# 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. Hypocholesterolemic / 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; GLUT1, 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; SREBPs, 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 (Rutherfurd-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 $IC_{50}$ = 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 (Table1) (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 suggestes 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 $kcal \times mol^{-1}$), 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 $kcal \times mol^{-1}$, respectively, are the most active, whereas the shortest and the least hydrophobic peptide LPYP (+6.2 $kcal \times mol^{-1}$) is only moderately active.

In order to function as a competitive inhibitor of HMGCoAR, a peptide should mimic the hydroxymethylglutaryl moiety. 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 nicotinamide 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, Pripp, & 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, NSLTLPILRYL, 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 $IC_{50}$ 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 $IC_{50}$ = 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 ($IC_{50}$ = 0.15 mg/mL on DPPH free radical), reducing power (0.125 at 0.05 mg/mL), and ACE inhibitory activity ($IC_{50}$ = 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 $IC_{50}$ 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 SHRs at a dose of 5 mg $kg^{-1}$ 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 *Saccharomyces cerevisiae* and porcine DPP-IV) *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 $Fe^{2+}$/mg, respectively. It was also a strong inhibitor of ACE ($IC_{50}$ = 10.1 μg/mL) and DPP-IV ($IC_{50}$ = 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 $Fe^{2+}$/mg). YIEAVNKVSPRAGQF was the strongest ACE inhibitor with an $IC_{50}$ = 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 $Fe^{2+}$/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 ($IC_{50}$ = 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. FFRSKLLSDGAAAAKGALLPQYW (CSP1), RCMAFLLSDGAAAAQQLLPQYW (CSP2), and DPAQPNYPWTAVLVFRH (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 (DDDDDDDDD), 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

388 enterocytes and express brush border peptidases and transporters, are a useful *in vitro* model of this
389 barrier that may be used to investigate peptide stability and transport. Differentiated Caco-2 cells
390 grown on filters create a two-compartments system, where the apical (AP) side of the cell monolayer
391 (*in vivo* corresponding to the intestinal lumen) is separated from the BL side (*in vivo* corresponding
392 to the intestinal vascular and lymphatic circulation). The hydrolysate under investigation is incubated
393 in the AP compartment and, after a suitable time, the solution in the BL chamber is removed and
394 analyzed by LC-ESI-MS/MS in order to detect and quantify absorbed peptides. In our experience,
395 even starting from a very complex hydrolysate, only a relatively small number of peptides is identified
396 in the BL solution (Lammi, et al., 2016). In practice, in this approach the Caco-2 monolayer is used
397 as a "natural sieve of bioavailable species" that permits to concentrate the further research exclusively
398 on absorbable peptides, using before *in silico* docking simulations and then suitable bioassays
399 performed on pure synthetic samples. The use of this procedure permitted the identification of
400 hypocholesterolemic peptides (Lammi, et al., 2016; Lammi, Zanoni, Aiello, Arnoldi, & Grazioso,
401 2016; Lammi, Zanoni, Arnoldi, & Vistoli, 2016).
402 The Caco-2 model is also useful to evaluate the stability of bioactive peptides. For example, it allowed
403 to study the trans-epithelial transport and the brush-border degradation of ACE inhibitory peptides
404 derived from dry-cured ham (Gallego, Grootaert, Mora, Aristoy, Van Camp, & Toldra, 2016), and to
405 investigate the *in situ* degradation and/or cellular internalization followed by degradation which are
406 faster than the transport rate of soy peptides IAVPGEVA, IAVPTGVA, and LPYP (see section 2.1
407 for activity) (Aiello, et al., 2018).
408 Although the Caco-2 cell line has been extensively used during the past 35 years as the best available
409 *in vitro* model for performing absorption studies, the use of this approach is certainly new in the field
410 of bioactive peptides from food proteins. The standardization of a common procedure to differentiate
411 the cells is required, in order to reduce the variability among laboratories and to permit the comparison
412 of the results. To achieve this goal, some parameters should be taken into account, such as the degree
413 of differentiation and polarization of the intestinal cells, by measuring the cell monolayer
414 permeability, which can be assessed by electrical (trans-epithelial electrical resistance (TEER)) or
415 functional measurements (passage of molecules confined to the extracellular space). In facts, if the
416 Caco-2 cell monolayer is not well differentiated and tight junctions are not well formed, absorption
417 experiments of food-derived peptides are significantly altered. In particular, the intestinal monolayer
418 should not leak, otherwise the transport process becomes unspecific and therefore very far from
419 normal physiology.
420 The Virtual Screening approach is a relatively unexploited area in the field of bioactive peptides from
421 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**

