MULTIFUNCTIONAL PEPTIDES FOR THE PREVENTION OF CARDIOVASCULAR DISEASE: A NEW CONCEPT IN THE AREA OF BIOACTIVE FOOD-DERIVED PEPTIDES

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Abstract
Bioactive peptides derived from food proteins are increasingly recognized as useful tools for improving health. In this dynamic field, multifunctional peptides represent an emerging area: this definition indicates those peptides which have the capacity to impart more than one physiological outcome by affecting different targets. They may be considered an improvement in respect to monofunctional peptides, owing to lower negative side effects and reduced costs. This review discusses the current information on multifunctional peptides useful in the area of cardiovascular disease prevention. Hypcholesterolemic / anti-diabetic peptides were identified in soybean and lupin protein hydrolysates, whereas hypotensive / anti-diabetic peptides in milk proteins hydrolysate. Antioxidant peptides with at least another biological activity (hypotensive peptides, anti-diabetic and hypocholesterolemic) were purified from hempseed, lentils, rice bran, milk, egg yolk, and cumin protein hydrolysates. The polypeptide lunasin is hypocholesterolemic, antioxidant, and anti-inflammatory. Finally, the current hurdles in view of their practical exploitation are discussed in detail.

Keywords: ACE, antioxidant, bioactive peptides, hypocholesterolemic, hypoglycemic, milk, soybean.

Abbreviations
ACE, angiotensin converting enzyme; ADME-T, absorption, distribution, metabolism, excretion and toxicity; Akt, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; AP, apical; AT1R, angiotensin type I receptor; AT2R, angiotensin type II receptor; BL, basolateral;
BLASTp, protein Basic Local Alignment Search Tool; BP, blood pressure; CN, casein; CVD, cardiovascular disease; DOE, Quality by Design of experiments; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; DPP-IV, dipeptidyl peptidase-IV; ESI, electro-spray ionization; FRAP, ferric-reducing antioxidant power; GIT, gastrointestinal tract; GLU1, glucose transporter type 1; GLUT4, glucose transporter type 4; GS, glycogen synthase; GSK3, glycogen synthase kinase-3β; HMGCoAR, HMGCoA reductase; HNF-1α, hepatocyte nuclear factor-1α; IL-6, interleukine-6; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; LDLR, LDL receptor; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ORAC, oxygen radical absorbance capacity; PCAF, P300/CBP-associated factor; PCSK9, proprotein convertase subtilisin/kexintype 9; Q-TOF-MS, quadrupole time-of-flight mass spectrometer QSAR, quantitative structure activity relationship; RAS, renin-angiotensin system; ROS, reactive oxygen species; RP, reverse-phase; RSM, response surface methodology; SBP, systolic blood pressure; SHR, hypertensive rats; SREBP, sterol-responsive element binding protein; TAEC, trolox equivalent antioxidant capacity; TEER, trans-epithelial electrical resistance; TNF-α, tumor necrosis factor-α
1. Introduction

In the area of functional foods and dietary supplements, bioactive peptides are increasingly recognized as useful tools for improving health and preventing chronic diseases (Udenigwe & Aluko, 2012). In fact, food proteins do not only supply nutrients, but also provide numerous health benefits through their impact on specific biochemical pathways. Most of these activities are due to peptides encrypted in the parent protein sequences, which are delivered by digestion, absorbed intact by intestinal cells, and transported to their target organs where they exert their biological activity (Rutherford-Markwick, 2012). Over the years, numerous bioactive peptides have been identified in protein hydrolysates from various foods. In addition, bioactive peptides may also derive from food processing, especially during fermentation (Sanjukta & Rai, 2016; Toldrá, Reig, Aristoy, & Mora, 2018). For example, the peptide with the sequence HHL was found in soy paste (Nakahara, Sano, Yamaguchi, Sugimoto, Chikata, Kinoshita, et al., 2010) and MAP and MKP in cheese (Yamada, Sakurai, Ochi, Mitsuyama, Yamauchi, & Abe, 2013). Sometimes, instead they may be naturally present. This is the case of lunasin, a polypeptide initially identified in soybean (Hernández-Ledesma, Hsieh, & de Lumen, 2009) and then in other legumes (Jeong, Lee, Jeong, Park, Cheong, & de Lumen, 2009).

The very diversified structures explain the wide range of functional activities performed by food peptides: in fact, literature reports anticancer, anti-inflammatory, hypotensive, hypocholesterolemic, anti-diabetic, antioxidant, immunomodulatory, and antibacterial activities (Daliri, Oh, & Lee, 2017). In this dynamic field, multifunctional peptides represent an emerging area with numerous potential applications (Meisel, 2004; Daliri, Oh, & Lee, 2017). This definition indicates the peptides that have the capacity to impart more than one physiological outcome by affecting different targets. They may be considered improvements in respect to monofunctional peptides which provide one single activity, owing to lower negative side effects and reduced costs.

In this area, two approaches are possible: the former is based on food protein hydrolysates, where the multifunctional activities are due to different peptides, each one endowed with a specific activity, whereas the latter is based on peptides able to interfere with two or more biological pathways. This review is focused only on the second approach, i.e. on peptides that are intrinsically multifunctional, with specific reference to the area of cardiovascular disease (CVD) prevention. Hypertension, hypercholesterolemia, diabetes, and overweight are the main risk factors for developing this multifactorial disease. Many of these causes are related to atherosclerosis, which is also strictly connected with oxidative stress and inflammatory processes (Wu, Xia, Kalionis, Wan, & Sun, 2014). This review takes in consideration all studies reporting peptides that exert at least two of the following activities: hypocholesterolemic, anti-diabetic, hypotensive, anti-diabetic, or antioxidant, and
discusses available data in order to highlight the intrinsic strength and potentiality of multifunctional peptides to prevent the CVD. The last part of this review is dedicated to the current challenges to overcome for their practical exploitation highlighting two relevant aspects: the methodological approaches for the production of bioactive peptides and the impressive heterogeneity of the assays and approaches used up-to-now to evaluate their biological activity. Multifunctional peptides are a new challenging topic in which many efforts should be addressed for a future exploitation in dietary supplements and functional foods. To go in this exciting direction, a paradigmatic shift from monofunctional peptides to multifunctional ones is necessary.

