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Title:

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Date:

2021-03-01

Citation:

Xue, L., Yin, R., Howell, K. & Zhang, P. (2021). Activity and bioavailability of food protein-derived angiotensin-I-converting enzyme-inhibitory peptides. Comprehensive Reviews in Food Science and Food Safety, 20 (2), pp.1150-1187. <https://doi.org/10.1111/1541-4337.12711>.

Persistent Link:

<https://hdl.handle.net/11343/337957>

Activity and bioavailability of food protein-derived angiotensin-I-converting enzyme inhibitory peptides

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Word count of text: 15078

Choice of Journal: Comprehensive review in food science and food safety

Running title: Bioactivity of ACE-inhibitory peptides

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/1541-4337.12711](https://doi.org/10.1111/1541-4337.12711).

Abstract

Angiotensin-I-converting enzyme (ACE) inhibitory peptides are able to inhibit the activity of ACE, which is the key enzymatic factor mediating systemic hypertension. ACE-inhibitory peptides can be obtained from edible proteins and have the function of antihypertension. The amino acid sequences and the secondary structures of ACE-inhibitory peptides determine the inhibitory activities and stability. The resistance of ACE-inhibitory peptides to digestive enzymes and peptidase affect their antihypertensive bioactivity *in vivo*. In this paper, the mechanism of ACE-inhibition, sources of the inhibitory peptides, structure-activity relationships, stability during digestion, absorption and transportation of ACE-inhibitory peptides, and consumption of ACE-inhibitory peptides are reviewed, which provide guidance to the development of new functional foods and production of antihypertensive nutraceuticals and pharmaceuticals.

Key Words : ACE-inhibitory Peptides, Protein, Structure-activity Relationship, Bioaccessibility, Bioavailability, Functional Food

1. Introduction

Angiotensin-I-converting enzyme (ACE, EC 3.4.15.1) inhibitors have been widely investigated for their use in the prevention and management of hypertension (Aluko, 2015; Fang et al., 2008; Iwaniak, Minkiewicz, & Darewicz, 2014; Wu, Liao, & Udenigwe, 2017). ACE inhibitors have been suggested to reduce relative mortality by 10% and cardiovascular mortality by 12 % in clinical studies (Ferrari & Boersma, 2013). Synthetic ACE-inhibitory medicines, such as Captopril, Enalapril, Benzapril, Fosinopril and Lisinopril have been produced for the treatment of hypertension (Hernández-Ledesma, del Mar Contreras, & Recio, 2011). However, these synthetic ACE inhibitors have been associated with undesirable side effects, including a dry cough, hypotension, taste disturbance, angioedema, and skin rashes (Lee & Hur, 2017).

ACE-inhibitory peptides from food are considered as a natural and relatively safe supplement to manage blood pressure (Fang et al., 2008; He, Liu, & Ma, 2013). These peptides are derived from food proteins and do not have the side effects like other blood pressure management medicines (Li, Le, Shi, & Shrestha, 2004; Phelan, Aherne, FitzGerald, & O'Brien, 2009). The length of most natural ACE-inhibitory peptides range from 2 to 20 amino acid residues (Nongonierma & Fitzgerald, 2015). Peptides isolated from different types of food sources have been reported with ACE-inhibitory activity, such as chicken meat (Sangsawad, Choowongkamon, et al., 2018), beef (Jang & Lee, 2005), loach (Li, Zhou, Huang, Sun, & Zeng, 2012), skate (Ngo et al., 2015), shrimp (Krichen et al., 2018), soybean (Hanafi et al., 2018), egg (Yu, Zhao, Liu, Lu, & Chen, 2011), dairy (Tagliazucchi, Shamsia, Helal, & Conte, 2017; Xue et al., 2018), and potato (Fu, Alashi, Young, Therkildsen, & Aluko, 2017). These peptides are either derived from natural proteins present in food or from microbial activity through three main mechanisms, enzymatic hydrolysis (Hanafi et al., 2018; Ishak & Sarbon, 2017; Liu et al., 2017), fermentation (Guo et al., 2019; Pan & Guo, 2010) and genetic recombination in bacteria and yeast (Jeong, Shin, Ahn, Song, & Lee, 2007; Lv, Huo, & Fu, 2003; Zhuang et al., 2019). Depending on the method of food processing, the same protein may produce different types of peptides with different amino acid sequence, structure and bioactivity (Chicon, López-Fandiño, Quirós, & Belloque, 2006; Majumder & Wu, 2009).

Previous research has demonstrated the ACE-inhibitory activities of natural food peptides both *in vitro* and *in vivo* conditions (Aluko, 2015). Some researchers suggested that ACE-inhibitory peptides with a food origin have the potential to be used as an alternative therapy for patients with hypertensive or related conditions (Fang et al., 2008; H. He et al., 2013; Li et al., 2004), while others only considered ACE-inhibitory peptides as a functional food ingredient or a disease prophylactic

agent to aid the treatment and not substitute for drug therapy (Nongonierma & Fitzgerald, 2016). This is mainly due to ACE-inhibitory peptides having a lower effectiveness than the synthesised drug. For example, captopril is about 1000-fold more potent than the equivalent food-derived ACE-inhibitory peptide (Vermeirssen, Van Camp, & Verstraete, 2004). In addition, food-derived ACE-inhibitory peptides may undergo structural transformations during digestion and absorption process, which could further reduce their bioavailability and bioactivity (Miguel, Aleixandre, Ramos, & López-Fandiño, 2006; Sánchez, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014; Udenigwe & Fogliano, 2017).

This review summarises the ACE-inhibitory peptides derived from different food sources, especially the peptides identified in the past decade, and compares these peptides in their production methods and structure-activity relationships (**Figure 1**). Importantly, this review summarises the influence of digestion and absorption on bioavailability and thus bioactivity of the food-derived peptides. This review provides guidance for future research on ACE-inhibitory peptides, especially the potential integration of these peptides into the food system to support general health.

2. Classification, Structure, Function and Inhibition of ACE

ACE, also called peptidyl-dipeptidase A or kininase II, is a zinc-dependent dipeptidyl carboxypeptidase, which can drive blood vessels to constrict to increase blood pressure (Aluko, 2015; Lee & Hur, 2017). ACE exists widely in mammalian tissues, especially in the vascular endothelial cells, absorptive epithelial, neuroepithelial, and male germinal cells (Li et al., 2004). There are different types of ACE with different protein sequences and 3 dimensional structures, which can exert different bioactivities in blood pressure management systems.

2.1 Classification of ACE

Longer somatic ACE (sACE) and shorter germinal or testicular ACE (tACE or gACE) are two different isoforms of ACE distinguished by size. sACE can be biosynthesised in numerous types of endothelial cells, such as kidney, small intestine and epididymis, and particularly strong in the capillaries of the lung (Riordan, 2003), while gene sequence responsible for tACE is expressed only in spermatids and spermatozoa and is testosterone dependent (Atanassova, Lakova, Bratchkova, Krasteva, & Donchev, 2009). A 21 kb length gene with 26 exons and 25 introns on chromosome 17 at q23 is responsible for encoding both tACE and sACE, except that sACE is transcribed from exons 1-12, while tACE is

transcribed from exons 13-26 (Hubert, Houot, Corvol, & Soubrier, 1991; Li et al., 2004; Riordan, 2003; Warner, Smith, Hooper, & Turner, 2004).

The structure of sACE and tACE are very different (**Table 1**). sACE is a membrane-bound glycoprotein with a single polypeptide chain composed of 1306 amino acids (140-170 kDa). It consists of a C-terminal cytosolic domain with 28 residues (from Met1 to Ala29), a heavily glycosylated extracellular domain with 1227 residues (from Leu30 to Arg1256), and a hydrophobic transmembrane domain with 22 residues (from Gln1278 to Ser1306). The extracellular domain consists of two homologous domains, including the N domain of 612 residues and the C domain of 600 residues connected by a sequence of 15 residues. Each of the homologous domain contains a catalytically active site in the middle, which is characterised by a consensus zinc-binding motif – HEXXH, where X can be any amino acid (Table 1) (Coates, 2003; Deddish et al., 1994; Riordan, 2003). tACE is a membrane-bound glycoprotein of 732 amino acids with the same cytosolic and transmembrane domains and the same extracellular domain as sACE except for its first 36 residues of the N-terminal (Conrad, Schwager, Carmona, & Sturrock, 2016; Woodman et al., 2005). The substrate preference of tACE is still unknown and this isoenzyme does not generate angiotensin II *in vivo* (Atanassova et al., 2009; Corradi, Schwager, Nchinda, Sturrock, & Acharya, 2006). sACE is used as the model ACE in nearly all studies of ACE-inhibitory peptides as it regulates blood pressure (Atanassova et al., 2009). Although the C and N domain of these two types of ACE show a high degree of similarity in both amino acid sequence and spatial conformation, the binding ability and hydrolysis rate of different substrates to the C and N-domains are different (Binevski, Sizova, Pozdnev, & Kost, 2003; Turner & Hooper, 2002). It is widely accepted that the C domain of ACE is the main site of angiotensin I cleavage *in vivo* (Fuchs et al., 2008; Junot et al., 2001; Vermeirssen et al., 2004). The C-terminal hydrophobic sequence of both types of ACE can anchor to the cell membrane, allowing the enzyme attached to the surface of cell membrane.

2.2 The function of ACE in blood pressure management

ACE is related to two blood pressure management systems, the renin-angiotensin system (RAS) and the kallikrein-kinin system (KKS) (**Figure 2**). The RAS mainly presents in adrenal cortex, kidney, liver, pancreas, heart, retina, vascular smooth muscle, brain and the adipose tissue. The initial enzymatic reaction of the RAS involves the cleavage of liver generated glycoprotein prohormone named angiotensinogen (AGT, amino acid sequence as DRVYIHPFHLVI) into decapeptide angiotensin I (ANG I, amino acid sequence as DRVYIHPFHL) by renin (EC.3.4.23.25), which is an enzyme secreted by the juxtaglomerular cells of the kidney. ANG I could be transported to the lungs, kidney and other organs

via the circulatory system, where it is further converted to the bioactive ANG II by ACE. This step is the most important regulatory mechanism in the RAS system, where the C-terminal dipeptide HL is cleaved by ACE. The ANG II is the central component of RAS and could induce the release of aldosterone, which further leads to the increase in sodium concentration and vasoconstriction, and ultimately result in the increase of systolic blood pressure (SBP). ANG II can be further converted to angiotensin III when its active (2–8) fragment is cleaved by amino-peptidase A, which also promotes the release aldosterone (Aluko, 2015; Hernández-Ledesma et al., 2011; Lee & Hur, 2017; Li et al., 2004; Manikkam, Vasiljevic, Donkor, & Mathai, 2016; Warner et al., 2004).

It is widely assumed that renin levels can be controlled by ANG II as a critical step of the negative feedback loops of the RAS system, where ANG II could rapidly inhibit renin secretion in kidney by binding to the AT1 (ANG II type 1) receptors secreted by juxtaglomerular cells in the kidney, therefore reduce the rate of AGT and AGN I conversion (Neubauer et al., 2018). This concept mostly originates from *in vitro* studies, but not yet confirmed *in vivo*. Manipulations of ANG II *in vivo* are not suitable to validate the concept of a direct negative feedback, as this process could be confounded by side effects associated with renin secretion, such as changes of blood pressure or sodium balance (Neubauer et al., 2018). Inhibition of ACE raises plasma renin activity by suppressing the ANG II level, which further promotes renin production due to the lack of negative feedback by ANG II. For instance, the plasma renin activities were reported to increase after the oral administration of ACE-inhibitory peptide IPP and VPP in spontaneously hypertensive rats (SHRs), (Sipola, Finckenberg, Korpela, Vapaatalo, & Nurminen, 2002). Additionally, retention of salt and water, increase in circulating volume and the perfusion of the juxtaglomerular apparatus could also provide inhibitory signal to renin (Lee & Hur, 2017).