This work was supported in part by Cariplo Foundation, project "SUPER-HEMP: Sustainable Process for Enhanced Recovery of Hempseed Oil", and in part by Fondazione Banca del Monte di Lombardia, project "Inibitori peptidici di PCSK9 e PCSK9 mutato: un nuovo approccio al trattamento dell'ipercolesterolemia".

# REFERENCES

Acharya, C., Kufareva, I., Ilatovskiy, A. V., & Abagyan, R. (2014). PeptiSite: a structural database of peptide binding sites in 4D. *Biochemical and Biophysical Research Communications, 445*(4), 717-723.

Agyei, D. (2015). Bioactive Proteins and Peptides from Soybeans. *Recent Patents on Food, Nutrition & Agriculture, 7*(2), 100-107.

Aiello, G., Ferruzza, S., Ranaldi, G., Sambuy, Y., Arnoldi, A., Vistoli, G., & Lammi, C. (2018). Behavior of three hypocholesterolemic peptides from soy protein in an intestinal model based on differentiated Caco-2 cell. *Journal of Functional Foods, 45*, 363-370.

Boots, J.-W. P. (2006). Protein hydrolysate enriched in peptides inhibiting DPP-IV and their use. Europen Patent EP1831361B1, Priority date 2004-12-23.

Boschin, G., Scigliuolo, G. M., Resta, D., & Arnoldi, A. (2014). ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. *Food Chemistry, 145*, 34-40.

Bär, J., Weber, A., Hoffmann, T., Stork, J., Wermann, M., Wagner, L., Aust, S., Gerhartz, B., & Demuth, H. U. (2003). Characterisation of human dipeptidyl peptidase IV expressed in Pichia pastoris. A structural and mechanistic comparison between the recombinant human and the purified porcine enzyme. *Biological Chemistry, 384*(12), 1553-1563.

Cam, A., & de Mejia, E. G. (2012). RGD-peptide lunasin inhibits Akt-mediated NF-κB activation in human macrophages through interaction with the αVβ3 integrin. *Molecular Nutrition & Food Research, 56*(10), 1569-1581.

Chay Pak Ting, B. P., Mine, Y., Juneja, L. R., Okubo, T., Gauthier, S. F., & Pouliot, Y. (2011). Comparative composition and antioxidant activity of Peptide fractions obtained by ultrafiltration of egg yolk protein enzymatic hydrolysates. *Membranes (Basel), 1*(3), 149-161.

Contreras, M. D., Hernandez-Ledesma, B., Amigo, L., Martin-Alvarez, P. J., & Recio, I. (2011). Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. *Lwt-Food Science and Technology, 44*(1), 9-15.

Daliri, E. B., Oh, D. H., & Lee, B. H. (2017). Bioactive Peptides. *Foods, 6*(5).

De Mejia, E. G., Vasconez, M., De Lumen, B. O., & Nelson, R. (2004). Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. *Journal of Agricultural and Food Chemistry, 52*(19), 5882-5887.

Di, L. (2015). Strategic approaches to optimizing peptide ADME properties. *The AAPS Journal, 17*(1), 134-143.

Dia, V. P., Torres, S., De Lumen, B. O., Erdman, J. W., & De Mejia, E. G. (2009). Presence of lunasin in plasma of men after soy protein consumption. *Journal of Agricultural and Food Chemistry, 57*(4), 1260-1266.

Espejo-Carpio, F. J., De Gobba, C., Guadix, A., Guadix, E. M., & Otte, J. (2013). Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions. *International Dairy Journal, 32*(2), 175-183.