2. Multifunctional peptides in CVD prevention

2.1 Peptides with hypocholesterolemic and hypoglycemic activities

These multifunctional peptides were identified in soybean and lupin (Table 1). Two peptides, LPYP and IAVPGEVA, were isolated and characterized after digesting soy glycinin with trypsin and pepsin, whereas the alignment of IAVPGEVA with the glycinin sequence permitted the identification of peptide IAVPTGVA (Pak, Koo, Kasymova, & Kwon, 2005). Experiments performed using the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) showed that IAVPGEVA, IAVPTGVA, and LPYP act as competitive inhibitors with IC50 = 222 µM, 274 µM, and 300 µM, respectively, whereas assays in HepG2 cells showed that the inhibition of HMGCoAR enhances the low-density lipoprotein receptor (LDLR) protein levels, by activation of the sterol-responsive element binding protein 2 (SREBP-2) pathway, and the low-density lipoprotein (LDL)-uptake (Table 1) (Lammi, Zanoni, & Arnoldi, 2015a). Moreover, they increase the phosphorylation level of HMGCoAR on Ser 872 (the inactive form of HMGCoAR), via the activation of the adenosine monophosphate-activated protein kinase (AMPK)-pathway. Interestingly, since this activation suggests that they might be potentially active also on glucose metabolism, using the same cell model, other experiments provided evidence that they modulate glucose metabolism and uptake through the activation of the Akt and AMPK pathways (Lammi, Zanoni, & Arnoldi, 2015b). Through an increase of the phosphorylation level at Ser 473, the activation of Akt leads to the inhibition of glycogen synthase kinase-3β (GSK3), which in turn produces a positive glycogen synthase (GS) regulation and formation of hepatic glycogen. In parallel, the glucose transporter type 4 (GLUT4) and glucose transporter type 1 (GLUT1) protein levels increase leads to an improvement of glucose uptake by HepG2 cells, mainly due to the GLUT1 transporter activity on cellular membranes (Table 1). These evidences support the hypothesis that the dual ability of these peptides to modulate glucose and cholesterol metabolism may be due to the synergic activation of Akt and AMPK. Using an in vitro
tool based on the purified catalytic domain of recombinant human dipeptidyl peptidase-IV (DPP-IV), it was demonstrated that IAVPGEVA, IAVPTGVA, and LPYP inhibit the DPP-IV activity with IC_{50} equal to 94.6 μM, 106.0 μM, and 164.3 μM, respectively (Aiello, Ferruzza, Ranaldi, Sambuy, Arnoldi, Vistoli, et al., 2018; Lammi, Zanoni, Arnoldi, & Vistoli, 2016).

Lupin proteins are another source of multifunctional peptides. In an absorption experiment performed on pepsin and trypsin protein hydrolysates using differentiated Caco2 cells as a model of the intestine (Lammi, Aiello, Vistoli, Zanoni, Arnoldi, Sambuy, et al., 2016), eleven tryptic peptides and eight peptic peptides were detected by LC-ESI-MS/MS analysis in the basolateral (BL) chamber. Out of these, the most interesting was LTFPGSAED, the first multifunctional peptide that was also shown to be potentially bioavailable. This nonapeptide, obtained by the pepsin hydrolysis of lupin β-conglutin, inhibits the HMGCoAR activity in vitro with a concentration-response curve and an IC_{50} value equal to 68.7 μM. Treatments of HepG2 cells with LTFPGSAED produce a rise of the SREBP2 protein levels and a subsequent increase of the LDLR protein levels, whereas, from a functional point of view, it enhances the ability of HepG2 cells to uptake LDL from the extracellular environment (Zanoni, Aiello, Arnoldi, & Lammi, 2017). This peptide is also an inhibitor of human recombinant DPP-IV activity with an IC_{50} equal to 228 μM (Lammi, Zanoni, Arnoldi, & Vistoli, 2016).

Interestingly, soybean peptides (IAVPTGVA, IAVPGVEA and LPYP) are more active as DPP-IV than as HMGCoAR inhibitors, whereas the peptide lupin (LTFPGSAED) shows an opposite behavior. This difference may be explained considering their amino acidic sequences. In fact, to be an effective DPP-IV inhibitor, a peptide should display a hydrophobic character, should have a length varying from 2 to 8 amino acid residues, and should contain a Pro residue located at the first, second, third, or fourth N-terminal position. Besides, the Pro residue should be flanked by Leu, Val, Phe, Ala, and Gly (Boots, 2006). Indeed, LPYP contains a Pro as the second N-terminal residue and IAVPGEVA, IAVPTGVA, and LTFPGSAED a Pro as the fourth N-terminal residue. In addition, this Pro is flanked by a Leu residue in LPYP, by a Val residue in IAVPTGVA and IAVPGEVA, and by a Phe residue in LTFPGSAED. Moreover, these peptides are mostly composed of hydrophobic amino acid residues, such as Ala, Gly, Ile, Leu, and Pro. However, the biological activity of LTFPGSAED, which is the most hydrophobic one (+14.7 kcal×mol^{-1}), is probably impaired by its length (9 amino acid residues). IAVPTGVA and IAVPGEVA, which display intermediate hydrophobic values equal to 8.4 and 11.8 kcal×mol^{-1}, respectively, are the most active, whereas the shortest and the least hydrophobic peptide LPYP (+6.2 kcal×mol^{-1}) is only moderately active.

In order to function as a competitive inhibitor of HMGCoAR, a peptide should mimic the hydroxymethylglutaryl moity. To achieve this goal, the conformation and the side chain groups play a more important role than the total hydrophobicity. Moreover, the correlation of the inhibitory
activity with the peptide length has not been established yet. Based on these considerations, it was assessed that VPTG and VPGE fragments acquire a bioactive “turn” conformations. The Pro residue in each soybean and lupin peptide mimics the nicotinamid moiety of NADPH, which is the enzyme co-factor (Pak, Koo, Lee, Kim, & Kwon, 2005; Pak, Koo, Kwon, & Yun, 2012). Moreover, it was established that a Leu, Ile and/or Tyr residue at the N-terminus and a Glu residue at the C-terminus play important roles for the peptide inhibitory property (Pak, Koo, Lee, Kim, & Kwon, 2005; Pak, Koo, Kwon, & Yun, 2012). Indeed, all these peptides satisfy these features. However, only peptide LTFPGSAED comprises two negative charged side chains at C-terminal tail that improve its ability to interact with the receptor site and make it the best HMGCoAR inhibitor.

### 2.2 Peptides with hypotensive and hypoglycemic activity

Although milk proteins are among the most extensively investigated sources of bioactive peptides, literature reports only a few multifunctional peptides from this material. Caseins (CNs) appear to be the best source of peptides with angiotensin converting enzyme (ACE) inhibitory activity (Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013; Otte, Shalaby, Zakora, Prripp, & Ei-Shabrawy, 2007). The different casein fractions from ovine milk were separated, hydrolyzed, and peptide fractions were separated by a multistep procedure based on reverse phase (RP) semi preparative HPLC and their activity tested. This traditional procedure allowed the identification of the pentapeptide LPYPY, obtained by hydrolyzing kappa-casein with pepsin and then with corolase PP. The IC$_{50}$ value of the in vitro ACE inhibitory activity was equal to 28.9 µM (Gomez-Ruiz, Ramos, & Recio, 2007). The alignment using BLASTp tool revealed that this peptide is conserved among several species, such as bovine, ovine, and goat caseins, but not in donkey, camel, and pig caseins.

Another study, based on a more rational approach, showed that LPYPY is also a DPP-IV inhibitor (Nongonierma, Mooney, Shields, & FitzGerald, 2014). The in silico digestion of milk protein with gastrointestinal enzymes permitted to predict the release of five peptides (LPYPY included) with a proline residue at position 2 from the N-terminus, known as the preferred DPP-IV substrates. In vitro experiments using the porcine DPP-IV enzyme, confirmed that LPYPY function as an inhibitor with an IC$_{50}$ equal to 90.8 µM. Interestingly, working on goat casein trypsin/chymotrypsin hydrolysates, it was possible to identify peptide INNQFLPYPY, which was 2-folds more active than LPYPY as DPP-IV inhibitor, displaying an IC$_{50}$ = 40.08 µM (Zhang, Chen, Ma, & Chen, 2015). An investigation on the ACE inhibitory activity would assess whether this peptide is multifunctional too.