The kallikrein-kinin system (KKS) is also known as the nitric oxide system (NOS). In this system, bradykinin (BK, amino acid sequence as RPPGFSPFR), a vasodilatory peptide, can bind to the β -receptors present in a variety of tissues and organs, such as bronchial and vascular smooth muscle, to increase intracellular Ca^{2+} level and to stimulate endothelial nitric oxide synthase (eNOS) to convert L-arginine to nitric oxide (NO) (Aluko, 2015; Dhaval, Yadav, & Purwar, 2016; Lee & Hur, 2017). NO can maintain regular blood vessel dilation thus blood pressure. ACE can break down and inactivate BK, leading to an insufficient level of NO (especially under high levels of angiotensin II), which result in insufficient blood vessel relaxation and therefore, raise blood pressure (Lee & Hur, 2017; Wu et al., 2017). ACE inhibitors reduce the inactivation of BK and therefore reduce blood pressure.

2.3 The enzymatic active sites of ACE and mechanisms of inhibition

Medicines used for the treatment of hypertension achieve the effect by one of the following mechanisms (1) to inhibit activities of renin and/or ACE; (2) to reduce and/or inhibit the binding between angiotensin II and its receptors; (3) to increase the NO level in blood (Aluko, 2015). ACE inhibitors could reduce blood pressure by reducing ANG II formation and BK degradation through RAS and KKS. sACE has three main active site pockets (subsites), S1, S1' and S2. The S1 pocket includes three residues, Ala354, Glu384 and Tyr523, while the S2 pocket includes five residues, Gln281, His353, Tyr520, His513 and Lys511 residues, and the S1' only contains Glu162 residue (Wu, Jia, Yan, Du, & Gui, 2015). ACE is a metallo-protease with a zinc ion (Zn^{2+}) interacting with His383, His387 and Glu411 (Pina & Roque, 2009). These regions are responsible for the bioactivity of the ACE, and are also the regions that ACE inhibitors bind (Natesh, Schwager, Sturrock, & Acharya, 2003). Importantly, the natural conformation of sACE is dominated by spiral structures and forms a stereoscopic structure, which contains 61 α -helix, 18 β -sheet, and 14 β -turn. Four disulfide bonds present between Cys157-Cys165, Cys757-Cys763, Cys957-Cys975 and Cys1143-Cys1155, contribute to the structural stability (**Figure 3**).

Most ACE inhibitors and bioactive peptides interact with and inhibit both the C- and N-domains of sACE and tACE by forming hydrogen bonds and hydrophobic interactions at the S1, S2, S1' subsites of ACE and/or binding directly to the catalytic Zn^{2+} . Multiple hydrogen bonds between peptides and ACE could stabilise the structure of non-catalytic enzyme-peptide complex, and as a result, lead to the inhibition of ACE enzyme activity (Abdelhedi & Nasri, 2019). To completely inhibit the cleavage of ANG I and BK *in vitro*, active sites located at both the N- and C-domains of ACE need to be blocked. The conversion of ANG I to ANG II could be prevented when either the N-domain or the C-domain of ACE is inhibited, however, it is necessary to inhibit both sites of ACE to protect BK (Georgiadis et al., 2003).

ACE can be inactivated by competitive, non-competitive or uncompetitive mechanisms. The competitive inhibitors act by competing with normal substrates or ligands for the same binding site of the enzyme protein, reducing the affinity between the enzyme and the substrate. In this case, the K_m (the concentration of the substrate when the reaction reaches half of the maximum rate) of the enzyme reaction increases, but the V_{max} (the highest reaction rate when the enzyme is saturated under a certain reaction condition) does not change. Most of the known ACE-inhibitory peptides are competitive inhibitors (Rawendra et al., 2013; Wu et al., 2019; Xue et al., 2018). Non-competitive inhibitors are able to bind with ACE at a site rather than the ACE active sites to inactivate the whole

complex. Whether or not the substrate binds to the enzyme, the inhibitor can bind to the enzyme (Fu, Young, et al., 2016; Lee, Qian, & Kim, 2010). Presence of a non-competitive inhibitor will decrease $V_{\max}$ and will not affect K_m . Rarely, an ACE-inhibitory peptide binds to ACE only when the substrate is bound to ACE. Uncompetitive inhibitors of this type decrease both $V_{\max}$ and K_m (Nakagomi K et al., 1998).

2.4 ACE-inhibitory peptides activity

There are different parameters used in research to quantify the activity of ACE-inhibitory peptides both in vitro and in vivo. IC_{50} , the concentration of a peptide that reduce ACE activity by 50%, was introduced as a key indicator of inhibitory potency. The lower the IC_{50} value, the higher the ACE-inhibitory effect, and this indicator is mostly used in in vitro experiments.

For in vivo studies, the ACE levels in the plasma of animal and human subjects can directly reflect the effect of peptides treatment. The concentration of ACE-inhibitory peptide in plasma can reflect whether the peptide can reach the blood circulatory system in intact form and play an ACE-inhibitory role. This measurement is commonly used in conjunction with direction measurement of the arterial, systolic or diastolic blood pressure in animal and human studies. A recent study investigated both the ACE activity/ACE-inhibitory peptides in plasma and blood pressure, where human subjects were provided 50 g whey protein hydrolysate, containing two ACE-inhibitory peptides IW and WL (Martin et al., 2020). Results suggested that the plasma ACE activity of healthy volunteers decreased to $75.1 \pm 6.9\%$ of the original level, while their plasma concentrations of IW and WL increased from 2.8 ± 0.7 nM to 99.1 ± 58.7 nM and from 10.1 ± 1.8 nM to 34.9 ± 19.4 nM respectively, while no blood pressure reduction was observed.

More *in vivo* studies on ACE-inhibitory peptides were carried out in animals, mostly in SHR_s rather than human subjects. Hemp seed meal protein hydrolysate (HMH) showed strong antihypertensive effects in SHR_s, where the plasma ACE activity in HMH-fed rats was significantly ($p < 0.05$) inhibited ($0.047\text{--}0.059$ U/mL) and plasma renin level was also decreased ($0.040\text{--}0.054$ $\mu\text{g/mL}$) (Girgih, Alashi, He, Malomo, & Aluko, 2014). Feeding 1% chicken skin protein hydrolysates (CSPHs) can significantly ($P < 0.05$) lower SBP of SHR_s (~ 36 mmHg) by decreasing the plasma ACE level (Onuh et al., 2016). Additionally, oral administration of corn germ peptide at dosage of 1 g/kg body weigh (BW) could not only inhibit the ACE activities in artery (1474.6 mU/mg), kidney (758.4 mU/mg), lung (810.3 mU/mg), and heart (666.4 mU/mg) of SHR_s, but also significantly lowered ($p < 0.01$) the levels of both renin (15.1 ng/L) and Ang II (41.3 ng/L), which further led to lower blood pressure (Guo et al.,

2020). Another animal study reported significantly ($p < 0.05$) reduction in plasma ACE activity and the ANG II level when SHR were orally administered 100 mg/kg BW grass carp peptides fractions for 6 weeks and the SBP of SHR was also decreased by 20.7 mmHg 4 weeks after treatment (Wang et al., 2014).

However, quantification of ACE activity and ACE-inhibitory peptides in plasma is quite challenging due to their low concentrations. The level of ACE-inhibitory peptides in plasma might be in the ng ml^{-1} or pg ml^{-1} range, and thereby requiring highly sensitive and accurate methods (Van Platerink, Janssen, Horsten, & Haverkamp, 2006). Therefore, for most *in vivo* studies, the antihypertensive activity of peptides was only measured by examining the arterial, systolic or diastolic blood pressure, although it may not be able to tell whether it was the original peptides or their metabolites that exerted antihypertension effect. For instance, injection of IVY (isolated from wheat germ hydrolysate) to mice at 5 mg/kg body weight resulted in a transient antihypertensive effect of 10% reduction in arterial blood pressure after 8 mins of injection and the pressure returned to normal after 20 mins (Matsui et al., 2000). The same study also found that IVY was metabolized in human and rat plasma into a new ACE inhibitor, VY. Subsequential study found that VY level in human plasma could increase with the VY intake dosage, where oral administration of VY resulted in a significant reduction of blood pressure in both mild essential hypertensive and high-normal subjects, however no blood pressure changes were observed in normotensive subjects (Matsui et al., 2002a; Matsui et al., 2002b).

2.5 Classification of ACE-inhibitory peptides

Based on the inhibitory mechanism, ACE-inhibitory peptides can be categorised into three groups: the inhibitor type, the substrate type and the prodrug type (Fujita, Yokoyama, & Yoshikawa, 2000; Li et al., 2004; Vermeirssen et al., 2004). Inhibitor peptides are peptides that cannot be hydrolysed by ACE, therefore can keep their intact structure and activity when preincubated with ACE. Common inhibitor type ACE-inhibitory peptides include IKP (IC_{50} 1.6 μM) (Fujita et al., 2000), VPP (IC_{50} 9 μM) (Satake et al., 2002), MRW (IC_{50} 0.6 μM) (Yang, Marczak, Yokoo, Usui, & Yoshikawa, 2003), VF (IC_{50} 120 μM) (Vercruysse et al., 2010), and IPP (IC_{50} 5 μM) (Gleeson, Brayden, & Ryan, 2017) (Table 2).

The substrate type peptides can temporarily connect to ACE and be hydrolysed into smaller peptides, then the inhibition activity of peptides may decrease or even disappear completely. Therefore, the substrate peptides can not show antihypertensive effect *in vivo*, which need long-lasting inhibition. This is one of the reasons why ACE inhibitory activities of peptides *in vitro* are not

always parallel to their antihypertensive effects *in vivo*. AVYPYQR (IC_{50} 15 μ M) from casein is the first example of substrate peptide (Susumu, Nakagomi, Tomizuka, & Suzuki, 1985). Another example would be FFGRCVSP (IC_{50} 0.4 μ M) from ovalbumin, which can be hydrolyzed by ACE into FF, GR, CV, and SP with IC_{50} increased to 4.6 μ M, however has no antihypertensive effect (Fujita et al., 2000).

The prodrug peptides show no- or low-inhibitory activity themselves, but can be converted to active ACE inhibitor by ACE or by gastrointestinal proteases, and their antihypertensive effects last longer than the original peptides (Fujita et al., 2000). LKPNM is a typical prodrug type ACE-inhibitory peptide derived from thermolysin digesta of dried bonito with IC_{50} value of 2.4 μ M. It could be hydrolysed by ACE to produce a new peptide LKP (IC_{50} 0.32 μ M), which has stronger ACE-inhibitory activity than the original peptide. Intravenous injection of LKPNM and IKP at a dose of 10 mg/kg could lead to 80 mmHg and 70 mmHg reduction in the SBP of SHR respectively, while oral administration of these two peptides at 60 mg/kg resulted in 23 mmHg and 20 mmHg respectively (Fujita et al., 2000; Fujita & Yoshikawa, 1999). Despite of the similar effects in blood pressure reduction, there is a clear delay in the time of maximum blood pressure reduction between these two peptides, where was 2 hours slower and this delay may due to the time for LKPNM to be hydrolyzed into IKP. These studies also reported another prodrug-type ACE-inhibitory peptide, IWHHT (IC_{50} 5.8 μ M), which could be hydrolysed by ACE and released IWH (IC_{50} 3.5 μ M). Although the blood pressure reducing effect in SHR were similar between IWHHT and IWH, a significant difference in the time of maximum pressure research was also observed between these two peptides (-26 mm Hg, 6 h for IWHHT, -30 mm Hg, 4 h for IWH after oral administration), demonstrating the possible hydrolysis from IWHHT to IWH. LRPVA released from bovine lactoferrin is another example of the pro-drug type ACE inhibitor, which is converted by ACE itself to LRP showing higher inhibitory activity and longer anti-hypertensive effect (Yoshikawa, 2015).

3. Natural sources of novel ACE-inhibitory Peptides

The first natural ACE-inhibitory peptide was isolated from snake venom (Ferreira, Bartelt, & Greene, 1970). Since then, researchers have been trying to identify new peptides from different food sources, including animal origin peptides from dairy, fish, meat and egg products, plant origin peptides from soybean and rice, and microbially-derived peptides (Table 2).