Fosgerau, K., & Hoffmann, T. (2015). Peptide therapeutics: current status and future directions. *Drug Discovery Today, 20*(1), 122-128.

Gallego, M., Grootaert, C., Mora, L., Aristoy, M. C., Van Camp, J., & Toldra, F. (2016). Transepithelial transport of dry-cured ham peptides with ACE inhibitory activity through a Caco-2 cell monolayer. *Journal of Functional Foods, 21*, 388-395.

Galvez, A. F. (2012). Identification of lunasin as the active component in soy protein responsible for reducing LDL cholesterol and risk of cardiovascular disease. *Circulation, 126*(21).

Galvez, A. F., Chen, N., Macasieb, J., & de Lumen, B. O. (2001). Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. *Cancer Research, 61*(20), 7473-7478.

García-Mora, P., Martín-Martínez, M., Angeles Bonache, M., González-Múniz, R., Peñas, E., Frias, J., & Martinez-Villaluenga, C. (2017). Identification, functional gastrointestinal stability and molecular docking studies of lentil peptides with dual antioxidant and angiotensin I converting enzyme inhibitory activities. *Food Chemistry, 221*, 464-472.

Girgih, A. T., He, R., Malomo, S., Offengenden, M., Wu, J., & Aluko, R. E. (2014). Structural and functional characterization of hemp seed (*Cannabis sativa* L.) protein-derived antioxidant and antihypertensive peptides. *Journal of Functional Foods, 6*, 384-394.

Gomez-Ruiz, J. A., Ramos, M., & Recio, I. (2007). Identification of novel angiotensin-converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques. *Electrophoresis, 28*(22), 4202-4211.

Gu, L., Wang, Y., Xu, Y., Tian, Q., Lei, G., Zhao, C., Gao, Z., Pan, Q., Zhao, W., Nong, L., & Tan, S. (2017). Lunasin functionally enhances LDL uptake via inhibiting PCSK9 and enhancing LDLR expression. *Oncotarget, 8*(46), 80826-80840.

Hashemi, P., Shamizadeh, M., Badiei, A., Ghiasvand, A. R., & Azizi, K. (2009). Study of the essential oil composition of cumin seeds by an amino ethyl-functionalized nanoporous SPME fiber. *Chromatographia, 70*(7-8), 1147-1151.

Hernández-Ledesma, B., Hsieh, C. C., & de Lumen, B. O. (2009). Lunasin, a novel seed peptide for cancer prevention. *Peptides, 30*(2), 426-430.

633 Iqbal, J., & Hussain, M. M. (2009). Intestinal lipid absorption. *American Journal of Physiology-*
634 *Endocrinology and Metabolism, 296*(6), E1183-1194.

635 Ishak, N. H., & Sarbon, N. M. (2018). A review of protein hydrolysates and bioactive peptides
636 deriving from wastes generated by fish processing. *Food and Bioprocess Technology, 11*(1),
637 2-16.

638 Jeong, H. J., Lee, J. R., Jeong, J. B., Park, J. H., Cheong, Y. K., & de Lumen, B. O. (2009). The
639 Cancer Preventive Seed Peptide Lunasin From Rye Is Bioavailable and Bioactive. *Nutrition*
640 *and Cancer-an International Journal, 61*(5), 680-686.

641 Jiang, Y. Z., Noh, S. K., & Koo, S. I. (2001). Egg phosphatidylcholine decreases the lymphatic
642 absorption of cholesterol in rats. *Journal of Nutrition, 131*(9), 2358-2363.

643 Lacroix, I. M., & Li-Chan, E. C. (2015). Comparison of the susceptibility of porcine and human
644 dipeptidyl-peptidase IV to inhibition by protein-derived peptides. *Peptides, 69*, 19-25.

645 Lammi, C., Aiello, G., Vistoli, G., Zanoni, C., Arnoldi, A., Sambuy, Y., Ferruzza, S., & Ranaldi, G.
646 (2016). A multidisciplinary investigation on the bioavailability and activity of peptides from
647 lupin protein. *Journal of Functional Foods, 24*, 297-306.