Three egg white ovotransferrin peptides (IRW, IQW, and LKP) were identified as ACE inhibitory (Majumder & Wu, 2011; Majumder & Wu, 2010), but only IRW showed a multifunctional behavior. Treating 16–17 weeks old male spontaneously hypertensive rats (SHRs) for 18 days with a low daily
dose (3 mg/Kg BW) or a high daily dose (15 mg/Kg BW) of IRW, the mean blood pressure (BP) were reduced by ~10 mmHg and ~40 mmHg, respectively, compared to untreated SHRs (Majumder, Chakrabarti, Morton, Panahi, Kaufman, Davidge, et al., 2013; Majumder, Chakrabarti, Morton, Panahi, Kaufman, Davidge, et al., 2015). IRW was also able to ameliorate insulin resistance in rat muscle L6 cells (Son, Chan, & Wu, 2018). Treatment of these cells with angiotensin II significantly decreased insulin-stimulated glucose uptake, impaired insulin signaling pathway and GLUT4 translocation, while adding IRW significantly reversed these outcomes. The improvement in insulin sensitivity was mediated by the downregulation of angiotensin II stimulated angiotensin type I receptor (AT1R) expression. These results underline that the renin-angiotensin system (RAS) system may become a complementary therapeutic target for studying the potential beneficial effect of food-derived bioactive peptides for the metabolic syndrome prevention (Son, Chan, & Wu, 2018).

2.3. Antioxidant peptides displaying multifunctional behavior

Several antioxidant peptides (AntiOxPeps), identified in different food protein hydrolysates, are also endowed with other biological activities. Hypotensive AntiOxPeps were characterized from hempseed, lentils, rice bran, and milk protein hydrolysates, hypoglycemic ones from egg yolk and cumin hydrolysates, and hypocholesterolemic ones from cumin hydrolysates. Lunasin represents a very peculiar case, since it is a polypeptide with antioxidant, cholesterol-lowering, and anti-inflammatory properties naturally present in some seeds.

2.3.1 AntiOxPeps with hypotensive activity

Peptides with antioxidant and antihypertensive features were identified in hempseed, lentils, rice bran, and milk proteins (Table 3). A hempseed protein hydrolysate, produced through simulated gastrointestinal tract digestion, was fractionated with a traditional procedure including consecutive fractionation by RP-HPLC followed by tandem mass spectrometry analysis of the active fractions leading to the identification of 23 short-chain peptides (< 5 amino acids long) (Girgih, He, Malomo, Offengenden, Wu, & Aluko, 2014). At the tested concentration (0.5 mg/mL), WVYY and PSLPA were the most active antioxidants, having 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activities equal to 67% and 58% and metal chelation activities equal to 94.0% and 96.0%, respectively, whereas WYT, SVYT, and IPAGV were less active (DPPH radical scavenging activities falling in the range 22.0-42.0% and metal chelation activities in the range 55.0-75.0%). Investigated in vivo using SHRs treated with 30 mg/kg body weight, WVYY showed a maximum systolic blood pressure (SBP) reduction by 34 mmHg (at 2 h), PSLPA of 40 mmHg (at 4 h), WYT of 13 mmHg (at 2 h), SVYT of 24 mmHg (at 6 h), and IPAGV of 36 mmHg (at 4 h). Biochemical investigations,
performed to get an insight of the mechanism of action, showed that WYT, SVYT, and IPAGV inhibited either the ACE and renin systems, whereas WVYY and PSLPA were active only on ACE one. The abundance of hydrophobic, acidic, branched-chain amino acids may positively contribute to enhance the antioxidant and antihypertensive potentials of these peptides. The low SBP-lowering effect of WYT could result from poor binding to the target enzymes, rapid inactivation in the gastrointestinal tract (GIT) or within the blood circulatory system or inefficient absorption. In contrast, the longer-lasting SBP-lowering effect of SVYT, IPAGV, and PSLPA indicated a more efficient absorption coupled with strong binding to target enzymes and resistance to structural inactivation by GIT or blood proteases (Girgih, He, Malomo, Offengenden, Wu, & Aluko, 2014).

Lentil proteins treated with Savinase® are another source of peptides with antioxidant and ACE inhibitory activities. The most abundant peptides identified in these protein hydrolysates by LC-ESI-MS/MS were fragments from vicilin, convicilin and legumin. LLSGTQNQPSFLSGF, NSLTLPIRLY, TLEPNSVFLPVLLH showed the highest antioxidant activities, equal to 0.013, 1.432, and 0.139 µmol Trolox eq/µmol peptide, respectively, and also the best ACE inhibitory activities, with IC50 values equal to 120, 77.1, and 117.8 µM, respectively (García-Mora, Martín-Martínez, Angeles Bonache, González-Múniz, Peñas, Frias, et al., 2017). Interestingly, the gastrointestinal digestion greatly improved the dual activity of these peptides (antioxidant activity 10–14 µmol Trolox eq / µmol peptide; ACE inhibitory activity IC50 = 11–21 µM), indicating that the release of smaller peptide fragments and amino acids might result in additive and synergistic biological effects. As for the relationship between the structure and the antioxidant/antihypertensive activity, the C-terminal heptapeptide is crucial for their dual activity. In particular, the ACE inhibition relies on the formation of hydrogen bonds between peptides C-terminal residues and residues of the ACE catalytic site. The ability of these peptides to inhibit ACE is consistent with earlier studies showing that hydrophobic or aromatic amino acid residues or proline residue at the C-terminus positively contribute to the improvement of ACE inhibitory potency (Wu, Aluko, & Nakai, 2006).

Rice bran is another potential source of bioactive peptides possessing antioxidant and ACE inhibitory activities (Wang, Chen, Fu, Li, & Wei, 2017). Rice bran protein was hydrolyzed using trypsin and the hydrolysate was then separated by a membrane bioreactor system, gel filtration, and RP-HPLC. With this procedure peptide YSK was identified which exhibited high DPPH free radicals scavenging activity (IC50 = 0.15 mg/mL on DPPH free radical), reducing power (0.125 at 0.05 mg/mL), and ACE inhibitory activity (IC50 = 76.0 µM). A molecular docking study revealed that its ACE inhibition is mainly attributed to the formation of very strong hydrogen bonds with the S2 pocket (Gln281, Lys511 and Tyr520) and the S10 pocket (Glu162) of the enzyme.
Finally, novel peptides with ACE-inhibitory and antihypertensive activity were identified in peptic hydrolysates from purified CN fractions that had been separated by semi-preparative HPLC and analyzed by ion trap mass spectrometry. This procedure permitted the identification of 44 peptides, among which three sequences, corresponding to αs1-CN f(90–94) (RYLGY), αs1-CN f(143–149) (AYFYPEL), and αS2-CN f(89–95) (YQKFPQY), showed IC\textsubscript{50} values equal to 0.7 μM, 6.58 μM, and 20.08 μM, respectively. These peptides also exert antihypertensive activity when they are orally administered to SHR\textsuperscript{s} at a dose of 5 mg kg\textsuperscript{-1} of body weight. Moreover, they also exert an ABTS radical scavenging activity with ORAC values equal to 2.83, 3.22, and 2.03 μmol Trolox equiv / μmol peptide (Miguel, Contreras, Recio, & Aleixandre, 2009).