3.1. ACE-inhibitory peptides from animal origins

ACE-inhibitory peptides have been identified from various animal products, such as chicken, beef, egg, and milk (**Table 2**). In most cases, peptides are present in the hydrolysate of animal muscle and connective tissue. For instance, a hexapeptide VLAGTL was identified in the hydrolysate of beef sarcoplasmic protein treated with thermolysin and proteinase A, and this peptide was demonstrated to have ACE-inhibitory activity (IC_{50} 32.06 μ M) both *in vitro* and *in vivo* (Jang et al., 2004; Jang & Lee, 2005). Up to date, IKW (IC_{50} 0.21 μ M) from chicken muscle is the most potent peptide reported in this field (Fujita et al., 2000). Sangsawad, Roytrakul, and Yongsawatdigul (2017) reported three peptides identified in the digesta of chicken breast, KPLLCS, ELFTT and KPLL with IC_{50} value of 0.37 μ M, 6.35 μ M and 11.98 μ M, respectively. Sangsawad, Choowongkamon, et al. (2018) further examined the permeability for these peptides across intestinal epithelium and demonstrated the possible breakdown of peptides during absorption process. Among these peptides, KPLL retained the highest ACE-inhibitory activity after passing the intestinal epithelium cell and maintained 56% inhibitory activity compared to that before passing the cell, while KPLLCS retained 26.87% and ELFTT retained 1.78% activity. Another peptide IWHHT was identified in the thermolysin digesta of both Spent Hen (IC_{50} 9.93 μ M) and bonito (IC_{50} 5.8 μ M) (Fujita et al., 2000; Gu et al., 2019).

Fish protein is another rich source of ACE-inhibitory peptides. Li et al. (2012) identified a peptide AHLL from loach fish treated with bromelain. The ACE-inhibitory activity of this peptide was found to be 33.7-fold higher compared to the original loach protein hydrolysate. Krichen et al. (2018) identified four new peptides SSSKAKKMP, HGEGGRSTHE, WLGHGGRPDHE and WRMDIDGDMISEQEAHQ from the shrimp protein. Gelatin from both bovine and fish is one of the main sources of ACE-inhibitory peptides. GPL (IC_{50} 2.55-2.6 μ M) could be found in the hydrolysate of both bovine skin gelatin (Kim, Byun, Park, & Shahidi, 2001) and Alaska pollack skin gelatin (Byun & Kim, 2001). Ngo et al. (2015) hydrolysed skate skin gelatin and obtained two peptides MVGSAPGVL (IC_{50} 3.09 μ M) and LGPLGHQ (IC_{50} 4.22 μ M), which also showed antihypertension effect *in vivo*. Clearly, the sequence GPL also exists in LGPLGHQ from skate skin hydrolyte and the difference in peptides identified from these two studies may be due to the different proteases used. Additionally, there is a chance LGPLGHQ may be further hydrolysed into GPL in *in vivo* conditions. Further, two peptides VPAAPPK (IC_{50} 0.45 μ M) and NGTWFEPP (IC_{50} 0.63 μ M) were released from an haruan myofibrillar treated with proteinase K and thermolysin (Ghassem, Arihara, Babji, Said, & Ibrahim, 2011). Samaranayaka, Kitts, and Li-Chan (2010) reported that the protein hydrolysate of pacific hake fish could inhibit ACE with IC_{50} of 161 μ M, however, they didn't isolate the bioactive peptide.

Compared to other food sources, more studies have isolated ACE-inhibitory peptides from chicken egg whites and cow milk proteins. For example, three peptides, QIGLF, TNGIIR, RVPSL, were identified from egg white protein hydrolysed by alcalase with IC_{50} values of 75 μ M, 70 μ M, 20 μ M (Lee, Jeon, & Byun, 2011; Yu et al., 2012; Yu, Yin, et al., 2011). ACE-inhibitory peptides (YV, IC_{50} 214.42 μ M, IVRDPNGMGAW, IC_{50} 4.39 μ M) were also identified from ostrich eggs (Khueychai et al., 2018) and soft-shelled turtle eggs (Rawendra et al., 2013). Several peptides obtained from dairy products have been found with high ACE-inhibitory activity. The first ACE-inhibitory peptides derived from bovine casein was reported as early as in 1980s, including FFVAPFPEVFGK (CEI₁₂), FFVAP (CEI₅), and AVYPYQR (CEI₈₇) (Maruyama & Suzuki, 1982; Susumu et al., 1985). Many other casein derived peptides were also reported, such as RYLGY (IC_{50} 0.71 μ M), AYFYPEL (IC_{50} 6.58 μ M) and YQKFPQY (IC_{50} 20.08 μ M) (Mar Contreras, Carrón, Montero, Ramos, & Recio, 2009). Importantly, a bovine casein derived peptide MKP was reported with very strong ACE-inhibitory activity *in vitro* (IC_{50} 0.43 μ M). At 4 hours after oral administration of MKP (1 mg/kg), the SBP of SHRs decreased from 180.4 ± 0.6 to 152.2 ± 0.3 mmHg (Yamada et al., 2015). At least 14 different types of peptides derived from casein have been reported with different ACE-inhibitory activities (**Table 2**). These peptides were hydrolysed from casein by different proteases under different conditions, and therefore have different cleavage sites. For example, when treated with pepsin and trypsin, a novel peptide YQKFPQYLQY (IC_{50} 11.1 μ M) could be isolated in casein hydrolysate (Xue et al., 2018). Whey protein is also a source of ACE-inhibitory peptides. Several whey protein derived peptides, such as LIWKL (Ruiz-Giménez et al., 2012) and RWQ (Fernández-Musoles et al., 2013), have been found with strong ACE-inhibitory activity *in vitro* and antihypertensive activity *in vivo*. Further, immobilised *Lactobacillus helveticus* proteinase was found able to break down whey protein into smaller peptides with ACE-inhibitory activity (Guo et al., 2019).

Insect proteins may also be used to produce ACE-inhibitory peptides, however this has not been well investigated. A novel tripeptide, ASL (IC_{50} 102.15 μ M) was identified from silkworm pupa hydrolysed by pepsin, trypsin, and α -chymotrypsin (Q. Wu et al., 2015). Insects protein is a promising alternative protein source for human consumption and new peptides derived from insect protein are a promising new research area.

3.2. ACE-inhibitory peptides from plant origins

Plant proteins from soybean, rice, peanut, hazelnut, wheat, barley and hemp are also good source of ACE-inhibitory peptides. Lin et al. (2017) identified an antihypertensive peptide, LSW (IC_{50} 2.7 μ M), from soybean protein hydrolysed by thermolysin. Another study analysed rice hydrolysate and

identified two novel peptides of VNP (IC_{50} 6.4 μ M) and VWP (IC_{50} 4.5 μ M) (Chen et al., 2013). In addition to enzymic hydrolysis of plant protein, studies have also investigated the impacts of fermentation on the production of ACE-inhibitory peptides. For instance, peptide LVQGS was identified in soybean fermented by *Aspergillus oryzae*, which has an IC_{50} value of 43.7 μ M (Rho, Lee, Chung, Kim, & Lee, 2009).

Many different plant materials were found able to produce ACE-inhibitory peptides. Li et al. (2014) reported the diastolic blood pressure (DBP) of SHR decreased around 16 mmHg and SBP decreased 22 mmHg 4 hours after the oral administration of pistachio kernel hydrolysate. Then a novel peptide ACKEP (IC_{50} 126 μ M) was identified from hydrolysate. Furthermore, barley hydrolysate contain peptides FQLPKF (IC_{50} 28.2 μ M) and GFPTLKIF (IC_{50} 41.2 μ M) with ACE-inhibitory activities (Gangopadhyay et al., 2016). Two peptides WG and PRY were obtained from potato hydrolysed by ficain, with IC_{50} values of 231.22 μ M and 97.59 μ M, respectively (Fu et al., 2017; Fu, Wu, Zhu, & Xiao, 2016). A novel tripeptide YSK was obtained from trypsin hydrolysed rice bran with an IC_{50} value of 76 μ M (Wang, Chen, Fu, Li, & Wei, 2017). The inhibition effect of YSK is attributed to formation of strong hydrogen bonds with ACE catalytic site. In a similar way, Garcia-Mora et al. (2017) reported peptides LLSGTQNQPSFLSGF, NSLTLPILRYL and TLEPNSVFLPVLLH (IC_{50} 77 – 119 μ M) from lentil protein.

Nuts and seeds are also potential source of ACE-inhibitory peptides. Liu, Fang, Min, Liu, and Li (2018) reported three novel peptides AVKVL, YLVR, TLVGR derived from hazelnuts with IC_{50} values of 73.06 μ M, 15.42 μ M and 249.3 μ M, respectively, while peptide EPNGLLLPQY was identified in walnut protein derivative (IC_{50} 233.178 μ M) (Wang et al., 2018). Orio et al. (2017) identified three peptides GVLV, LGY and RVR derived from hemp seed with IC_{50} values of 16 μ M, 154 μ M and 526 μ M, respectively. Peptides with ACE inhibitory activity were also identified in zein (Miyoshi et al., 1991), flaxseed (Maramba, Shand, & Wanasundara, 2011), canola (Wu, Aluko, & Muir, 2008), and rapeseed (Marczak et al., 2003) (Table 2).

Overall, less number of ACE-inhibitory peptides were identified from plant origin protein in comparison to animal origin protein, nevertheless, these bioactive peptides have been identified in a diverse range of plant based food, suggesting much more novel ACE-inhibitory peptides could present in plant food materials, and warrant further investigation.

3.3. ACE-inhibitory peptides from microorganisms and others

Algae, as a human food supplement, has been commercially produced widely, and research has observed ACE-inhibitory activity of algae hydrolysates. For instance, tripeptide IQP (IC_{50} 5.77 μ M)

derived from *Spirulina platensis* could lower both systolic and diastolic blood pressure *in vivo* (Lu, Ren, Xue, et al., 2010). VECYGNRPQF (IC₅₀ 26.9 µM) derived from *Chlorella vulgaris* (Sheih, Fang, & Wu, 2009), YMGLDLK (IC₅₀ 36.1 µM) from *Isochrysis galbana* (Wu, Xu, Sun, Yu, & Zhou, 2015), LVTVM (IC₅₀ 18 µM) from *Nannochloropsis Oculata* (Qian et al., 2013) were also found to have relatively strong ACE-inhibitory activity.

Yeast protein is another source of ACE-inhibitory peptide. Several peptides, including YGKPVAVPAR and LPESVHLDK have ACE-inhibitory activity and are derived from *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* (Mirzaei, Mirdamadi, Ehsani, & Aminlari, 2018; Mirzaei, Mirdamadi, Ehsani, Aminlari, & Hosseini, 2015). Two peptides, KVLPVP and VLPVP, (IC₅₀ 4.6 and 1.7 µM respectively), were isolated from *Escherichia coli* with strong ACE-inhibitory activity (Lei, Sun, Liu, Liu, & Li, 2008; Liu, Sun, Zhang, & Li, 2007).

4. Methods to Produce ACE-inhibitory Peptides

The ACE-inhibitory peptides can be obtained from food by a number of methods, including enzymatic hydrolysis and fermentation. Once the sequence and structure are known, peptides can also be synthesised using chemical and enzymatic methods, and through recombinant DNA technology.

4.1 Enzymatic protein hydrolysis

Enzymatic hydrolysis is the most common method to obtain ACE-inhibitory peptides from their original protein source and may involve one or multiple enzymes. Many natural peptides are produced from food proteins during normal human digestion process, hydrolysed by gastrointestinal enzymes, such as pepsin, pancreatin, trypsin, α-chymotrypsin, and peptidases. Enzymes from bacteria, fungi and plants, commercial enzymes, and chemicals such as acids or alkali are also commonly used to produce peptides from various proteins (Manikkam et al., 2016). For instance, cysteine protease from *Actinidia* can release ACE-inhibitory peptides from different plant proteins such as oat, soy and rice (Zhang, Sun, Liu, Li, & Jiang, 2017).

The type of ACE-inhibitory peptide produced after protein hydrolysis depends on the type of protease selected, as different enzymes have different cleavage sites and could produce different peptides even from the same substrate. Combination of different enzymes would influence the peptides produced further (Manikkam et al., 2016). For instance, when casein is hydrolysed by pepsin, trypsin or the combination of pepsin and trypsin, peptides with different amino acid

sequence and ACE-inhibitory activity are produced (**Table 2**). Chiang, Tsou, Tsai, and Tsai (2006) compared the soy protein hydrolysed by five different enzymes, alcalase, flavourzyme, trypsin, chymotrypsin and pepsin, and observed different ACE-inhibitory activities, whereas the hydrolysate produced by flavourzyme showed the lowest ACE-inhibitory activity while the hydrolysate produced by alcalase showed the highest inhibitory activities (Chiang et al., 2006). Similar study was conducted with wild almond digested with alcalase, flavourzyme, trypsin, chymotrypsin and pepsin. The ACE inhibition of hydrolysate vary from 88.7% (flavourzyme) to 99.9% (alcalase); alcalase produces hydrolysates with the highest ACE-inhibitory activities (Mirzapour, Rezaei, & Sentandreu, 2017).

