lim, Decker, & McClements, 2011). Dynamic models include division into subcompartments, mimicking different structures and functionalities in the gastrointestinal tract, and account for physical forces and changes in kinetics during digestion (Thuenemann, 2015).

Dynamic models represent a novel approach and have significant advantages over static models, including accounting for the effects of peristalsis and transition of proteins between different compartments of the gastrointestinal tract (Dupont & Mackie, 2015; Picariello, Mamone, Nitride, Addeo, & Ferranti, 2013). Both static and dynamic models neglect specific issues such as correlation with *in vivo* models, accounting for different digestion times in individual compartments depending on type of food, predigestion processes in the mouth, and, very significantly, "matrix effects" (McClements et al., 2015; Rein et al., 2013). Matrix effects are crucial not only in determining the cleavage sites, but also the kinetics of proteolysis (Barbé et al., 2014). Complex matrices, which are degraded slowly, will release more bioactive peptides (Hernández-Ledesma et al., 2004).

However, the biggest problem with *in vitro* models is correlation with *in vivo* studies. *In vitro* systems, for standardization purposes, employ purified enzymes, often alone or in combination with no more than 2 or 3 additional enzymes. However, under physiological conditions, factors such as pH, concentration of salts, presence of isoforms or inhibitors, and activators of enzymes influence not only speed of reaction, but also type of peptide produced. Different conditions will result in release of different types of bile salt emulsifiers, producing changes in emulsion structure and creating problems of correlation with *in vitro* models (Marciani et al., 2007). It is also necessary to consider the interaction between different food components (for example, addition of sugars or lipids) and component characteristics (for example, phosphorylation), which can lead to changes in both digestibility and stability (Rein et al., 2013).

Prediction of peptide release may also be carried out *in silico* using the reverse genome engineering approach as an alternative to testing a target protein for peptide formation and bioactivity (Panchaud, Affolter, & Kussmann, 2012). The first step is to screen the protein databases for matches based on molecular similarities and then perform virtual hydrolysis using tools such as ExPASy Peptide Cutter and/or PoPS (Cavallo, 2003; Gasteiger, 2003). However, when mixtures of enzymes are used, it can be unclear which of these enzymes has been responsible for hydrolyzing the sample. Therefore, an additional tool, EnzymePredictor, was developed by Vijayakumar et al. (2012), which can identify the protein source of a particular peptide and the enzymes that could have produced the peptide. The algorithms used to develop this and other similar tools (such as CutDB and PROSPER) have been reviewed and compared (duVerle & Mamitsuka, 2012).

Two general approaches for predicting bioactivity of peptides exploit structural similarities with source proteins and peptides of known activity and use matrices to correlate AA sequences with individual effects. Some of the examples of peptide coming from hormone degradation and/or digestion of well-known proteins (such as lactoferrin or caseins) are given in Table S2. Structural similarities between source proteins are mainly based on the presence of evolutionary conserved domains and motifs (Iwaniak & Dziuba, 2009). Databases, such as BioPep that allow linking domains to major protein classes, could help in narrowing down potential protein sources for peptides with desired bioactivity. Motif detection and classification is also possible using genetic programming (Tomita, Kato, Okochi, & Honda, 2008). Gu and Wu (2013) employed structure–function relationships for screening protein sources that could give peptides having a desired health effect. They used quantitative structure–activity relationship models to predict food protein sources that could give antihypertensive peptides. Additionally, it is possible to predict the occurrence of a bioactive peptide sequence in a source protein using the PeptideLocator software (Mooney, Haslam, Holton, Pollastri, & Shields, 2013). Structural analysis is not limited only to the source protein. For example, the PeptideRanker server can be used to determine the probability that a specific peptide will be bioactive, based on structural similarity, and with scoring into groups based on likelihood for the particular bioactivity. This corresponds to the "integrated approach" suggested by Udenigwe (2014).