2.3.2 AntiOxPeps with hypotensive and hypoglycemic activities

When egg yolk is used for the extraction of phospholipids, the main by-products are defatted egg yolk proteins that possess limited biological and technological value, because the defatting process, which requires the use of ethanol and hexane, impairs protein functionality (Chay Pak Ting, Mine, Juneja, Okubo, Gauthier, & Pouliot, 2011; Jiang, Noh, & Koo, 2001). Yolk protein by-products, however, can be converted into added-value products with improved functional and biological properties by enzymatic hydrolysis. In an interesting paper (Zambrowicz, Pokora, Setner, Dąbrowska, Szołtysik, Babij, et al., 2015), yolk proteins were hydrolyzed with pepsin, the hydrolysate was fractionated by ion-exchange chromatography and RP-HPLC and the isolated peptides were identified using MALDI-TOF and the Mascot Search Results database. Four peptides were identified, corresponding to fragments of apolipoprotein B (YINQMPQKSRE and YINQMPQKSREA), vitellogenin-2 (VTGRFAGHPAAQ) and apovitellenin-1 (YIEAVNKVSPRAGQF). They were synthesized and shown to be antioxidant, ACE inhibitory, and antidiabetic (inhibitory of α-glucosidase from \textit{Saccharomyces cerevisiae} and porcine DPP-IV) \textit{in vitro} (Table 4). Peptide YINQMPQKSRE revealed the strongest antioxidant activity, since the DPPH free radical scavenging and iron chelating activities reached 2.3 μM Trolox eq/mg and 37.4 μg Fe\textsuperscript{2+)/mg, respectively. It was also a strong inhibitor of ACE (IC\textsubscript{50} = 10.1 μg/mL) and DPP-IV (IC\textsubscript{50} = 222.8 μg/mL). Peptide YINQMPQKSREA, differing only for the presence of alanine at the C-terminal sequence, had the highest ferric reducing activity (76.0 μg Fe\textsuperscript{2+)/mg). YIEAVNKVSPRAGQF was the strongest ACE inhibitor with an IC\textsubscript{50} = 9.4 μg/mL and was also a very good antioxidant, since the DPPH free radical scavenging, ferric reducing and iron chelating activities reached 2.2 μM Trolox eq/mg, 61.0 and 25.0 μg Fe\textsuperscript{2+)/mg, respectively, but it had no impact on the activity of α-glucosidase or DPP-IV. Peptide VTGRFAGHPAAQ was instead the best inhibitor of α-glucosidase (IC\textsubscript{50} = 365.4 μg/mL).
2.3.3 AntiOxPeps with hypoglycemic and hypocholesterolemic activities

Cumin (*Cuminum cyminum*) is an annual herbaceous plant belonging to the family of *Apiaceae*, whose seeds are well-known aromatic and culinary spices, generally used as condiment or flavoring. Cumin seeds are traditionally used as anti-diarrheal, carminative, stimulant, stomachic, and diuretic agents in traditional remedies (Hashemi, Shamizadeh, Badiei, Ghiasvand, & Azizi, 2009). Its high quality proteins were investigated as potential precursors of bioactive peptides (Table 5). Three novel peptides (CSPs), i.e. FFRSKLLSDGAAAAAKGALLPQYW (CSP1), RCMAFLLSGDAAAAAQQLLPQYW (CSP2), and DPAQPNYPTAVLVFRH (CSP3), were identified and demonstrated to possess antioxidant and anti-α-amylase activities (Siow & Gan, 2016). Tested at 100 µg, CSP1 showed the highest ferric-reducing antioxidant power (FRAP) activity (36.7 mM) and α-amylase inhibition (24.5%), but relatively low DPPH radical scavenging activity. CSP2 was an effective DPPH radical scavenger (58.6%) with a FRAP value of 29.16 mM, but was a poor inhibitor of porcine α-amylase. CSP3 was the less active in all assays. A structure–activity relationship study indicated that the active amino acid residues within the peptide sequence are important structural entities for the anti-oxidant and anti α-amylase activities. Subsequently, it was shown that these peptides mediate a hypocholesterolemic effect through their capacity to inhibit the formation of cholesterol micelles, which were measured *in vitro* creating an environment able to simulate the intestinal tract (Siow & Gan, 2016). In fact, in the intestine cholesterol is absorbed as cholesterol-mixed micelles, whereas released cholesterol from disrupted micelles is excreted in feces with a consequent hypocholesterolemic effect (Iqbal & Hussain, 2009).

2.3.4 AntiOxPeps with hypocholesterolemic and anti-inflammatory activities

Lunasin is a unique 43-amino acid polypeptide sequence encoded within the soybean *Gm2S-1* gene (Odani, Koide, & Ono, 1987). Soybean is a rich source of lunasin, with a concentration ranging from 4.4 to 70.5 mg lunasin/g of protein in different genotypes (Hernández-Ledesma, Hsieh, & de Lumen, 2009). Its concentration increases during seed maturation and decreases during sprouting depending on soaking time (Park, Jeong, & de Lumen, 2005). The interaction of cultivars, growing temperature, and soil moisture conditions significantly affects the lunasin concentration (Wang, Dia, Vasconez, de Mejia, & Nelson, 2008). The possibility of a selective breeding to produce lunasin rich cultivars was suggested (De Mejia, Vasconez, De Lumen, & Nelson, 2004). Moreover, recent studies demonstrated the presence of lunasin in cereals and other plants, such as wheat, barley, oat, rye, quinoa, and amaranth seeds (Jeong, Lee, Jeong, Park, Cheong, & de Lumen, 2009). The carboxyl terminus of lunasin contains nine aspartic acid residues (DDDDDDDDDD), a cell adhesion motif composed of a
RGD sequence and a predicted helix with structural homology to a conserved region of chromatin binding proteins (Galvez, Chen, Macasieb, & de Lumen, 2001).

Although lunasin has been investigated mostly for its anticancer activity (Galvez, Chen, Macasieb, & de Lumen, 2001), evidences suggest that this polypeptide is also antioxidant, hypocholesterolemic, and anti-inflammatory. The antioxidant/anti-inflammatory properties were assayed in different manners: (a) inhibition of the 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt radical cation, (b) inhibition of reactive oxygen species (ROS) production, (c) inhibition of the release of proinflammatory cytokines (tumor necrosis factor-α [TNF-α] and interleukine-6 [IL-6]) (Hernández-Ledesma, Hsieh, & de Lumen, 2009). Moreover, the RGD motif in lunasin regulates inflammatory-related pathologies by inhibiting Protein Kinase B (Akt)-mediated NF-κB pathways, through interaction with αVβ3 integrin in lipopolysaccharide (LPS)-induced human THP-1 macrophages, thus involving antagonism of integrin signaling and downstream proinflammatory cascades (Cam & de Mejia, 2012).