Food processing techniques, such as thermal treatment, high hydrostatic pressure and ultrasonic, may induce changes in protein steric structure and enhance exposure to enzymes, which could increase protein digestibility and peptide release. These different treatments may result in peptides with different ACE-inhibitory activities. Majumder and Wu (2009) reported that boiled egg hydrolysate showed lower ACE-inhibitory activities compared with the stir-fried egg hydrolysate. The reason might be that the treatment temperature (170 °C) for fried eggs was higher than that of boiled eggs (100 °C), leading to higher degrees of protein denaturation and release of different bioactive peptides. The thermal gradient from the outside to the center of the egg might result in yolk protein not been extensively denatured in boiled egg, on the other hand, stir-fried egg was almost uniformly heated during the frying process to achieve complete denaturation. This can result in higher digestibility and ACE-inhibitory activity of fried eggs. Quiros et al. (2007) reported that high pressure treatment up to 400 MPa together with chymotrypsin, trypsin and pepsin could enhance ovalbumin hydrolysis by enhancing the accessibility of enzymes to the hydrophobic regions of the ovalbumin. Although no significant improvement in ACE-inhibitory activity was observed in hydrolysates treated under 200-400 MPa, pressure treatment accelerated the proteolysis and release of three peptides, YAEERYPIL, FRADHPFL and RADHPFL, which were demonstrated with antihypertensive effects *in vivo* by other studies (Table 2) (Quirósa, Chichon, Recio, & López-Fandiño, 2007). Ultrasonic treatments during proteolysis can also promote the hydrolysis of wheat germ protein and release more ACE-inhibitory peptides by increasing surface hydrophobicity of the germ protein and loosening of the protein tissue (Jia et al., 2010).

Other factors, such as the duration of hydrolysis, pH, degree of protein hydrolysis, enzyme-substrate ratio, and various types of pre-treatments would also influence the production of peptides (Chiang et al., 2006; Manikkam et al., 2016). Optimization of the hydrolysis process has been covered in

previous reviews and is not discussed here (Chen, Wang, Shu, & Chen, 2018; Ishak & Sarbon, 2017; Moayed et al., 2018; Shu, Shi, Chen, Ji, & Meng, 2019; Zhang et al., 2017).

4.2 Fermentation

ACE-inhibitory peptides can be produced during microbial fermentation. Proteases secreted by bacteria, yeast or mould species can hydrolyse protein of different food sources to produce ACE-inhibitory peptide (Aluko, 2015), such as those from bovine milk (Pan & Guo, 2010), camel milk (Mati et al., 2017), meat (Escudero et al., 2013), and soy beans (Singh & Vij, 2017; Zhu et al., 2008).

The hydrolysis of proteins during fermentation is a result of combined actions of enzymes. The microbial strain used in fermentation is important, where lactic acid bacteria, including *Lactobacillus helveticus*, *Leuconostoc mesenteroids*, *Lactobacillus reuteri*, *Lactobacillus fermentum*, *Lactobacillus johnii*, *Lactobacillus plantarum* and *Lactobacillus casei*, are commonly used as starter cultures for dairy products and also releasing ACE-inhibitory peptides from milk proteins (Aspri, Leni, Galaverna, & Papademas, 2018; Beltran-Barrientos, Hernandez-Mendoza, Torres-Llanez, Gonzalez-Cordova, & Vallejo-Cordoba, 2016; Pan & Guo, 2010; Singh & Vij, 2017). Fermentation time influences the peptide produced, which may take from hours to several days, depending on the strains of bacteria or yeast used (Aluko, 2015). Tripeptides VPP ($IC_{50}=9\mu M$) (Foltz et al., 2008; Kawaguchi, Nakamura, Kamiie, Takahashi, & Yamamoto, 2012; Nakamura et al., 1995; Ohsawa et al., 2008; Panayotova, Pashova-Baltova, & Dimitrov, 2018; Satake et al., 2002), and IPP ($IC_{50}=5\mu M$) (Foltz et al., 2008; Gleeson et al., 2017; Gleeson, Heade, Ryan, & Brayden, 2015; Kawaguchi et al., 2012; Nakamura et al., 1995; Ohsawa et al., 2008; Panayotova et al., 2018) are the two most studied peptides with strong ACE-inhibitory activities, and both of them are released from β -casein by *L. helveticus*, *L. bulgaricus*, *S. thermophilus* fermentation. Additionally, the autolysate of spent brewer's yeast was reported with moderate ACE-inhibitory activity (IC_{50} 146 $\mu g/mL$), which suggests an alternative use of this brewery by-product (Amorim, Pinheiro, & Pintado, 2019).

4.3 Genetic recombination in bacteria and yeast

Genetic recombination can be performed to biosynthesize the target peptide in microorganism. This has allowed the industrial production of ACE-inhibitory peptide at higher yield and lower cost compared with the traditional enzymatic methods. For example, Liu et al. (2007) successfully produced 170 mg/L of pure recombinant peptide KVLPPV (IC_{50} 4.6 μM) in *Escherichia coli* (*E. coli*). Further, the maximum drop in the systolic blood pressure of rats can be up to 21.4 ± 7.2 mmHg after oral intake of the recombinant peptide in a dose-dependent manner between 0.3 to 0.6 mg/kg of

body weight. The tripeptide HHL is commonly used to test the ACE inhibition activity of a peptide in laboratory conditions (HHL method). Jeong et al. (2007) reported that this HHL could be produced in *E.coli* with a final yield of 6.2 mg/L. Further, 8 ACE-inhibitory peptides could be produced simultaneously in *Pichia pastoris* SMD1168H with a combined IC_{50} value of 88.2 μM , and these peptides could further be hydrolysed by pepsin to produce stronger peptides with an IC_{50} value of 0.69 μM (Tai, Li, Hung, & Yin, 2018). One of the challenges to produce peptides through genetic engineering is the low molecular weight of ACE-inhibitory peptides, which are susceptible to proteases hydrolysis in the host bacteria or yeast (Liu et al., 2007). The same study also suggests that the biosynthesis rate of peptides with low molecular weight is lower than that with higher molecular weight. Most ACE-inhibitory peptides discussed in previous session have short peptide chains and lower molecular weight. Further, peptides produced in the host microorganism might be harmful for the host and affect the normal expression of host gene (Hernández-Ledesma et al., 2011). Despite of these challenges, genetic recombination technique represents an efficient and cost-effective method for the mass production of ACE-inhibitory peptide. Future researches may focus on producing more types of peptides via this technique and improving production efficiency.

4.4 Purification of ACE-inhibitory peptides

The direct product of enzyme hydrolysis, fermentation or genetic engineering is usually a mixture of multiple peptides, where separation and purification steps need to be applied to obtain high purity ACE-inhibitory peptides. Methods used to separate and purify peptides include membrane filtration, size exclusion, ion exchange and reverse-phase chromatography, which separate peptides according to their size, charge, affinity, polarity and hydrophobicity/hydrophilicity characteristics (Abdelhedi & Nasri, 2019; Aluko, 2015; Lee & Hur, 2017). These methods are usually combined to achieve better purification purpose with centrifugation normally as the first step, followed by other steps such as membrane filtration and freeze-drying. In a study on brewers' spent grain protein hydrolysate with proteases, gel permeation HPLC, tangential flow filtration, semi-preparative HPLC were used to separate and purify two peptides IVY and LDL (Connolly, O'Keeffe, Piggott, Nongonierma, & FitzGerald, 2015). Ultrafiltration at 10 kDa and 3 kDa was used to separate the trypsin hydrolysate of *Gracilariopsis lemaneiformis*, followed by Sephadex G-25 gel filtration column, Sephadex G-15 gel filtration column and ÄKTA pure system with C18 column, to obtain a tetrapeptide of QVEY with an IC_{50} value of 474.36 μM (Cao et al., 2017). Wild almond protein hydrolysate was purified by sequential ultrafiltration at 10 and 3 kDa and further purified by reversed phase high-performance liquid chromatography (RP-HPLC), to obtain 3 novel ACE-inhibitory peptides with sequences VVNE,

VVTR, and VVGVD (Mirzapour et al., 2017). Similarly, Haruan myofibrillar protein hydrolysate was ultrafiltered by 10 and 3 kDa molecular weight membranes in succession, followed by gel filtration on polyacrylamide Bio-Gel P-2 column and RP-HPLC on C18 column to obtain two peptides, VPAAPPK ($IC_{50} = 0.45 \mu M$) and NGTWFEPP ($IC_{50} = 0.63 \mu M$) (Ghassem et al., 2011). Most of these reported purification methods were for laboratory research purpose with limited studies on mass production of pure peptides. To allow the possible wide application of ACE-inhibitory peptides as functional food and/or supplements, future research may explore the possible cost-effective and efficient purification methods of peptides suitable for industrial application.

5. Structure-Activity Relationships of ACE-inhibitory Peptides

ACE is a zinc metallopeptidase, hence the interactions between peptide and the Zn^{2+} plays an important role in enzyme inhibition (Mirzaei, Mirdamadi, Safavi, & Hadizadeh, 2019). Peptides can interact with ACE in many ways, including hydrogen bonds, hydrophobic interactions, hydrophilic interactions, van der Waals force, electrostatic force and metal acceptor interactions (Li et al., 2014), and the inhibitory activities of peptides are highly dependent on their primary and secondary structures and interactions with the ACE-protein complex.

5.1 Molecular weight and primary structure impact

Most ACE-inhibitory peptides are short with less than 12 amino acids (Ni, Li, Liu, & Hu, 2012). Mirzapour et al. (2017) suggested that smaller peptides less than 3 kDa have higher ACE-inhibitory activity compared to the larger peptides, which is also supported by many studies summarised in the section 5.4 of this article. This may be because larger peptide may not fit into the active site of the ACE (Natesh et al., 2003).

The inhibitory ability of peptides is also related to its amino acid sequence, especially the content of hydrophobic amino acids. Hydrophobic amino acids, such as aromatic or branched side chains usually lead to higher ACE-inhibitory activity (Wu, Aluko, & Nakai, 2006). For instance, peptides with hydrophobic amino acid residues Val in the sequence, such as VPP, VVNE, VVTR, VVGVD, VAP, VKK and VPAAPPK were reported to have strong ACE inhibition activity (Ghassem et al., 2011; Mirzapour et al., 2017; Satake et al., 2002). Some researchers have suggested that the ACE-inhibitory activity of a peptide is strongly associated with its three amino acid residues on both C- and N-terminals of peptides, since they can interact with the hydrophobic subsites, S1, S1', and S2 on ACE (Ni et al., 2012). For instance, peptides contain Trp, Phe, Tyr, or Pro at the C-terminal, and Ile and Val at the N-terminal have higher ACE-inhibitory activities potential because these amino acids can enhance the

binding of peptides with ACE (Li et al., 2004). Other researchers suggested that the ACE-inhibitory activity is more related to the C-terminal amino acids of the peptide, since ACE could cleave the C-terminal amino acids. More precisely, ACE prefers inhibitors with hydrophobic amino acid at the three C-terminal positions, which could have stronger affinity to the S1, S1' and S2' pockets (Hernández-Ledesma et al., 2011; Li et al., 2004). Additionally, the positive charge on the side chain of the Arg and Lys residues at the C-terminal could also increase the ACE-inhibitory potential of peptides (Vermeirssen et al., 2004). Xie, Choung, Cao, Lee, and Choi (2015) modified four ACE-inhibitory peptide VK, YA, KY and TAY with additional Trp at the C-terminal and obtained new peptides with significantly higher ACE-inhibitory activities. Importantly, the ACE-inhibitory activity of VKW was increased by 1450 times compared to the original VK. Another study reported high ACE-inhibitory activity of two peptides derived from Haruan myofibrillar protein, named VPAAPPK with IC₅₀ of 0.45 µM and NGTWFEPP with IC₅₀ of 0.63 µM. Both of these peptides have two proline residues at the C-terminal (Ghassem et al., 2011). In clinical trial, hydrophobic ACE-inhibitory drugs were found superior compared to the hydrophilic ones, as the former one could bind to both the C- and N- terminal catalytic sites to better block ANG II production, while hydrophilic drugs only bind to the C-terminal (Moskowitz, 2002).