648 Lammi, C., Zanoni, C., Aiello, G., Arnoldi, A., & Grazioso, G. (2016). Lupin peptides modulate the
649 protein-protein interaction of PCSK9 with the low density lipoprotein receptor in HepG2
650 cells. *Scientific Reports, 6,* 29931 (pp. 13).

651 Lammi, C., Zanoni, C., & Arnoldi, A. (2015a). IAVPGEVA, IAVPTGVA, and LPYP, three peptides
652 from soy glycinin, modulate cholesterol metabolism in HepG2 cells through the activation of
653 the LDLR-SREBP2 pathway. *Journal of Functional Foods, 14*, 469-478.

654 Lammi, C., Zanoni, C., & Arnoldi, A. (2015b). Three peptides from soy glycinin modulate glucose
655 metabolism in human hepatic HepG2 cells. *International Journal of Molecular Sciences,*
656 *16*(11), 27362-27370.

657 Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. (2015). Two peptides from soy beta-conglycinin
658 induce a hypocholesterolemic effect in HepG2 cells by a statin-Like mechanism: comparative
659 in vitro and in silico modeling studies. *Journal of Agricultural and Food Chemistry, 63*(36),
660 7945-7951.

661 Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. (2016). Peptides Derived from Soy and Lupin
662 Protein as Dipeptidyl-Peptidase IV Inhibitors: In Vitro Biochemical Screening and in Silico
663 Molecular Modeling Study. *Journal of Agricultural and Food Chemistry, 64*(51), 9601-9606.

664 Lin, S. H., Huang, K. J., Weng, C. F., & Shiuan, D. (2015). Exploration of natural product ingredients
665 as inhibitors of human HMG-CoA reductase through structure-based virtual screening. *Drug,*
666 *Design, Development and Therapy, 9*, 3313-3324.

667 Liu, Y. F., Eichler, J., & Pischetsrieder, M. (2015). Virtual screening of a milk peptide database for
668 the identification of food-derived antimicrobial peptides. *Molecular Nutrition & Food Research,*
669 *59*(11), 2243-2254.

670 Majumder, K., Chakrabarti, S., Morton, J. S., Panahi, S., Kaufman, S., Davidge, S. T., & Wu, J.
671 (2013). Egg-derived tri-peptide IRW exerts antihypertensive effects in spontaneously
672 hypertensive rats. *PLoS One, 8*(11), e82829.

673 Majumder, K., Chakrabarti, S., Morton, J. S., Panahi, S., Kaufman, S., Davidge, S. T., & Wu, J. P.
674 (2015). Egg-derived ACE-inhibitory peptides IQW and LKP reduce blood pressure in
675 spontaneously hypertensive rats. *Journal of Functional Foods, 13*, 50-60.

676 Majumder, K., & Wu, J. (2011). Purification and characterisation of angiotensin I converting enzyme
677 (ACE) inhibitory peptides derived from enzymatic hydrolysate of ovotransferrin. *Food*
678 *Chemistry, 126*(4), 1614-1619.

679 Majumder, K., & Wu, J. P. (2010). A new approach for identification of novel antihypertensive
680 peptides from egg proteins by QSAR and bioinformatics. *Food Research International, 43*(5),
681 1371-1378.

682 Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. *Biofactors, 21*(1-4), 55-61.

683 Miguel, M., Contreras, M. M., Recio, I., & Aleixandre, A. (2009). ACE-inhibitory and
684 antihypertensive properties of a bovine casein hydrolysate. *Food Chemistry, 112*(1), 211-214.

685 Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., & Darewicz, M. (2008). BIOPEP database and
686 other programs for processing bioactive peptide sequences. *Journal of the Association of Official*
687 *Analytical Chemists International, 91*(4), 965-980.

688 Naik, L., Mann, B., Bajaj, R., Sangwan, R. B., & Sharma, R. (2013). Process Optimization for the
689 Production of Bio-functional Whey Protein Hydrolysates: Adopting Response Surface
690 Methodology. *International Journal of Peptide Research and Therapeutics, 19*(3), 231-237.