Where sequences of bioactive peptides are already known, matrices can be used to assign values (indexes) to AA residues, based on their properties including hydrophobicity, molecular weight, number of atoms, and so on. These indexes can then be used for prediction using comparisons with sequences of known activity. Though prediction of protein activity and subcellular localization based on Chou's pseudo AA composition (psAAC) is fairly simple (Chou, 2001), the approach is more complicated when applied to bioactive peptides. Georgiou, Karakasidis, Nieto, and Torres (2009) made an attempt to modify Chou's psAAC for the classification of AAs, using the fuzzy clustering technique and 2 variables: Minkowski distance and NTV metric, as proposed by Nieto, Torres, and Vazquez-Trasande (2003). Other researchers evaluated the use of a replacement matrix to estimate the effect of substituting one AA with another (Le & Gascuel, 2008). However, although each AA had been classified using an index based on its physiochemical properties (Kawashima et al., 2008), it was difficult to find any predictor that could take into account both the type and the position of a particular AA. The position of an AA is relevant because some AAs give rise to different secondary structures (Malkov, Zivković, Beljanski, Hall, & Zarić, 2008; Sahu & Panda, 2010) and these changes may affect protein function (Ng & Henikoff, 2006). ReplacementMatrix software is available that carries out maximum likelihood estimation of AA replacement rates (Dang, Lefort, Le, Le, & Gascuel, 2011).

Also, algorithms and methods to assess specific bioactivities have been developed. Combining bioinformatics with high throughput sequencing has led to the generation of algorithms, such as IgRepertoireConstructor (Safonova et al., 2015). Immunorepertoires useful not only in prediction of immunomodulatory activity, but also for a better quantification and comparison of peptides from different sources (Greiff, Miho, Menzel, & Reddy, 2015; Shlemov, Bankevich, Bzikadze, & Safonova, 2016). Q14

Conclusions

We have assessed current understanding of structure–function relationships for bioactive peptides of animal origin and show that these peptides contain high levels of proline, which influence their stability and uptake. We have also discussed the influence of processing on activity of these peptides, as well as methods for predicting their release from originating proteins. Interest in functional foods is growing fast, but several key issues are still unresolved. Standardization in digestion models is badly needed, not only to compare scientific results, but especially to ensure reproducibility and correlation between *in vitro* and *in vivo* results. Additionally, some peptide activities, in particular antioxidative and antimicrobial, have been tested only rarely *in vivo*, which raised questions and doubts about their effects in humans. Methods for testing peptide bioactivities are also extremely heterogeneous, not only in the experimental methods, but also in the expression of results. In addition, better uniformity in production is of crucial importance if bioactive peptides are to become more useful as components of functional foods.

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Authors' Contribution

Authors E. Maestri and M. Pavlicevic critically reviewed previous data and performed statistical analysis. Authors M. Montorsi and N. Marimoli participated in study design and critically reviewed the manuscript draft.

Conflict of Interest

Authors declare that they are free of any conflicts of interest.

List of Abbreviations

AA	amino acid
ACE	angiotensin-converting enzyme
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
ET	electron transfer
FXase	intrinsic factor tenase
HAT	hydrogen atom transfer
MS	mass spectrometry
NK	natural killer
psAAC	pseudo amino acid composition
RP-HPLC	reverse-phase HPLC
SHR	spontaneously hypertensive rats

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of databases for bioactive peptides (accessed March 2018).

Table S2. List of bioactive peptides from animal sources with indication of *in vivo* or *in vitro* activity (IC50).

Table S3. Scores of the index "normalized van der Waals volume" for the different amino acids.

Table S4. Non parametric analysis of variance for the distribution of amino acids in the different positions of bioactive peptides, classified according to main classes of activity.

Figure S1. Box-plot distribution of amino acid scores for "normalized van der Waals volume", according to the different positions in the sequence.

Figure S2. Box-plot distribution of amino acid scores for "normalized van der Waals volume", according to the main category of activity.

Figure S3. WebLogo representation of the distribution of individual amino acid residues in bioactive peptides of 2 to 10 AAs.

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