The final two amino acids at the C-terminal are considered as a factor influencing ACE-inhibitory activity, as they can be hydrolysed by ACE. The dipeptide residues of three peptides, Hip-His-Leu, Hip-Phe-Arg, and Hip-Ala-Pro, at C-terminal are the same as those of ANG I, BK, and BPP_{5a}(EKWAP), respectively (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Studies on these peptides showed that ACE has no affinity to inhibitors with dicarboxylic amino acids at C-terminal, which indicates that ACE is highly specific with regard to the terminal dipeptide residues of peptide substrates or competitive inhibitors, and peptides with a Pro residue at the penultimate position may bind to ACE more weakly than those with Pro at the ultimate position.

The stereochemical conformation of amino acids may also affect the ACE-inhibitory activity of peptides. Previous studies have suggested that ACE has a higher affinity to the L-configuration amino acid at position three from the C-terminal (Hernández-Ledesma et al., 2011). The transformation of proline from *trans* to *cis* at the C-terminal position led to significant changes in peptide structure and its interaction with ACE, therefore decreased inhibitory activity (Gómez-Ruiz, Recio, & Belloque, 2004). Two peptides derived from casein, VPP and IPP showed excellent ACE-inhibitory activity both *in vitro* and *in vivo* (Table 1). These two peptides share a similar structure with *cis* configuration at both the first aliphatic and the second Pro amino acid residues, which suggests that the *cis*

configuration of the first peptide bond is a more effective geometric form for stronger ACE inhibition (Siltari, Viitanen, Kukkurainen, Vapaatalo, & Valjakka, 2014). Small peptides cannot form secondary and tertiary structures but many of them fold to different geometric forms under appropriate conditions. It is generally assumed that the *cis/trans* configurations are the main geometric forms at the active site, and one configuration may have higher affinity than the other.

However, there are some special conditions that do not conform to the previous descriptions. Although previous finding stated that Pro residue at one or more position in the C-terminal position would promote the ACE-inhibitory activities of peptides (Cheung et al., 1980; Ibrahim, Ahmed, & Miyata, 2017), Mirzaei et al. (2019) showed that the replacement of Pro in the sequence of YGKPVAVPAR with His residue leads to a 55.63% increase in antihypertensive effect. This could be due to the Zn-chelating activity of the introduced imidazole ring. Moreover, Liu et al. (2018) suggested that a peptide such as YLVR has stronger inhibition activities due to its stronger covalent cation- π interaction with ACE in comparison to similar peptides. To sum up, the hydrophilicity / hydrophobicity and stereochemical conformations of amino acids affect the activity of ACE-inhibitory peptides.

5.2 Structural/conformational properties

In addition to the primary structure of peptides, some secondary structure and spatial conformation could also influence the inhibitory activity of peptides. For smaller peptides (between 2 and 8 amino acids), the primary structure - amino acid sequence is the main factor determining its ACE-inhibitory activity, while the secondary structure could form and influence the activity for larger peptides with more than 9-10 amino acids. The secondary structure of longer peptides consists of α -helix and β -sheets, as well as β -turn and random coil structures, formed by the hydrogen bonds between different amino acids. The secondary structure can be influenced by the primary structure, which further affects the binding to ACE and their ACE-inhibitory ability. For instance, the secondary structure of casein-derived peptide, NMAINPSKENLCSTFCK, contains a random coil, α -helix and a β -sheet conformations, without any β -turns (Tu et al., 2018). The same study suggested that the β -sheets account for $32\% \pm 0.96\%$ of the secondary structure and could contribute to the ACE-inhibitory activities. On the other hand, Yu, Zhao, et al. (2011) and Cian, Vioque, and Drago (2015) reported that an α -helix played a vital role for the ACE-inhibitory activities of peptide QIGLF and gluten hydrolysate extracts. However, the detailed mechanisms on how the secondary structure of peptides influence ACE-inhibitory activity require further investigation.

Some peptides may have spatial interactions and together form a tertiary-like complex with synergistic effect on ACE inhibition. For example, simultaneous presence of both SR and GNGSGYV allows more space of the ACE active site to be occupied and therefore higher antihypertensive activities, and both of these peptides are degraded fragments of GNGSGYVSR (Wu et al., 2019). The SR dipeptide could attack the catalytic Zn of ACE and form a coordinate bond, followed by GNGSGYV binding with the arginine of SR and other residues on ACE active site, blocking other substrates from entering the active pocket. Future research may further investigate into this interesting area and explore more combination of peptides in synergistic ACE-inhibitory activities.

5.3 Molecular simulations to uncover structure-activity relationships

The tertiary structure of proteins is formed on top of the secondary structures through the interactions of the side chain groups of α -helix, β -sheet, β -turn, random coil by hydrogen bond, Van der Waals' force, hydrophobic interaction, and electrostatic interaction. ACE is a protein with tertiary structure, while the peptide inhibitors may have the secondary structure or only primary structure. How ACE and these inhibitor's form steric-structural complexes need to be considered for each peptide interaction as the conformation is unique. There are two methods to predict the mechanism of ACE-inhibition, firstly by classical exploration of the Michaelis–Menten equation, and secondly by *in silico* molecular simulation (Abdelhedi & Nasri, 2019). Here, we focus on molecular simulations to analyse the mechanism of ACE-inhibitory peptide against their primary and secondary structure, to include molecular docking (Fu et al., 2017; Jiang, Wang, Che, & Tian, 2014; Liu et al., 2018; Yu et al., 2018), molecular dynamics simulation (Anekthanakul, 2019; Fang et al., 2019; Jiang et al., 2019; Qi, Zhang, Huang, & Wu, 2017) and quantitative structure-activities relationship (QSAR) (Foltz, Van Buren, Klaffke, & Duchateau, 2009; Gu & Wu, 2013; Majumder & Wu, 2010).

Molecular docking has been used to predict the bonding and interaction between peptide fragment and ACE with steric structures. The molecular interaction mechanism of four dairy derived peptides (IPA, FP, GKP (Pan, Guo, Zhao, & Cao, 2011), and LL (Pan, Cao, Guo, & Zhao, 2012)) was investigated by flexible molecule docking, and observed hydrogen bonds, hydrophobic, hydrophilic, electrostatic and Zn^{2+} interactions between ACE and these peptides. Six hydrogen bonds were found between YSK (IC_{50} 76 μM) and ACE at residues Glu162, Gln281, Asp415, Tyr520 and Lys511 (Wang et al., 2017). Molecular docking further suggested the formation of strong hydrogen bonds between ASL peptide with the S1 (Ala354) and S2 (Gln281 and His353) pockets of ACE (Q. Wu et al., 2015). Fu et al. (2019) investigated two ACE-inhibitory peptides, ANSEVAQWR (IC_{50} 89.58 μM) and EALVSQLTR (IC_{50} 91.48 μM) derived from *T. lepturus* myosin using molecular docking and indicated that the

stereochemical structure of ANSEVAQWR form 11 hydrogen bonds with ACE, while EALVSQLTR only forms 9 hydrogen bonds. This suggested that higher number of hydrogen bonds with ACE could be related to greater inhibition ability of the peptide. Further, EALVSQLTR formed a direct coordinate bond with the Zn^{2+} of ACE, while ANSEVAQWR did not, suggesting weak correlation between ACE inhibition and peptide linkage with Zn^{2+} . In the case of EPNGLLLPQY (IC_{50} 233.178 μM), an ACE-inhibitory peptide from walnut, molecular docking showed that its ACE inhibition was not only due to the hydrophobic interaction but also hydrogen bonds and electrostatic interaction (Wang et al., 2018).

Molecular dynamics simulation investigates the motion of molecular systems and calculate thermodynamic quantities and other macroscopic properties of the system to understand the overall stability of inhibitor-bound ACE mainly using three parameters, solvent accessible surface area, root mean square deviations and radius of gyration. Molecular dynamics simulations and docking study suggested that the binding mechanism between peptide GEF and ACE was due to hydrogen bonds, electrostatic and van der Waal interactions, and the strength of electrostatic interactions with GEF peptide was larger with the N-domains of ACE than the C-domains (Qi, Zhang, Liu, Zheng, & Wu, 2018). The same study further indicated that GEF peptide formed higher numbers of hydrogen bonds with the N-domain (6) compared to the C-domain (3). Additionally, the bonding mechanism at the C-domain and N-domain was slightly different, where GEF peptide interacts with the Lys475 residue of the C-domain and form chelation with the zinc ion, while this peptide only interact with the Lys489 residue at the N-domain (Qi et al., 2018).

A quantitative structure activity relationship (QSAR) model is used to evaluate the relationship between chemical structures and biological activity of chemicals based on existing data and to predict the activities of new chemicals (Fahimeh, Mehridehnavi, Pérez-Garrido, & Pérez-Sánchez, 2018) and can prioritise lead optimization and virtual screening (Roy, Kar, & Das, 2015). When the chemical property is modeled against the structure, the model is called quantitative structure property relationship (QSPR) model. For the modelling work of QSAR/QSPR, the datasets need to be firstly selected for the structural/empirical descriptors, followed by variable selection, model construction and validation process (Yousefinejad & Hemmateenejad, 2015). For instance, QSAR modelling was able to accurately predict three potent peptides as the hydrolysis products of ovotransferrin treated with thermolysin-pepsin *in silico* (Majumder & Wu, 2010). Additionally, QSAR modelling could be utilised in conjunction with analytical chemistry techniques, such as LC-MS/MS, to identify novel tripeptides (Gu & Wu, 2013). Further, machine learning technique, such as artificial

neural network (ANN) approach could be used to construct QSAR model based on the structural and activity data of existing peptides, including ACE-inhibitory activity, 3D-shape, size, amino acids structure, and electrical parameters and this model was successfully applied in the isolation of peptides from defatted wheat germ protein (He et al., 2012). However, the application domain of the QSAR model is highly dependent on the characteristics of the training dataset. For peptides with structures that deviate too far from the application domain, the prediction performance of QSAR model is poor (Tong et al., 2004). Therefore, QSAR models may not be useful to find new structural molecules, but only to guide researchers to optimise existing inhibitory peptides and to find similar peptide on the basis of one or more known skeletons.

Molecular simulation is increasingly accepted and used by researchers, especially for drug development, but has not been widely applied in ACE resistant peptides research. With the development of molecular simulation technique, artificial intelligence and big data accumulated from previous studies, researchers could optimise simulation models to better predict ACE-inhibitory activity of new or untested peptides, or to design new peptides (Ren, Wang, Chen, & Cao, 2014). This is undoubtedly a promising area of future research.

6. Digestion, Transportation and Absorption of ACE-inhibitory Peptides

The ACE-inhibitory ability of peptides *in vitro* do not guarantee their antihypertensive effects *in vivo*, since there are several physical and biological barriers that need to be overcome before they can reach blood circulation system (Wang, Xie, & Li, 2019). The antihypertension effect of ACE-inhibitory peptides *in vivo* can be induced only if these peptides are absorbed into blood circulation in an active form with sufficient quantity and can reach their target organs and tissues. As a result, the antihypertensive effect of peptides depend on their resistance to gastrointestinal digestion and their permeability into blood circulatory system in the active form (Sangsawad, Choowongkamon, et al., 2018). The two major barriers for intact absorption of peptides include the hydrolysis by pepsin and pancreatic enzymes and the physical permeability barrier of the intestinal epithelium, where brush border peptidases are also present (Vermeirssen et al., 2004). Depending on the primary and/or secondary structure of the peptide, some ACE-inhibitory peptides may enter the blood circulation in intact forms or degraded forms after hydrolysis by gastrointestinal protease and/or epithelium membrane peptidase to exert antihypertensive effects.

6.1 Gastrointestinal digestion

The digestive enzymes in the gastrointestinal tract may act upon the ACE-inhibitory peptides, and the resistance of a peptide to digestive enzymes depends on whether the amino acid sequence has

cleavage sites for digestive enzymes and whether these cleavage sites are exposed (Martinez-Maqueda, Miralles, Recio, & Hernandez-Ledesma, 2012). For this purpose, *in vitro* digestion systems can be used to study the resistance of ACE-inhibitory peptides to gastrointestinal degradation. Enzymes, including pepsin, trypsin, pancreatic protease, elastase, α -chymotrypsin, and carboxypeptidase A and B are commonly used to mimic the human digestion process (Vermeirssen et al., 2004).