691 Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E., & Uchida, R.
692 (2010). Antihypertensive Effect of Peptide-Enriched Soy Sauce-Like Seasoning and
693 Identification of Its Angiotensin I-Converting Enzyme Inhibitory Substances (vol 58, pg 821,
694 2010). *Journal of Agricultural and Food Chemistry, 58*(9), 5858-5858.

695 Naqash, S. Y., & Nazeer, R. A. (2013). Antioxidant and functional properties of protein hydrolysates
696 from pink perch (Nemipterus japonicus) muscle. *Journal of Food Science and Technology,*
697 *50*(5), 972-978.

698 Nongonierma, A. B., Maux, S. L., Esteveny, C., & FitzGerald, R. J. (2017). Response surface
699 methodology applied to the generation of casein hydrolysates with antioxidant and dipeptidyl
700 peptidase IV inhibitory properties. *Journal of the Science of Food and Agriculture, 97*(4),
701 1093-1101.

702 Nongonierma, A. B., Mazzocchi, C., Paolella, S., & FitzGerald, R. J. (2017). Release of dipeptidyl
703 peptidase IV (DPP-IV) inhibitory peptides from milk protein isolate (MPI) during enzymatic
704 hydrolysis. *Food Research International, 94*, 79-89.

705 Nongonierma, A. B., Mooney, C., Shields, D. C., & FitzGerald, R. J. (2014). In silico approaches to
706 predict the potential of milk protein-derived peptides as dipeptidyl peptidase IV (DPP-IV)
707 inhibitors. *Peptides, 57*, 43-51.

708 Odani, S., Koide, T., & Ono, T. (1987). Amino-acid-sequence of a soybean (*Glycine max*) seed
709 polypeptide having a poly(L-aspartic acid) structure. *Journal of Biological Chemistry,*
710 *262*(22), 10502-10505.

Otte, J., Shalaby, S. M., Zakora, M., Pripp, A. H., & Ei-Shabrawy, S. A. (2007). Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme and time of hydrolysis. *International Dairy Journal, 17*(5), 488-503.

Pak, V. V., Koo, M. S., Kasymova, T. D., & Kwon, D. Y. (2005). Isolation and identification of peptides from soy 11S-globulin with hypocholesterolemic activity. *Chemistry of Natural Compounds, 41*(6), 710-714.

Pak, V. V., Koo, M., Lee, N., Kim, M. S., & Kwon, D. Y. (2005). Structure-activity relationships of the peptide Ile-Ala-Val-Pro and its derivatives revealed using the semi-empirical AM1 method. *Chemistry of Natural Compounds, 41*(4), 454-460.

Pak, V. V., Koo, M., Kwon, D. Y., & Yun, L. (2012). Design of a highly potent inhibitory peptide acting as a competitive inhibitor of HMG-CoA reductase. *Amino Acids, 43*(5), 2015-2025.

Park, J. H., Jeong, H. J., & de Lumen, B. O. (2005). Contents and bioactivities of lunasin, Bowman-Birk inhibitor, and isoflavones in soybean seed. *Journal of Agricultural and Food Chemistry, 53*(20), 7686-7690.

Pripp, A. H., Isaksson, T., Stepaniak, L., & Sorhaug, T. (2004). Quantitative structure-activity relationship modelling of ACE-inhibitory peptides derived from milk proteins. *European Food Research and Technology, 219*(6), 579-583.

Pripp, A. H., Isaksson, T., Stepaniak, L., Sorhaug, T., & Ardo, Y. (2005). Quantitative structure activity relationship modelling of peptides and proteins as a tool in food science. *Trends in Food Science & Technology, 16*(11), 484-494.

Pripp, A. H. (2007). Docking and virtual screening of ACE inhibitory dipeptides. *European Food Research and Technology, 225*(3-4), 589-592.

Riordan, J. F. (2003). Angiotensin-I-converting enzyme and its relatives. *Genome Biology, 4*(8), 225.