As far as the hypocholesterolemic activity is involved, the transcriptional activation of HMGCoAR via specific acetylation of histone H3 by P300/CBP-associated factor (PCAF) is an essential step in hepatic cholesterol biosynthesis. In relation to this, the capacity of lunasin of reducing serum LDL cholesterol levels is based on different mechanisms. Lunasin selectively reduces the acetylation of the histone H3 tail at K14 position by PCAF, thus lowering the HMGCoAR gene expression and making HMGCoAR unavailable for cholesterol biosynthesis, and also increases the expression of the LDLR gene, which raises the amount of LDLR to clear LDL-cholesterol from bloodstream. In the presence of lunasin, the levels of SP1 proteins, the coactivator of SREBP, increase two times more than without lunasin (Galvez, 2012). Furthermore, a study revealed that a casein diet supplemented with a lunasin-enriched soy extract (LSE) lowered the LDL-cholesterol (LDL-C) levels more than a simple casein diet in pigs carrying mutated LDLR gene (Galvez, 2012). Finally, lunasin down-regulates the proprotein convertase subtilisin/kexintype 9 (PCSK9) via the down-regulation of the hepatocyte nuclear factor-1α (HNF-1α) (Gu, Wang, Xu, Tian, Lei, Zhao, et al., 2017). Interestingly, the final effects of lunasin are very similar to those of some soybean (IAVPGEVA, IAVPTGVA, and LPYP) and lupin peptides (LILPKHSDAD and LTFPGSAED), although the modes of action are quite different. In particular, lunasin inhibits the expression of HMGCoAR, which leads to an increased LDLR expression at transcriptional level (Galvez, 2012), whereas the other peptides produce a direct inhibition of HMGCoAR activity leading to an increase of the LDLR protein level and finally to an improved ability of HepG2 cells to clear extracellular LDL-cholesterol (Lammi, Zanoni, Arnoldi, & Vistoli, 2015). On the contrary, the behavior of lupin peptide LILPKHSDAD is similar to lunasin, since it down-regulates the PCSK9 protein level through reduction of HNF-1α.
(Zanoni, Aiello, Arnoldi, & Lammi, 2017), although only LILPKHSDAD inhibits the protein-protein interaction between PCSK9 and LDLR (Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016). A study provided evidence that lunasin is absorbed in the intestine, since it was found intact in plasma of volunteers after soybean consumption (Dia, Torres, De Lumen, Erdman, & De Mejia, 2009). The high bioavailability was explained with the simultaneous presence of protease inhibitors, which allow 30% of lunasin to reach the target tissues.

3. Multifunctional peptides: challenges and perspectives

The multifunctional behavior of individual peptides opens a scenario for their exploitation in dietary supplements and functional foods. However, the paradigmatic shift from monofunctional peptides to multifunctional peptides requires certainly a change in the methodologies used to perform research on food protein hydrolysates that are complex mixtures of peptides where only a few are biologically active. In order to address successfully this issue, some critical issues must be underlined and new integrated approaches should be developed. Some of these issues are addressed in this section.

3.1. New frontiers in the discovery of bioactive peptides

The classical approach to the discovery of bioactive peptides is based on the selection of protein sources of particular interest, either from plant or animal organisms, often starting from by-products or less valuable materials. More or less specific proteases are then selected for the hydrolysis (see section 3.2 for a discussion), conditions are optimized and the obtained protein hydrolysates are tested for the target activities after analysis by LC-ESI-MS/MS mass spectrometry. When a sufficiently active hydrolysate is found, a very time consuming work is started that includes multistep procedures (ultrafiltration, preparative HPLC on different phases, etc.) to obtain more and more purified fractions, whose retained activity must be confirmed after each single stage. The isolation of pure active peptides may require many months of work and, in our experience, success is not always guaranteed.

In a more rational approach, the biological assays may be guided by preventive in silico docking simulations between low energy conformations of the identified peptides and the target enzymes catalytic sites (HMGCoAR, ACE, DPP-IV, etc.), in order to select the best potential candidates for activity that are afterwards experimentally tested. Although permitting to save time and money, these tools do not resolve the very crucial issue of bioavailability.

In order to fill this gap, we have recently proposed an innovative multidisciplinary approach (Lammi, et al., 2016). The intestinal epithelial is the first major barrier to absorption encountered by any food component. Differentiated Caco-2 cells, which maintain the morphology and function of mature
enterocytes and express brush border peptidases and transporters, are a useful in vitro model of this barrier that may be used to investigate peptide stability and transport. Differentiated Caco-2 cells grown on filters create a two-compartments system, where the apical (AP) side of the cell monolayer (in vivo corresponding to the intestinal lumen) is separated from the BL side (in vivo corresponding to the intestinal vascular and lymphatic circulation). The hydrolysate under investigation is incubated in the AP compartment and, after a suitable time, the solution in the BL chamber is removed and analyzed by LC-ESI-MS/MS in order to detect and quantify absorbed peptides. In our experience, even starting from a very complex hydrolysate, only a relatively small number of peptides is identified in the BL solution (Lammi, et al., 2016). In practice, in this approach the Caco-2 monolayer is used as a “natural sieve of bioavailable species” that permits to concentrate the further research exclusively on absorbable peptides, using before in silico docking simulations and then suitable bioassays performed on pure synthetic samples. The use of this procedure permitted the identification of hypocholesterolemic peptides (Lammi, et al., 2016; Lammi, Zanoni, Aiello, Arnoldi, & Grazioso, 2016; Lammi, Zanoni, Arnoldi, & Vistoli, 2016). The Caco-2 model is also useful to evaluate the stability of bioactive peptides. For example, it allowed to study the trans-epithelial transport and the brush-border degradation of ACE inhibitory peptides derived from dry-cured ham (Gallego, Grootaert, Mora, Aristoy, Van Camp, & Toldra, 2016), and to investigate the in situ degradation and/or cellular internalization followed by degradation which are faster than the transport rate of soy peptides IAVPGEVA, IAVPTGVA, and LPYP (see section 2.1 for activity) (Aiello, et al., 2018).

Although the Caco-2 cell line has been extensively used during the past 35 years as the best available in vitro model for performing absorption studies, the use of this approach is certainly new in the field of bioactive peptides from food proteins. The standardization of a common procedure to differentiate the cells is required, in order to reduce the variability among laboratories and to permit the comparison of the results. To achieve this goal, some parameters should be taken into account, such as the degree of differentiation and polarization of the intestinal cells, by measuring the cell monolayer permeability, which can be assessed by electrical (trans-epithelial electrical resistance (TEER)) or functional measurements (passage of molecules confined to the extracellular space). In facts, if the Caco-2 cell monolayer is not well differentiated and tight junctions are not well formed, absorption experiments of food-derived peptides are significantly altered. In particular, the intestinal monolayer should not leak, otherwise the transport process becomes unspecific and therefore very far from normal physiology.