For some peptides, their ACE-inhibitory activity was decreased or even completely lost after hydrolysis by digestive enzymes. For instance, two peptides derived from ovalbumin, YAEERYPIL and RADHPFL, were susceptible to proteolytic degradation, and their ACE-inhibitory activity *in vitro* dropped nearly 100-fold after digestion with pepsin and pancreatic extract (IC_{50} of YAEERYPIL increased from 5.4 ± 0.8 to $446 \pm 1.8 \mu\text{g/mL}$, IC_{50} of RADHPFL increased from 5.3 ± 1.02 to $521 \pm 31.4 \mu\text{g/mL}$) (Miguel et al., 2006). In another study, the ACE-inhibitory activities of two peptides from Manchego cheese, VRYL and KKYNVPQL, significantly decreased after simulated digestion (IC_{50} of VRYL increased from 0.009 to 0.03 mg/mL, and IC_{50} of KKYNVPQL increased from 0.07 to >0.8 mg/mL) (Gómez-Ruiz, Ramos, & Recio, 2004). This was due to the changes in peptides structure, where VRYL was hydrolysed into Y, L, VR, VRY, YL, and VRYL, and KKYNVPQL was hydrolysed into Y, L, KK, KKY, KKYNVPQ, KKYNVP. Chen and Li (2012) demonstrated peptides tended to be hydrolysed by intestinal digestion instead of inactivation in the stomach, which was further supported by Ding, Wang, Yu, Zhang, and Liu (2016) and who stated that pancreatin was responsible for the degradation of peptides in the intestine. For instance, Gu and Wu (2013) suggested that the IC_{50} of soybean protein hydrolysate digested by thermolysin, pepsin and trypsin ($IC_{50}=115.6 \mu\text{M}$) was significantly higher compared to that of thermolysin ($IC_{50} 51.8 \mu\text{M}$) and combined pepsin and thermolysin ($IC_{50} 53.6\mu\text{M}$), which implies further hydrolysis by trypsin occurred in small intestine.

On the other hand, gastrointestinal digestion may lead to increased ACE inhibition activities of some peptides. For instance, the peptide TQPKTNAIPY from Manchego cheese could be digested into Y, PY, TQPK, TNAIP, TQPKTNAIPY, AIPY and TNAIPY fragments, and exhibited 6 fold higher ACE-inhibitory activity (Gómez-Ruiz, Ramos, et al., 2004). Gastrointestinal digestion of several lentil-derived oligopeptides results in enhanced ACE-inhibitory activity due to the release of smaller peptide fragments. For instance, IC_{50} of DLAIPVNRPGQLQSF decreased from more than 500 to $11.4 \pm 0.78 \mu\text{M}$ (García-Mora et al., 2017).

The stability of peptides in gastrointestinal digestion depends on the length and molecular size of the peptides and their structural characteristics. Previous research has indicated that peptides with molecular weight above 3 kDa are more likely hydrolysed by the gastrointestinal enzymes compared with those below 3 kDa (Chen & Li, 2012). Mirzapour et al. (2017) further suggested that small peptides with 2-6 amino acid are less susceptible to the hydrolysis of digestive enzymes, likely due to less numbers of enzyme susceptible peptide bonds and lower structural flexibility. Structural factors, such as the hydrophobicity, acid-base properties, and C- and N-terminal amino acid composition all contribute to the digestive stability of peptides (Wang et al., 2019). Researches suggest that peptides with high content of Pro residues are more resistant to hydrolysis by digestive enzymes and proline-specific peptidases (Fitzgerald & Meisel, 2000; Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernandez-Escalante, 2011). Picariello et al. (2010) found that peptides containing neutral and basic amino acids, such as Arg, His and Lys were more easily degraded by gastrointestinal enzyme compared with peptide containing acidic amino acids (Agudelo, Gauthier, Pouliot, Marin, & Savoie, 2004; Ao & Li, 2013). Hydrophobicity is another factor influencing the susceptibility to enzymatic digestions, where larger number of hydrophobic amino acids, such as Val and Leu, lead to the lower stability. The tripeptide IPY identified in Manchego cheese exhibited a lower IC_{50} ($204.9 \mu M$) likely due to the presence of hydrophobic Tyr at the C-terminal (Gómez-Ruiz, Ramos, et al., 2004; Miguel et al., 2006). Wang, Yadav, Smart, Tajiri, and Basit (2015) evaluated the intestinal stability of 17 different peptides during digestion and suggested that the cyclization contributed by disulfide bond linkage would potentially protect susceptible peptide bonds from enzymatic cleavage and the effect of pH was negligible compared to the effect of enzyme in terms of peptide degradation in gastric condition.

6.2 Intestinal absorption

ACE-inhibitory peptide absorption has been investigated by many studies both *in vivo* and *in vitro*. Spontaneously hypertensive rats (SHRs) are the most commonly used animal model for *in vivo* studies, while Caco-2 cells derived from a human intestinal adenocarcinoma are used for *in vitro* studies. Caco-2 cells can be used to study peptides resistance to hydrolysis by brush border peptidases on intestinal cell membrane (Guo et al., 2018; Lacroix, Chen, Kitts, & Li-Chan, 2017). Three mechanisms for the transportation of ACE-inhibitory peptides through small intestinal epithelial cells have been investigated, including carrier mediated (Pep T1-mediated) transport, paracellular transport and transcytosis (Figure 1) (Vermeirssen et al., 2004).

6.2.1 PepT1-mediated transport

Di- and tri-peptides can be actively transported intact across the brush border membrane of the epithelial cell by a peptide transporter named PepT1, which is a transporter with a transmembrane electrochemical protein gradient (Hillgren et al., 2013). PepT1 locates on the apical membrane of enterocytes and is responsible for the transportation of small peptides less than 500 Da. PepT1 also helps the exit of small peptides from the enterocytes into the surrounding bloodstream (Segura-Campos et al., 2011). Studies have reported that 400 di-peptides and 8000 tri-peptides can be recognised and transported by the PepT1, without selecting for specific amino acid sequence (Wang et al., 2019). For example, both IW and IWH peptides hydrolysed from spent hen myofibrils could be transported via PepT1 (Fan, Xu, Hong, & Wu, 2018). Wang and Li (2017) examined the transportation of peptides YPY, IV and AP mediated by the PepT1 transporter and suggested that the brush border peptidases were the main enzyme leading to the degradation of peptides for PepT1 transportation. The affinity between PepT1 transporters and peptides is highly relevant to the peptide structural characteristics, including the charge, C- and N-terminal amino acid composition and side chain (Wang et al., 2019). The affinity between the PepT1 transporter and neutral peptides is usually stronger, followed by positive peptides and negative peptides (Guettou et al., 2014; Moore et al., 2000). The hydrophobicity of amino acids at the C- and N-terminals has a strong effect on the binding of peptides to PepT1. For instance, Nielsen et al. (2001) reported peptides with hydrophobic side chain could firmly bind to PepT1. Hydrophobic amino acid residues such as Ile165, Trp306, Trp440, and Phe441 in PepT1 binding site are likely to associate with the side-chains of dipeptide and tripeptide with stronger hydrophobicity than the backbone. The phenyl side chains at C-terminal can form hydrophobic interactions with hydrophobic amino acid residues of PepT1 and result in a hydrophobic pocket within the binding site (Newstead, 2015).

6.2.2 Paracellular transport

The paracellular transport is a passive and energy-independent method to absorb water soluble peptides and involves transporting peptides by water-filled channels between enterocytes. The hydrophilicity of the peptides is a key factor for paracellular transport (Salamat & Johnston, 2005; Wang et al., 2019). Proteins in the tight junction of paracellular transport pathway consist polar amino acids, which leads to the tight junction being negatively charged. However, impact of charge on molecule transportation is inconclusive. Additionally, other factors, such as the overall dimensions of peptides, calcium chelating agents, surfactants, bile salts, phosphate esters, and some cationic polymers can also affect peptide permeability (Fernández-Musoles et al., 2013; Salamat & Johnston, 2005). The paracellular route is the main pathway for the transportation of peptides with

500-1600 Da molecular weight (Wang & Li, 2017). For instance, Fernández-Musoles et al. (2013) suggested that paracellular transport was the main route for peptides RWQ and WQ to cross the cell layer. Peptide VPP derived from cheese (Basirico et al., 2015; Satake et al., 2002), HLPLP derived from casein (Quirósa, Dávalos, Lasunción, Ramos, & Recio, 2008), QIGLF (Ding et al., 2014), RVPSL (Ding, Wang, Zhang, & Liu, 2015) and TNGIIR (Ding et al., 2016) derived from egg whites could all be transported intact across the Caco-2 monolayer by paracellular transport. Additionally, Ding et al. (2016) suggested that the paracellular pathway may avoid the degradation of the TNGIIR by intracellular enzyme, even with the action of brush border membrane peptidase.

6.2.3 Transcytosis

Transcytosis is an energy-dependent transportation pathway and includes three steps: endocytic uptake, intracellular transport through transcytosis vesicle and basolateral secretion (Regazzo et al., 2010). Transcytosis is the preferred absorption route for lipophilic peptides, where peptides can be transported to the basolateral side in endosomes intact. The interactions between cell membrane lipid bilayer and lipophilic peptides are vital for the transcytosis process. Hence the affinity between peptides and phospholipid-rich cell membrane could increase with peptide hydrophobicity (Wang et al., 2019). For instance, bradykinin (RPPGFSPFR) and RLSFNP have been proved transported mainly via the transcytosis pathway (Vij, Reddi, Kapila, & Kapila, 2016; Zhang et al., 2019).

The transport route for different peptides depends on several factors, such as hydrophobicity, molecular size and charge (Vermeirssen et al., 2004; Wang & Li, 2018). The quantity of transportation through each route depend on the sequences of peptides and may vary greatly for each other, and usually below 10 ppm within the body (Wang et al., 2019). In general, transcytosis and PepT1 together are responsible for transporting hydrophobic peptides and positively charged peptides, while paracellular and PepT1 together are responsible for transporting hydrophilic peptides and negatively charged peptides (Wang & Li, 2018). During transport, the small peptides are usually absorbed faster than free amino acids and their transport is more favourable (Vermeirssen et al., 2004). The transport rate of the peptides are decided by the susceptibility to the brush border peptidase, which could further hydrolyse peptides into amino acids, di- and tripeptides (Vermeirssen et al., 2004). For children with incompletely developed intestinal tract barriers, peptides are more readily absorbed compared to adults (Segura-Campos et al., 2011).

6.2.4 Influences of other food substances on peptide transportation

The absorption of peptides by intestinal epithelium cells could be influenced by other food components and some chemicals may alter the transepithelial electrical resistance in cell culture

model (Vermeirssen et al., 2004). Active transportation of sugars with amino acids and peptides could create an increase of intracellular osmotic pressure, which could lead to a solvent drag through the membrane (Pappenheimer & Volpp, 1992). Importantly, the permeability of some peptides, such as IPP, LKP, could be significantly increased in the presence of medium chain fatty acids (MCFA) permeation enhancers, which suggests the potential to make oral intake forms of these peptides in combination with these compounds (Gleeson et al., 2015). The paracellular transport of nutrients after meal could induce the tight junction to open through osmotic forces, and lead to higher amount of peptides transported *in vivo* than *in vitro* (Salamat & Johnston, 2005). Food matrices have protective effects on ACE-inhibitory peptides during digestion and could delay the absorption of peptides. This not only allows peptides to exhibit antihypertensive effects for a longer period of time, but could also increase the total amount of peptides being absorbed (Grootaert et al., 2017). Have, Vac der Pijl, Kies, and Deutz (2015) investigated the bioavailability of milk-derived peptide, IPP, LPP and VPP in the presence of a macronutrient matrix (casein-hydrolysate, carbohydrate, protein, fat and fibres) in a pig study, and reported that the systemic availability of all three peptide was improved due to the prolonged absorption. Some ACE-inhibitory peptides may pass small intestine and enter the colon, where they can be metabolised by microbiota (Vermeirssen et al., 2004). Further researches are required to investigate the influences of different food matrix on the bioaccessibility of different peptides to identify the most suitable combination for efficient delivery of peptides from food to blood circulation.