Rutherfurd-Markwick, K. J. (2012). Food proteins as a source of bioactive peptides with diverse functions. *British Journal of Nutrition, 108*, S149-S157.

Sanjukta, S., & Rai, A. K. (2016). Production of bioactive peptides during soybean fermentation and their potential health benefits. *Trends in Food Science & Technology, 50*, 1-10.

Siow, H. L., & Gan, C. Y. (2016). Extraction, identification, and structure-activity relationship of antioxidative and alpha-amylase inhibitory peptides from cumin seeds (*Cuminum cyminum*). *Journal of Functional Foods, 22*, 1-12.

Son, M., Chan, C. B., & Wu, J. (2018). Egg White Ovotransferrin-Derived ACE Inhibitory Peptide Ameliorates Angiotensin II-Stimulated Insulin Resistance in Skeletal Muscle Cells. *Molecular Nutrition & Food Research, 62*(4).

Toldrá, F., Reig, M., Aristoy, M. C., & Mora, L. (2018). Generation of bioactive peptides during food processing. *Food Chemistry, 267*, 395-404.

Turpeinen, A. M., Järvenpää, S., Kautiainen, H., Korpela, R., & Vapaatalo, H. (2013). Antihypertensive effects of bioactive tripeptides-a random effects meta-analysis. *Annals of Medicine, 45*(1), 51-56.

752 Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production,
753 processing, and potential health benefits. *Journal of Food Science, 77*(1), R11-24.

754 Wang, W., Dia, V. P., Vasconez, M., de Mejia, E. G., & Nelson, R. L. (2008). Analysis of soybean
755 protein-derived peptides and the effect of cultivar, environmental conditions, and processing
756 on lunasin concentration in soybean and soy products. *Journal of the Association of Official*
757 *Analytical Chemists, 91*(4), 936-946.

758 Wang, X. M., Chen, H. X., Fu, X. G., Li, S. Q., & Wei, J. (2017). A novel antioxidant and ACE
759 inhibitory peptide from rice bran protein: Biochemical characterization and molecular
760 docking study. *Lwt-Food Science and Technology, 75*, 93-99.

761 Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural requirements of Angiotensin I-converting enzyme
762 inhibitory peptides: quantitative structure-activity relationship study of di- and tripeptides.
763 *Journal of Agricultural and Food Chemistry, 54*(3), 732-738.

764 Wu, H., Liu, Y., Guo, M., Xie, J., & Jiang, X. (2014). A virtual screening method for inhibitory
765 peptides of Angiotensin I-converting enzyme. *Journal of Food Science, 79*(9), C1635-1642.

766 Wu, J., Xia, S., Kalionis, B., Wan, W., & Sun, T. (2014). The role of oxidative stress and
767 inflammation in cardiovascular aging. *BioMed Research International, 2014*, 615312.

768 Yamada, A., Sakurai, T., Ochi, D., Mitsuyama, E., Yamauchi, K., & Abe, F. (2013). Novel
769 angiotensin I-converting enzyme inhibitory peptide derived from bovine casein. *Food*
770 *Chemistry, 141*(4), 3781-3789.

771 Zambrowicz, A., Pokora, M., Setner, B., Dąbrowska, A., Szołtysik, M., Babij, K., Szewczuk, Z.,
772 Trziszka, T., Lubec, G., & Chrzanowska, J. (2015). Multifunctional peptides derived from an
773 egg yolk protein hydrolysate: isolation and characterization. *Amino Acids, 47*(2), 369-380.

774 Zanoni, C., Aiello, G., Arnoldi, A., & Lammi, C. (2017). Investigations on the hypocholesterolaemic
775 activity of LILPKHSDAD and LTFPGSAED, two peptides from lupin beta-conglutin: Focus
776 on LDLR and PCSK9 pathways. *Journal of Functional Foods, 32*, 1-8.