The Virtual Screening approach is a relatively unexploited area in the field of bioactive peptides from food sources. Virtual screening can be complementary to in vitro, cellular, and in vivo studies in order
to predict and understand the relationship between the peptide structure, bioactivity, and formation during proteolysis (Pripp, Isaksson, Stepaniak, Sorhaug, & Ardo, 2005; Wu, Liu, Guo, Xie, & Jiang, 2014). Since virtual screening involves the use of specific and accurate databases, exhaustive food-derived bioactive peptide databases should be useful in that respect (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). They may be used in combination with screening food protein sequences for possible release of bioactive peptides, in silico (computer-predicted) proteolysis and quantitative structure–activity relationship (QSAR) modelling. Recently, this approach has been employed for the identification of ACE-inhibitory (Pripp, 2007) and antimicrobial (Liu, Eichler, & Pischetsrieder, 2015) peptides from milk proteins.

Typically, food-derived peptides are characterized by poor absorption, distribution, metabolism, excretion and toxicity (ADME-T). In fact, they show rapid clearance, short half-life, and low permeability. In silico, in vitro, and in vivo tools have been developed to address the ADME-T challenges of peptides in order to improve peptide exploitation for the market (Di, 2015). Therefore, it would be beneficial to combine the ADME-T to the challenging process leading to the screening and identification of bioactive peptides from food sources (Daliri, Oh, & Lee, 2017). Certainly, this integrated approach would allow the identification of bioactive food peptides with better ADME-T profiles, improving their use in the development of functional foods and or dietary supplements.

### 3.2. New methodological approaches for the production of bioactive peptides

Another critical issue is the methodological approach to hydrolyze food proteins that is still affected by some empirical factors that are generally not very well controlled. This applies either to multifunctional peptides or single-target peptides, since the methodology used to release bioactive peptides cannot be more selective for the former than for the latter. However, the use of “Quality by Design of Experiments” (DOE) and response surface methodology (RSM) together with a QSAR-driven approach are up-to-date approaches used for optimized release, separation and recovering of bioactive peptides. For example, DOE approaches were applied for the optimization of milk protein hydrolysates with a wide range of bioactivities, specifically antioxidant (Contreras, Hernandez-Ledesma, Amigo, Martin-Alvarez, & Recio, 2011; Naik, Mann, Bajaj, Sangwan, & Sharma, 2013; Nongonierma, Maux, Esteveny, & FitzGerald, 2017; Zhao, Wu, & Li, 2010), ACE inhibitory (Naik, Mann, Bajaj, Sangwan, & Sharma, 2013), and DPP-IV inhibitory (Nongonierma, Mazzocchi, Paolella, & FitzGerald, 2017). In particular, the process conditions, such as temperature and duration of hydrolysis or fermentation, may result in non-reproducible peptide profiles, especially when the substrate contains mixtures of differently expressed proteins. Critical hydrolysis parameters, such as pH and buffer solution, must be optimized for each protein/substrate couple and each selected enzyme
or enzymes combination should be maintained constant during the reaction to ensure an efficient peptide release. Commercial enzymes should be carefully checked for efficacy and reproducibility of action as well as intrinsic stability, considering that batch to batch variability may result in significant activity variations. For instance, some inconsistencies in CN hydrolysis catalyzed by flavourzyme were attributed to loss of endopeptidase activity during storage (Toldrá, Reig, Aristoy, & Mora, 2018). Naturally, each bioactive peptide is released with a different kinetics: larger peptides appear in the early stage of hydrolysis and are then often cleaved into smaller peptides showing different bioactivities. Therefore, multiple sequential hydrolysis may result in peptides with enhanced or reduced activities, the latter due to degradation as it is sometimes observed (Agyei, 2015; Naqash & Nazeer, 2013).

The activities of multifunctional peptides are related to the amino acid composition, sequence, and length. Shortest peptides may exhibit a wide range of bioactivities, such as the ACE inhibitory and antioxidant activity (Ishak & Sarbon, 2018), whereas those containing eight or more amino acid residues may be HMGCoAR inhibitors (Lin, Huang, Weng, & Shiuan, 2015). The control of the time of hydrolysis is therefore a key parameter to modulate the final results.

Since multifunctional peptides provide more than one activity, it is necessary to identify, within their sequences, the active domain in order to rationalize their mechanism of action towards the specific molecular targets. Molecular docking simulation and QSAR are currently used for supporting the experimental evidence or for predicting the release of potent peptides. More specifically, QSAR models were developed to predict the ACE inhibitory activity (Pripp, Isaksson, Stepaniak, & Sorhaug, 2004) or DPP-IV inhibitory activity (Nongonierma, Mooney, Shields, & FitzGerald, 2014) of milk peptides. Molecular docking analysis is used to ascertain specific interactions (i.e., hydrogen bonding, electrostatic and hydrophobic) involved in the binding into the active site. Similarly, molecular docking analyses were used to predict the key molecular interactions between some lupin and soy peptides and the DPP-IV catalytic domain (Lammi, Zanoni, Arnoldi, & Vistoli, 2016). However, most studies use the molecular docking approach to explain the experimental results, whereas only a few employ it to select peptides for further experimental tests. The dynamic conformational changes induced in the peptide and the target protein upon binding impose limitations on computational docking studies and advocated for a 4D structural database documenting these changes (Acharya, Kufareva, Ilatovskiy, & Abagyan, 2014). No direct correlation was found between the Vina scores (predicted affinity) obtained by molecular docking of tri-peptides to the active site of DPP-IV and their in vitro DPP-IV inhibitory properties (Nongonierma, Mooney, Shields, & FitzGerald, 2014). These results may reflect the fact that binding of a peptide to a protein (or enzyme) molecule may arise from non-specific interactions or else occur at a site that is associated with an
activity other than that of interest. These scenarios cannot be easily ascertained by molecular simulations alone.

The work-up procedure has also a main impact on the generation of either monofunctional or multifunctional peptides. Most studies are based on the identification of bioactive peptides within complex protein hydrolysates: in these cases, often the purification steps may induce the loss of their potential additive or synergistic effects. In addition, the elimination of the interaction with other food components, such as polyphenols, lipids and carbohydrates, may reduce their potency after purification (Lin, Huang, Weng, & Shiuan, 2015).

3.3 Characterization of the biological activities: a heterogeneous approach

A careful literature analysis highlights a great heterogeneity of the assays and approaches used to evaluate the biological activity of food-derived peptides and indicates that a more efficient and homogenous characterization of the molecular mechanisms through which they exert their biological effects would be certainly needed. This is especially true in the case of multifunctional peptides. In particular, most of the peptides described in this review were studied exclusively using in vitro approaches, whereas only a small number using cellular techniques, just a few using experimental animal models, and none in the clinics.

In most cases, the in vitro approach is based on biochemical assays in which the purified domain of the target enzyme is involved. Only rarely these enzymes are human, while in most cases they belong to other animal species. For example, the screening and characterization of the hypotensive activity of food-derived peptides is mostly carried using the ACE from rabbit lung (see Tables 2, 3, and 4), with the exception of some others performed using the porcine enzyme (Boschin, Scigliuolo, Resta, & Arnoldi, 2014). Although the ACE sequence is highly conserved among species, including chimpanzee, rabbit, mouse, pig, and rat (Riordan, 2003), the use of different enzymes impair the possibility to compare different studies.