6.3 Stability and transport of ACE-inhibitory peptides

The small intestine is the main organ responsible for peptide absorption, where the surviving or newly formed peptides from digestion would need to be transported into the blood stream. However, before they can be absorbed by intestinal epithelium, various proteases and brush border membrane peptidases might further hydrolyse the peptides into smaller fragments and amino acids (Samaranayaka et al., 2010; Sangsawad, Choowongkamon, et al., 2018; Wang et al., 2019) or further hydrolysed by the cytosol peptidases. The bioavailability and degradation of peptides are influenced by the brush border and cytosol peptidases present in the transport route, which is highly relevant to peptide characteristics, such as molecular weight, charge, hydrogen bonding potential and hydrophobicity (Wang & Li, 2017). Some peptides may be more resistant to the brush border membrane peptidases, and Regazzo et al. (2010) reported a 17 amino acid residue peptide with only 10% being hydrolysed likely due to its proline-rich sequence. On the other hand, some peptides are more susceptible to hydrolysis by brush border membrane peptidase, especially those containing a

N-terminal L residue (Fernández-Musoles et al., 2013). Sangsawad, Choowongkamon, et al. (2018) reported that peptide KPLL could be hydrolysed by the brush border membrane enzymes to produce smaller peptides, and both KPLL and its fragments could be transported across cell monolayer. In another study, all five peptides (LPYPY, IPIQY, LKPTPEGDL, IPI and WR) derived from milk protein can be hydrolysed by the peptidases on Caco-2 cell surface with 8-30% of the peptides hydrolysed after 2 hours (Lacroix et al., 2017). Guo et al. (2018) reported that RLSFNP was partially degraded to FNP, SFNP and RLSE in the process of transmembrane transport using the Caco-2 cells monolayer as model, and all these peptides exhibited ACE-inhibitory activity. While the breakdown of the peptides by intestinal membrane peptidase could potentially generate some smaller, potent and more easily absorbed molecules, most studies have shown that the degraded products may have lower ACE-inhibitory activities compared to the original peptide (Wang et al., 2019). For medical therapy, antihypertensive peptides with high resistance to digestive proteinases and membrane peptidase and high permeability through the intestinal epithelium are preferred, which would have relatively more consistent ACE-inhibitory activity *in vitro* and antihypertensive activity *in vivo* (Bhat, Kumar, & Bhat, 2017).

The Caco-2 cell has a large number of membrane peptidases, including endopeptidases, enteropeptidases, aminopeptidase A, aminopeptidase N/P/W and dipeptidyl peptidase IV, and it is generally considered as a good *in vitro* model to study peptides absorption (Lacroix et al., 2017). However, it remains difficult to establish a direct relationship between ACE-inhibitory activity *in vitro* and antihypertensive effect *in vivo*, as the Caco-2 cell monolayer is tighter than the normal mammalian intestinal tissue, and the activities of brush border membrane enzymes on Caco-2 cell are generally lower than *in vivo* condition. Peptides actually transported in the mammalian intestinal tissue *in vivo* are expected to be higher than that transported *in vitro* using the Caco-2 cell monolayer (Vermeirssen et al., 2004).

7 Other possible mechanisms of antihypertension using food proteins

Some of the antihypertensive peptides derived from food sources display higher *in vivo* antihypertensive ability than *in vitro* ACE-inhibitory activity due to other mechanisms exerted by these peptides in blood pressure regulation. These peptides reduce blood pressure by inhibiting the production of angiotensin II and promoting the degradation of bradykinin, and their vasorelaxing activity is mediated by nitric oxide (NO). For instance, Yamaguchi, Kawaguchi, and Yamamoto (2009) reported that 5-days repeated administration of IPP and VPP may influence the expression of

endothelial nitric oxide synthase gene and the connexin 40 gene in the abdominal aorta of rats and further affect blood pressure regulation.

Some peptides may exhibit ACE-inhibitory activity and antioxidant activity at the same time. For instance, egg white derived antihypertensive peptide IRW could also inhibit oxidative stress in endothelial cells, and show oxygen radical-scavenging effect (Huang, Chakrabarti, et al., 2010; Huang, Majumder, & Wu, 2010). Similarly, VLPVPQK showed both antioxidative (trolox equivalent antioxidant capacity 5.71 ± 0.59 mmol/mg) (Shanmugam, Kapila, Sonfack, & Kapila, 2015) and ACE-inhibitory activities (IC_{50} 15 μ M) (Vij et al., 2016), which allows to protect rat osteoblastic cells from hydrogen peroxide-induced dysfunction and cellular oxidative damage (Mada, Reddi, Kumar, Kapila, & Kapila, 2017). Because oxidative stress and associated oxidative damage are important factors in the development of cardiovascular disease, peptides with antioxidant property may exhibit a stronger antihypertension effect. YPI, the end products of YAEERYPIL from gastrointestinal hydrolysis, could significantly decrease blood pressure of SHR for more than 30 mmHg in comparison to its *in vitro* ACE-inhibitory activity (IC_{50} value > 2.339 mM) (Miguel et al., 2006; Miguel et al., 2008). It has been suggested that the radical scavenging activity of YPI could be responsible for extra hypotensive effects. The antihypertension mechanism of YPI need to be further confirmed as the YP derived from YPI also has strong antihypertension effect. Nevertheless, it seems a promising area to further investigate the interactions and synergetic effect between ACE-inhibitory peptides and antioxidants in blood pressure regulation.

In addition to isolated peptides, some protein hydrolysates also showed both antihypertensive and antioxidant effects. For instance, pepsin-pancreatin-hydrolyzed pigeon pea protein (PPHPp) exhibited ACE inhibition activity (61.82%) and ability to scavenge DPPH $\cdot$ (40%), while pancreatin-hydrolyzed pigeon pea protein showed renin inhibition activity (14.28%) (Olagunju, Omoba, Enujiugha, Alashi, & Aluko, 2018). The same study also demonstrated that PPHPp had strong *in vivo* antihypertensive effect with the SBP of SHR decreased for 26.12 mmHg at 2 hours after oral administration. Additionally, the hydrolysates of prolamin fraction from barley also exhibited antioxidant activity (77.7%) and ACE inhibitory activity (61.3%). Furthermore, the small peptide fraction (<1 kDa) of wonderful cola protein pancreatin hydrolysate were reported to have both *in vitro* ACE inhibition (80%) and *in vivo* antihypertensive effects (SBP, -33 mmHg; DBP, -30 mmHg) 6 h after oral administration to SHR (Ijarotimi et al., 2018). The same peptide fraction also had 2,2-diphenyl 1-1-picrylhydrazyl (DPPH) radical scavenging capacity (55–67%) and metal chelation ability

(83–93%). Another example would be the alcalase hydrolysate of *Actinopyga lecanora*, which is a kind of sea cucumber exhibited both ACE inhibitory activity (69.8%) and antioxidative activity measured by DPPH radical scavenging capacity (56.00%) (Ghanbari et al., 2015).

Importantly, some peptides exert antihypertensive effect by inhibiting renin. For instance, an octapeptide (HPFHLLVY, H.77 for short) was identified as a powerful *in vitro* inhibitor of canine renin (IC_{50} 24 nM) and exhibited an IC_{50} of 1 mM against human renin and 0.6 mM against rat renin (Szelke et al., 1982). The peptide IRLIIVLMPILMA from the macroalgae had an IC_{50} value of 3.344 mM for renin (Fitzgerald et al., 2012), while flaxseed protein hydrolysate showed *in vitro* inhibitive activities of ACE (IC_{50} 0.0275–0.151 mg/ml) and renin (IC_{50} 1.22–2.81 mg/ml) (Udenigwe, Lin, Hou, & Aluko, 2009). However, it was not clear which peptides in these protein hydrolysates exhibited both antioxidant activity and ACE or renin inhibitory activity, and therefore need to be further studied. Three peptides (LY, TF and RALP) purified from rapeseed protein have different abilities in inhibiting ACE and renin (R. He et al., 2013). Among the three peptides, LY was found to have the highest ACE inhibition ability (LY IC_{50} 0.11 mM, TF IC_{50} 0.81 mM, and RALP IC_{50} 0.65 mM), while RALP was most potent ($p < 0.05$) against renin activity (RALP IC_{50} 0.97 mM, LY IC_{50} 1.87 mM and TF IC_{50} 3.1 mM). The same study also found that LY (-26 mmHg) was the most effective antihypertensive peptide compared to RALP (-16 mmHg) and TF (-12 mmHg) when they were orally administrated (30 mg/kg BW) to SHRs.

The rest peptides obtain their antihypertensive ability through other mechanisms. The vasorelaxing and hypotensive activities of MRW (rubimetide, derived from spinach rubisco) were mediated by the FPR2 (formyl peptide receptor-2) and prostaglandin D₂ (PGD₂), but not by NO (Zhao, Usui, Ohinata, & Yoshikawa, 2008). The same study suggested that although MRW had ACE inhibition ability, its hypotensive activity was mainly caused by vasorelaxation rather than ACE-inhibition. This was the reason why MRW showed antihypertensive effects in old SHRs where ordinary food-derived ACE-inhibitory peptides are often ineffective. The vasorelaxing effect of the peptide, named ovokinin (FRADHPFL, derived from ovalbumin) was mediated by bradykinin B₁ receptor and the release of prostaglandin I₂ as relaxing factors (Fujita, Usui, Kurahashi, & Yoshikawa, 1995). Another peptide RIY (IC_{50} 28 μ M) derived from rapeseed showed antihypertensive effect even in old rats, and its maximal hypotensive effect was -11.3 ± 1.8 mmHg 4 hours after oral administration (Marczak et al., 2003). RIY exhibited the vasorelaxation effect via prostaglandin I₂ and cholecystokinin(CCK) release (Yamada, Ohinata, Lipkowski, & Yoshikawa, 2011).

8. Commercialisation of ACE inhibition peptides based functional food

8.1 ACE-inhibitory peptides containing products

For general consumers, the ACE-inhibitory peptides can be obtained from two ways. The first way is to consume specific types of food that naturally contain some peptides or their precursors. For example, some fermented foods, such as cheese, yogurt and soybean pasta contain ACE-inhibitory peptides as some of their proteins can be hydrolysed to release peptides by microbial enzymes during fermentation process as discussed in Section 4.1. Alternatively, some ACE-inhibitory peptides can be produced within human body from the proteins of daily diet by gastrointestinal digestive enzymes as mentioned earlier (Section 4.1, Section 6.1). A good example would be the Tuna frame protein hydrolysate containing a 21 amino acids peptide GDLGKTTTVSNWSPPKYKDTP (IC_{50} 11.28 μ M), which can be hydrolysed into smaller active peptides to decrease systolic blood pressure (Lee et al., 2010). Nevertheless, the total amount of active peptides obtained from food either in active or precursor forms can be limited and their hypotensive effect can hardly be quantified, mainly due to the uncertainty of peptide concentrations and irregular intake frequency (Girgih et al., 2014; Xue et al., 2018).

Despite of this, commercialisation effort has been made to produce ACE-inhibitory peptides enriched functional food following this philosophy. Fermentation of milk or soybean has been optimised to enhance the release of ACE-inhibitory peptides and therefore to produce fermented products as a type of functional food with antihypertensive function (Chen, Ma, Qi, Cao, & Tan, 2019) and few undesirable side effects (Flambard & Johansen, 2007). However, this concept has not officially been accepted by some regulatory bodies, as for instance, European Food Safety Authority (EFSA) suggested insufficient evidence for nutritional claims at the current stage. Nevertheless, commercial antihypertensive fermented milk products are available in the market, such as the Japanese brand Ameal S and Calpis (Calpis Co., Tokyo, Japan) that contain IPP and VPP (Beltrán-Barrientos, Hernández-Mendoza, Torres-Llanez, González-Córdova, & Vallejo-Córdova, 2016). Furthermore, some lactic acid bacteria able to produce ACE inhibitory peptides have been patented. For instance, *Lb. helveticus* Cardi-04 (Chr. Hansen, Hørsholm, Denmark) has been patented to prepare antihypertensive peptides and functional foods (WO 2003/082019 A3 and WO 2004/015125 A1). Additionally, a US patent includes utilisation of *Lc. lactis* (NRRL-B50571) for the production of fermented milk with antihypertensive peptides (US 8,865,155)(Beltran-Barrientos et al., 2016). Instead of increasing the concentration of ACE-inhibitory peptides in the food directly, another

possible commercialisation approach is to enrich the concentration of protein hydrolysate containing ACE-inhibitory peptides sequences, which will allow the later break down in human body display its hypotensive function. However, up to date, there is no commercial product produced using this approach. Future research efforts are required to further explore the impacts of fermentation process and the involvement of specific microbes and other potential processing methods on the production of ACE-inhibitory peptides/precursors in the food product, which can provide more scientific evidence for the production of antihypertensive functional foods and legislation in this area.