777 Zhang, Y., Chen, R., Ma, H., & Chen, S. (2015). Isolation and Identification of Dipeptidyl Peptidase
778 IV-Inhibitory Peptides from Trypsin/Chymotrypsin-Treated Goat Milk Casein Hydrolysates
779 by 2D-TLC and LC-MS/MS. *Journal of Agricultural and Food Chemistry, 63*(40), 8819-
780 8828.

781 Zhao, X. H., Wu, D., & Li, T. J. (2010). Preparation and radical scavenging activity of papain-
782 catalyzed casein plasteins. *Dairy Science & Technology, 90*(5), 521-535.

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**Captions of figures**

Figure 1. Strengths, Weaknesses, Opportunities, and Threats (SWOT) analysis of multifunctional peptides

**Table 1. Peptides with hypocholesterolemic and hypoglycemic activities**

| **Activity** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Hypocholesterolemic** | | | | | | | | | | |
| **PepSequ** | **Origin** | ***In vitro* test** | **cell protein level variation *vs* control cells** | | | | | | | | |
| | | **HMGCoAR activity ($IC_{50}$) µM** | **SREBP-2 (%)** | **LDLR (%)** | **HMGCoAR (%)** | **pHMGCoAR (S872) (%)** | **pAMPK (T174) (%)** | **Conc. tested** | **LDL-Uptake (%)** | **Conc. tested** | **References** |
| IAVPGEVA | Soybean | 222.0 *a)* | 85.0 | 53.0 | 161.0 | 155.0 | 79.0 | 500 µM | 23.0 | 50 µM | Lammi, C., Zanoni, C., & Arnoldi, A. 2015a |
| IAVPTGVA | Soybean | 274.0 *a)* | 120.0 | 61.0 | 236.0 | 191.0 | 51.0 | 500 µM | 31.0 | 50 µM | Lammi, C., Zanoni, C., & Arnoldi, A. 2015a a |
| LPYP | Soybean | 300.0 *a)* | 155.0 | 68.0 | 172.0 | 178.0 | 100.0 | 500 µM | 27.0 | 100 µM | Lammi, C., Zanoni, C., & Arnoldi, A. 2015a |
| LTFPGSAED | Lupin | 68.4 *a)* | 62.4 | 102.7 | 67.8 | 90.4 | 196.4 | 50 µM | 235.9 | 50 µM | Zanoni, C., Aiello, G., Arnoldi, A., & Lammi, C. 2017 |
| | **Hypoglycemic** | | | | | | | | | | |
| **PepSequ** | **Origin** | ***In vitro* test** | | **cell protein level variation *vs* control cells** | | | | | | | |
| | | **DPP-IV ($IC_{50}$) µM** | **References** | **pAkt (S473) (%)** | **pGSK3 (%)** | **GLUT1 (%)** | **GLUT4 (%)** | **Conc. tested** | **Glucose-Uptake (%)** | **Conc. tested** | **References** |
| IAVPGEVA | Soybean | 94.6 *b)* | 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 *b)* | Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. 2016 | 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 *b)* | Aiello, G., Ferruzza, S., Ranaldi, G., et al., 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 *b)* | Lammi, C., Zanoni, C., Arnoldi, A., & Vistoli, G. 2016 | Nd *c)* | nd | nd | nd | nd | nd | nd | |

*a)* human recombinant HMGCoAR enzyme (EC 1.1.1.88); *b)* human recombinant DPP-IV enzyme (EC 3.4.14.51); *c)* 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**

| PepSequ | Origin | Activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **ACE $IC_{50}$ (µM)** | **References** | **Animal tests (Systolic Blood Pressure Reduction)** | **References** | **DPP-IV[j] $IC_{50}$ (µM)** | **References** | **Glucose Uptake in Ang II Treated L6 Myotubes (%)** | **References** |
| LPYPY | milk | 28.9[a)] | Gomez-Ruiz, Ramos, & Recio, 2007 | nd | | 90.8[b)] | Nongonierma, Mooney, Shields, & FitzGerald, 2014 | nd | |
| IRW | egg | nd | | 40 mmHg | Majumder, et al., 2013 | nd | | 22.5 | Son, Chan, & Wu, 2018 |