Most in vitro studies investigating the effects on DPP-IV are conducted using the porcine enzyme (Tables 2 and 4), although the human enzyme is commercially available (Table 1). Though the sequence is highly conserved among mammalian species, human and porcine DPP-IV enzymes have only an 88% sequence identity and there are evidences that porcine and human DPP-IV differ in their susceptibility to inhibition by food-derived peptides (Bär, Weber, Hoffmann, Stork, Wermann, Wagner, et al., 2003; Lacroix & Li-Chan, 2015). Since the inhibition is stronger on the porcine DPP-IV enzyme than the human one, the employment of the former may lead to an overestimation of the actual potency or effectiveness of a substance (Lacroix & Li-Chan, 2015). This is a very relevant aspect that should be taken into account while selecting the best peptides for further in vivo and
clinical investigations. In addition, the exclusive use of biochemical tools is a great limitation and alternative and cost-effective cell-based strategies are certainly required for a more effective discovery of food-derived DPP-IV inhibitors, also considering that in vitro strategies completely disregard absorption, distribution, and the possible fast metabolism of peptides into inactive sequences. A very recent paper has provided a new assay based on undifferentiated Caco-2 cells helpful to evaluate the DPP-IV inhibition in situ (Lammi, Bollati, Ferruzza, Ranaldi, Sambuy, Arnoldi, 2018).

It must be underlined that the development of multifunctional peptides represents a further challenge, because the prediction of the in vivo results is more complex with dual or more target pharmacology versus single-target pharmacology. The most complete studies on multifunctional peptides are those dedicated to the hypotensive effect, since often the biochemical screening is followed by in vivo studies. In this case, the translation from in vitro to in vivo effects is addressed. Another challenging aspect of the translation is the potentially biased signaling that might arise from novel ligands aimed at two or more receptors (Fosgerau & Hoffmann, 2015). Discrepancies between in vitro and in vivo activities have been already reported. For example, a meta-analysis of 19 human clinical trials shows that the lactotripeptides IPP and VPP lower the SBP and DBP in prehypertensive or mildly hypertensive subjects, although favorable effects are not observed in all individual studies (Turpeinen, Järvenpää, Kautiainen, Korpela, & Vapaatalo, 2013). The translation of results from animal models to humans might also be associated with greater risk in the case of multifunctional peptides. Therefore, assessments initially in well-designed animal models and then in clinical trials are required to provide robust evidence of their multiple biological activities for supporting their future health claims.

4. CONCLUSIONS

For the first time, this review collects and discusses the great potentiality of multifunctional peptides from food proteins in the CDV prevention. As illustrated in the Strengths, Weaknesses, Opportunities, and Threats (SWOT) analysis shown in Figure 1, these peptides have some interesting strengths, such as the capacity to modulate more than one biological target, the possibility to provide more health benefits with a single bioactive component, cost-efficiency and safety. However, there are also some weaknesses in common with monofunctional peptides, such as the poor oral bioavailability owing to scarce absorption or rapid metabolism. In any case, this is certainly a rapid developing field of investigation, that is attracting the interest of many researchers.
Acknowledgement

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REFERENCES


Captions of figures

Figure 1. Strengths, Weaknesses, Opportunities, and Threats (SWOT) analysis of multifunctional peptides
<table>
<thead>
<tr>
<th>Activity</th>
<th>Hypcholesterolemic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PepSequ</strong></td>
<td><strong>Origin</strong></td>
</tr>
<tr>
<td></td>
<td><strong>HMGCoAR activity (IC50) μM</strong></td>
</tr>
<tr>
<td>IAVPGEVA Soybean</td>
<td>222.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IAVPTGVA Soybean</td>
<td>274.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPYP Soybean</td>
<td>300.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LTFPGSAED Lupin</td>
<td>68.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

| Hypoglycemic                  | |
| **PepSequ**                   | **Origin**                                                                        | **In vitro test**                                                                 | **cell protein level variation vs control cells** |
|                               | **DPP-IV (IC50) μM** | **References** | **pAkt (S473) (%)** | **pGSK3 (%)** | **GLUT1 (%)** | **GLUT4 (%)** | **Conc. tested** | **Glucose-Uptake (%)** | **Conc. tested** | **References** |
| IAVPGEVA Soybean             | 94.6<sup>b</sup> | Aiello, G., Ferruzza, S., Ranaldi, G., et al., 2018 | 76.0          | 57.0          | 80.0         | 19.0         | 500 μM         | 180.0        | 50 μM           | Lammi, C., Zanoni, C., & Arnoldi, A. 2015b |
| IAVPTGVA Soybean             | 106.0<sup>b</sup> | Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. 2016 Aiello, G., Ferruzza, S., Ranaldi, G., et al., 2018 | 96.0          | 53.0          | 106.0        | 34.0         | 500 μM         | 298.0        | 50 μM           | Lammi, C., Zanoni, C., & Arnoldi, A. 2015b |
| LPYP Soybean                 | 164.3<sup>b</sup> | Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. 2018 | 77.0          | 76.0          | 52.0         | 135.0        | 500 μM         | 158.0        | 100 μM          | Lammi, C., Zanoni, C., & Arnoldi, A. 2015b |
| LTFPGSAED Lupin              | 228.0<sup>b</sup> | Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. 2016 | Nd<sup>c</sup> | nd            | nd           | nd           | nd            | nd           | nd              | Lammi, C., Zanoni, C., & Arnoldi, A. 2015b |

<sup>a</sup> human recombinant HMGCoAR enzyme (EC 1.1.1.88); <sup>b</sup> human recombinant DPP-IV enzyme (EC 3.4.14.51); <sup>c</sup> nd: not detected

DPP-IV, dipeptidyl peptidase-IV; GLUT1, glucose transporter type 1; GLUT4, glucose transporter type 4; HMGCoAR, HMGCoA reductase; LDL, low-density lipoprotein; LDLR, LDL Receptor; pAkt, phospho-protein kinase B; pAMPK, phospho-adenosine monophosphate-activated protein kinase; pGSK3, phospho-glycogen synthase kinase-3β; SREBP-2, sterol-responsive element binding protein-2
Table 2. Peptides with hypotensive and hypoglycemic activity

<table>
<thead>
<tr>
<th>PepSeq</th>
<th>Origin</th>
<th>Activity</th>
<th>ACE IC₅₀ (µM)</th>
<th>References</th>
<th>Animal tests (Systolic Blood Pressure Reduction)</th>
<th>DPP-IV IC₅₀ (µM)</th>
<th>References</th>
<th>Glucose Uptake in Ang II Treated L6 Myotubes (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPYPY</td>
<td>milk</td>
<td>nd</td>
<td>28.9ᵃ</td>
<td>Gomez-Ruiz, Ramos, &amp; Recio, 2007</td>
<td>nd</td>
<td>90.8ᵇ</td>
<td>Nongoniera, Mooney, Shields, &amp; FitzGerald, 2014</td>
<td>nd</td>
<td>Son, Chan, &amp; Wu, 2018</td>
</tr>
<tr>
<td>IRW</td>
<td>egg</td>
<td>nd</td>
<td>40 mmHg</td>
<td>Majumder, et al., 2013</td>
<td>nd</td>
<td>22.5</td>
<td>Son, Chan, &amp; Wu, 2018</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵃ) Rabbit ACE enzyme (EC 3.4.15.1);
ᵇ) Porcine DPP-IV enzyme (EC 3.4.14.5);
ACE; angiotensin converting enzyme, Ang II; Angiotensin II peptide, DPP-IV; dipeptidyl peptidase-IV.
Table 3. AntiOxPep with hypotensive activities

<table>
<thead>
<tr>
<th>PepSequ</th>
<th>Origin</th>
<th>ACE</th>
<th>Animal tests (Systolic Blood Pressure Reduction)</th>
<th>AntiOx</th>
<th>Assay used for the antiOx activity evaluation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>WVYY</td>
<td>hempseed</td>
<td>34 mmHg (2h)</td>
<td>67 (%)</td>
<td>DPPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSLPA</td>
<td>hempseed</td>
<td>40 mmHg (4h)</td>
<td>58 (%)</td>
<td>DPPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WYT</td>
<td>hempseed</td>
<td>89.0%&lt;sup&gt;a)&lt;/sup&gt;</td>
<td>13 mmHg (2h)</td>
<td>&lt; 22-42 (%)</td>
<td>DPPH</td>
<td>Girgih et al., 2014</td>
</tr>
<tr>
<td>SVYT</td>
<td>hempseed</td>
<td>79.0%&lt;sup&gt;a)&lt;/sup&gt;</td>
<td>24 mmHg (6h)</td>
<td>&lt; 22-42 (%)</td>
<td>DPPH</td>
<td></td>
</tr>
<tr>
<td>IPAGV</td>
<td>hempseed</td>
<td>36 mmHg (4h)</td>
<td>&lt; 22-42 (%)</td>
<td>DPPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLSGTQNQPSFLSGF</td>
<td>lentil</td>
<td>120.0 µM&lt;sup&gt;a)&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>0.013 (µmol Trolox eq./µmol)</td>
<td>TEAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSLTLPIRLRYL</td>
<td>lentil</td>
<td>77.14 µM&lt;sup&gt;a)&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>1.432 (µmol Trolox eq./µmol)</td>
<td>TEAC</td>
<td></td>
<td>García-Mora et al., 2017</td>
</tr>
<tr>
<td>TLEPNVSFLPVLLH</td>
<td>lentil</td>
<td>117.81 µM&lt;sup&gt;a)&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>0.139 (µmol Trolox eq./µmol)</td>
<td>TEAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YSK</td>
<td>rice bran</td>
<td>76.0 µM&lt;sup&gt;a)&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>0.15 mg/mL (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>DPPH</td>
<td></td>
<td>Wang et al., 2017</td>
</tr>
<tr>
<td>RYLGY</td>
<td>milk</td>
<td>0.71 µM&lt;sup&gt;a)&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>2.83 (µmol Trolox eq./µmol)</td>
<td>ORAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AYFYPEL</td>
<td>milk</td>
<td>6.58 µM&lt;sup&gt;a)&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>3.22 (µmol Trolox eq./µmol)</td>
<td>ORAC</td>
<td></td>
<td>Miguel et al., 2009</td>
</tr>
<tr>
<td>YQKFPQY</td>
<td>milk</td>
<td>20.08 µM&lt;sup&gt;a)&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>2.03 (µmol Trolox eq./µmol)</td>
<td>ORAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a)</sup> Rabbit ACE enzyme (EC 3.4.15.1);
ACE, angiotensin converting enzyme; AntiOx, antioxidant; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; ORAC, oxygen radical absorbance capacity; TAEC, Trolox equivalent antioxidant capacity.
Table 4. AntiOxPeps with also hypotensive and hypoglycemic activities

<table>
<thead>
<tr>
<th>PepSeq</th>
<th>Origin</th>
<th>Enzymatic (IC\textsubscript{50}) (μg/mL)</th>
<th>Activity</th>
<th>Antioxidant</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ACE</td>
<td>α-Glucosidase</td>
<td>DPP-IV</td>
<td>DPPH \textsuperscript{d}</td>
</tr>
<tr>
<td>YINQMPQKSRE</td>
<td>Egg</td>
<td>10.1\textsuperscript{a}</td>
<td>1,694.3 \textsuperscript{b}</td>
<td>222.8 \textsuperscript{c}</td>
<td>2.3</td>
</tr>
<tr>
<td>YINQMPQKSREA</td>
<td>Egg</td>
<td>12.6\textsuperscript{a}</td>
<td>454.6 \textsuperscript{b}</td>
<td>355.8 \textsuperscript{c}</td>
<td>1.8</td>
</tr>
<tr>
<td>VTGRFAGHPAAQ</td>
<td>Egg</td>
<td>27.3\textsuperscript{a}</td>
<td>365.4 \textsuperscript{b}</td>
<td>1,402.2 \textsuperscript{c}</td>
<td>1.5</td>
</tr>
<tr>
<td>YIEAVNKVSPRAGQF</td>
<td>Egg</td>
<td>9.4 \textsuperscript{a}</td>
<td>NA</td>
<td>NA</td>
<td>2.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a) } Rabbit ACE enzyme (EC 3.4.15.1);  
\textsuperscript{b) } α-Glucosidase from \textit{Saccharomyces cerevisiae} (EC 3.2.1.20);  
\textsuperscript{c) } Porcine DPP-IV enzyme (EC 3.4.14.5);  
\textsuperscript{d) } μM Troloxeq/mg  
\textsuperscript{e) } and  
\textsuperscript{f) } μg Fe\textsuperscript{2+}/mg  

ACE, angiotensin converting enzyme; CHEL, iron chelating; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; DPP-IV, dipeptidyl peptidase-IV; FRAP, ferric-reducing antioxidant power;
Table 5. AntiOxPeps with hypoglycemic and hypocholesterolemic activities.

<table>
<thead>
<tr>
<th>PepSequ</th>
<th>Origin</th>
<th>Activity</th>
<th>Antioxidant</th>
<th>Hypoglycemic</th>
<th>References</th>
<th>Hypocholesterolemic</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DPPH (%)</td>
<td>FRAP (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFRSKLLSDGAAAAAKGALLPQYW (CSP1)</td>
<td>cumin</td>
<td></td>
<td>3.88</td>
<td>36.71</td>
<td></td>
<td>-71.2%</td>
<td></td>
</tr>
<tr>
<td>RCMAFLLSDGAAAAAQQQLPQYW (CSP2)</td>
<td>cumin</td>
<td></td>
<td>58.64</td>
<td>29.61</td>
<td></td>
<td>-82.0%</td>
<td>Iqbal, 2009</td>
</tr>
<tr>
<td>DPAQPNYPWTAVLVFRH (CSP3)</td>
<td>cumin</td>
<td></td>
<td>3.43</td>
<td>7.60</td>
<td></td>
<td>91.2%</td>
<td></td>
</tr>
</tbody>
</table>

a) Porcine pancreatic α-amylase enzyme (EC 3.2.1.1);
DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; FRAP, ferric-reducing antioxidant power.

* Values referred to the percentage of cholesterol micelle concentration, which is determined by the loss 8-9 or increase (+) in the concentration of cholesterol micelle with CSP addition over the control (w/o CSP addition)