*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**

| PepSequ | Origin | ACE | Animal tests (Systolic Blood Pressure Reduction) | AntiOx | Assay used for the antiOx activity evaluation | References |
|---|---|---|---|---|---|---|
| WVYY | hempseed | | 34 mmHg (2h) | 67 (%) | DPPH | |
| PSLPA | hempseed | | 40 mmHg (4h) | 58 (%) | DPPH | |
| WYT | hempseed | 89.0%[a)] | 13 mmHg (2h) | < 22-42 (%) | DPPH | Girgih et al., 2014 |
| SVYT | hempseed | 79.0%[a)] | 24 mmHg (6h) | < 22-42 (%) | DPPH | |
| IPAGV | hempseed | | 36 mmHg (4h) | < 22-42 (%) | DPPH | |
| LLSGTQNQPSFLSGF | lentil | 120.0 µM[a)] ($IC_{50}$) | | 0.013 (µmol Trolox eq./µmol) | TEAC | |
| NSLTLPILRYL | lentil | 77.14 µM[a)] ($IC_{50}$) | | 1.432 (µmol Trolox eq./µmol) | TEAC | García-Mora et al., 2017 |
| TLEPNSVFLPVLLH | lentil | 117.81 µM[a)] ($IC_{50}$) | | 0.139 (µmol Trolox eq./µmol) | TEAC | |
| YSK | rice bran | 76.0 µM[a)] ($IC_{50}$) | | 0.15 mg/mL ($IC_{50}$) | DPPH | Wang et al., 2017 |
| RYLGY | milk | 0.71 µM[a)] ($IC_{50}$) | | 2.83 (µmol Trolox eq./µmol) | ORAC | |
| AYFYPEL | milk | 6.58 µM[a)] ($IC_{50}$) | | 3.22 (µmol Trolox eq./µmol) | ORAC | Miguel et al., 2009 |
| YQKFPQY | milk | 20.08 µM[a)] ($IC_{50}$) | | 2.03 (µmol Trolox eq./µmol) | ORAC | |

*a)* 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**

| PepSequ | Origin | Activity | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Enzymatic ($IC_{50}$) (µg/mL) | | | Antioxidant | | | |
| | | ACE | α-Glucosidase | DPP-IV | DPPH [d)] | FRAP [e)] | CHEL [f)] | References |
| YINQMPQKSRE | Egg | 10.1 [a)] | 1,694.3 [b)] | 222.8 [c)] | 2.3 | 59 | 37 | |
| YINQMPQKSREA | Egg | 12.6 [a)] | 454.6 [b)] | 355.8 [c)] | 1.8 | 76 | 8.5 | Zambrowicz |
| VTGRFAGHPAAQ | Egg | 27.3 [a)] | 365.4 [b)] | 1,402.2 [c)] | 1.5 | 58 | NA | et al., 2015 |
| YIEAVNKVSPRAGQF | Egg | 9.4 [a)] | NA | NA | 2.2 | 61 | 25.2 | |

*a)* Rabbit ACE enzyme (EC 3.4.15.1);

*b)* α-Glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20);

*c)* Porcine DPP-IV enzyme (EC 3.4.14.5);

*d)* µM Troloxeq/mg

*e)* and

*f)* µg $Fe^{2+}$/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.**

| PepSequ | Origin | Activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | Antioxidant | | Hypoglycemic | References | Hypocholesterolemic | References |
| | | DPPH (%) | FRAP (mM) | α-amylase inhibition (%) | | Formation of cholesterol micelles* | |
| FFRSKLLSDGAAAAKGALLPQYW (CSP1) | cumin | 3.88 | 36.71 | 24.54[a)] | Siow, H. L., & Gan, C. Y., 2016 | -71.2% | Iqbal, 2009 |
| RCMAFLLSDGAAAAQQLLPQYW (CSP2) | cumin | 58.64 | 29.61 | 7.22[a)] | | -82.0% | |
| DPAQPNYPWTAVLVFRH (CSP3) | cumin | 3.43 | 7.60 | 12.52[a)] | | 91.2% | |

*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)