The alternative way for consumer to obtain ACE-inhibitory peptides is to consume food products fortified with high concentrations of peptides. High-purity ACE-inhibitory peptides have been successfully produced by the factory and added into food and beverages in the form of liquid or as a functional additive. These foods could also be considered as a type of functional food to regulate blood pressure. Lactotripeptides (AmealPeptide®, Asahi Calpis Wellness Co., Ltd., Japan) is certified as a “food for specified health use” (FOSHU) in Japan and has been granted GRAS (Generally Recognized as Safe) approval in the US. At present, tablets and dairy beverage made from lactotripeptides are commercially available in Japan and other countries. A lactotripeptide (LTP)-enriched yogurt beverage was prepared from the AmealPeptide powder (contains IPP, LPP and VPP peptides) to assess the bioavailability of these peptides when consumed in beverage (Foltz et al., 2007). This study confirmed that the plasma concentrations of IPP and LPP in human subjects increased significantly from picomolar concentrations to nanomolar concentrations after intaking 250 mL of yogurt beverage containing 57 mg of LTP (20.4 mg IPP, 20.0 mg VPP, and 16.5 mg LPP once a day. The same study also found that consuming LTP-enriched yogurt with a meal at the same dosage could further increase plasma concentration of IPP, which was likely due to the increase in the plasma elimination half-life. An obvious limitation with using high purity peptide to fortify food product is the high cost of preparing high purity peptides due to the complexity of the process and the inevitable loss of peptides in the purification process.

8.2 Challenges for the application of ACE-inhibitory peptides in food industry

To widely commercialise peptide-based function food mentioned above, it is necessary to investigate the influences of food processing on peptides’ ACE-inhibitory activity. Food processing condition such as heating, pH, pressure, and other components in food ingredients may affect the stability and activity of ACE-inhibitory peptides (Anantharaman & Finot, 1993; López-Fandiño, Otte, & van Camp, 2006). Commonly used food processing techniques, such as heat sterilisation, drying,

baking and ultrahigh pressure treatment may alter the structure of peptides, and therefore could change their ACE-inhibitory activity. For example, Hu et al. (2016) compared the effect of different drying methods including spray drying, fluid bed drying and vacuum freeze drying on the ACE-inhibitory activity of peptides derived from bovine casein hydrolysate. This study reported that the product obtained by vacuum freeze drying had the highest anti-hypertension activity *in vivo*, followed by the product obtained by fluid bed drying, while spray dried product had the maximum loss of anti-hypertensive activity. pH changes may cause the change of some specific amino acid, which in turn could affect the functional properties of peptides. For example, acidic treatments could destroy glutamine and asparagine, whereas alkaline treatments could alter cystine, serine and threonine and produce lysinoalanine and D-amino acids (Anantharaman & Finot, 1993). Other food components, such as divalent metals, can chelate peptides due to peptides' metal-binding properties. Chelation leads to the low solubility of the chelate and the decrease of the absorption rate of the peptide, which in turn leads to the decrease of the antihypertensive activity of the peptide (Wu et al., 2017). Similarly, polyphenols may bind to peptides and form insoluble aggregates, thereby affecting the activity and absorption of peptides (Charlton et al., 2002). The selection of peptides for food enrichment can be important as some peptides are more resistant to certain processing treatments than others. For example, ACE-inhibitory peptides from tuna cooking juice showed resistance to heat, high pressure and pH treatments (Hwang, 2010), and therefore may be suitable for the enrichment of food products requiring these processing treatments. Another ACE-inhibitory peptide derived from duck meat hydrolysate (MW<3 kDa) was found stable in NaCl and glucose solution and could maintain activity when heating up to 100°C, however its activity was significantly decreased under alkaline condition (Shi et al., 2019). Maillard reactions may occur between amino acids and reducing sugars during thermal food processing, and therefore could affect the activity of ACE-inhibitory peptides. For example, the ACE-inhibitory activity of douchi, a fermented soybean product, gradually increases ($p<0.05$) during the 60 h pre-fermentation process and the first 2 weeks of ripening, but decreased in the following 2 weeks of ripening due to Maillard reactions (Wang, Li, Cheng, Yin, & Li, 2013). Another peptide IPP can react with ribose through a Maillard reaction and the resultant product had lower ACE-inhibitory activity but strong antioxidant properties (Jiang, Rai, O'Connor, & Brodkorb, 2013). However, not all Maillard reactions lead to reduction in ACE-inhibitory activity, where elevated activity was observed in the casein hydrolysate–xylose system after Maillard reaction at pH 8.0, 110 °C (Hong, Meng, & Lu, 2015). In this case, the substance with higher ACE inhibitory activity may be melanoidins, the products of Maillard reaction (Rufián-Henares & Morales, 2007). Another study investigated the changes in the concentration of 8

ACE-inhibitory peptides (IQP, LQP, IIP, LIP, LLP, IPP, LPP, and VPP) present in steamed bread, baked bread, and soda crackers at different stages of the bread/cracker-making process (Zhao, Hu, Schieber, & Gänzle, 2013). These researchers found that the concentrations of IPP, LPP and VPP remained stable during dough preparation and decreased during thermal treatment, while the concentrations of other peptides decreased at the dough stage but remained relatively stable during baking. During the production process of these cereal products, it was not only the thermal treatment that affected the ACE-inhibitory activity, but due to a combined effect of protein hydrolysis by yeast protease, pH, temperature and the interaction between peptide and other components of dough such as Maillard reaction. This indicated the complexity of how food processing could alter the activity of ACE-inhibitory peptides. Overall, there are only a small number of researches looking into the impacts of food processing on the activity of ACE-inhibitory peptides, further researches are required to develop optimal food processing protocols to produce ACE-inhibitory peptide based functional foods.

Apart from the immature food processing protocols for the volume production of ACE-inhibitory peptide based functional foods, the sensory attribute of the peptides is another limiting factor for their wide applications, as these peptides can give a bitterness taste. Molecular simulation technology was applied not only to study structure-activity relationship of ACE-inhibitory peptides as discussed in Section 5.3, but also to find the methods of debittering these peptides. Several QSAR researches have showed that bulky basic amino acids at the N-terminal and bulky hydrophobic amino acids at the C-terminal were highly correlated to bitterness of ACE-inhibitory peptides (Kim & Li-Chan, 2006; Pripp & Ardo, 2007; Wu & Aluko, 2007). Based on this, ACE-inhibitory peptides with no bitterness or low bitterness can be screened (Pooja, Rani, & Prakash, 2017). Bitterness of these peptides can be reduced by using selective bacteria for fermentation or adding proteases to hydrolyse bitter peptides, such as the proline specific exo- and endopeptidases (Cheung, Aluko, Cliff, & Li-Chan, 2015; Fitzgerald & O'Cuinn, 2006). For instance, *Lb. helveticus* strains with the ability of proteolysis are frequently used in the manufacture of cheese and yoghurt, which contribute to both decreased bitterness by hydrolysing bitter peptides (Sieber et al., 2010) and production of ACE-inhibitory peptides (Nakamura et al., 1995). While the protease can debitter the protein hydrolysate, whether the ACE inhibitory activity of the hydrolysate is affected needs to be assessed case by case. For example, casein hydrolysate treated with FlavourzymeTM (Novozymes, Denmark) could significantly decrease bitterness, while its ACE-inhibitory activity was not affected (Wang, Hu, & Zhao, 2013). Treatment using exopeptidase such as carboxypeptidase, aminopeptidase, could

significantly lower the bitterness of whey protein hydrolysates, but at same time reduce the ACE-inhibitory activities (Cheung et al., 2015). Debittering of protein hydrolysates can also be achieved by physical methods to remove bitter peptides using activated carbon (Suh, Bae, & Noh, 2000) or by using either chromatographic procedures or solvent extraction (De Leo, Panarese, Gallerani, & Ceci, 2009). In addition, microcapsule technology can be used to reduce bitterness and improve the bioavailability of ACE-inhibitory peptides (Huang, Xiao, Hao, & Yang, 2017; Sarabandi, Mahoonak, Hamishekar, Ghorbani, & Jafari, 2018).

9. Conclusion

Due to the prevalence of hypertension and other related chronic diseases in the world's population, there is an increasing demand of nutraceuticals to manage blood pressure. ACE-inhibitory peptides have gained great attention from food industry and research to be utilised as a functional ingredient to prevent and support the treatment of hypertension.

ACE-inhibitory peptides have been identified from different food sources, and are generally categorised on origin, such as animal sources of dairy, fish, meat and egg products; plant origin, from soybeans, rice, and nuts; and microbial origin, from yeasts and algae. ACE-inhibitory peptides are released from the food by the food processing methods, including enzymatic hydrolysis, and fermentation. With advancement of molecular techniques, some peptides could also be produced via heterologous genetic recombination. Peptides derived from food and genetic recombination could further be purified to obtain high purity compounds for research and potentially industrial use. It should be noted that there are very few studies investigating the effects of other food components on the stability and activity of ACE-inhibitory peptides during food processing. Whether these components will combine with ACE-inhibitory peptides to form complexes molecules during processing, and whether they affect peptides' bioavailability and their antihypertensive ability need to be further investigated. Further, there are limited research on the effects of processing conditions, such as conditions of thermal sterilization, pH, pressure, on the changes of activity and sensory properties of peptides during processing. Further researches are required to optimise not only the production of ACE-inhibitory peptides, but also their integration into different food products while maintaining activity, and thus to facilitate commercial mass production of ACE-inhibitory peptides and ACE-inhibitory peptides based functional foods.

The activity of ACE-inhibitory peptides is highly dependent on its hydrophobic interaction, hydrophilic interaction, hydrogen bonds, van der Waals force, electrostatic force and metal acceptor

interaction with ACE. Molecular simulation has been widely used to study structure-activity relationships for the purpose to modify existing peptides, and to obtain new peptides with enhanced antihypertensive effects. The bioavailability of ACE-inhibitory peptides is influenced by digestion and absorption processes, which could modulate the integrity and bioactivity of these small peptides. The resistance of peptides to digestive enzymes, intestinal epithelial membrane peptidases and other enzymes depends on their physicochemical and structure properties, which further determine peptide absorption rate and therefore, bioavailability. The bioavailability of ACE-inhibitory peptides could also be influenced by other components in the food, such as medium chain fatty acids, sugar and proteins. With the assistant of molecular simulation, future research should develop new peptides with improved gastro-intestinal stability, higher absorption rates and extended active time *in vivo*, which may be consumed with specific food components to enhance their bioavailability. This will provide the fundamental knowledge and basis for food industry to produce peptide based functional foods to address the need of blood pressure management.

In addition to ACE-inhibitory peptides, other peptides derived from food sources may also exhibit antihypertensive activity, but via other mechanisms, such as inhibiting the production of angiotensin II and promoting the degradation of bradykinin, or inhibiting rennin, or mediated by FPR2 and prostaglandin D2. Importantly, some protein hydrolysates and peptides exhibit ACE-inhibitory activity and antioxidant activity at the same time, and some peptides with antioxidant property may exhibit a stronger antihypertension effect. Further research may investigate the interactions and synergetic effect between ACE-inhibitory peptides, antioxidants and other antihypertensive peptides in blood pressure regulation.

As an active ingredient of functional food, ACE-inhibitory peptides are mostly consumed as part of food since they are most commonly derived from natural food protein through processing. Despite of this, ACE-inhibitory peptides can be directly consumed as an oral supplement or fortified to certain food products. Limited research efforts have been made to investigate the changes in the activity of ACE-inhibitory peptides during different food processing, and further studies are required to optimise the manufacturing procedure of ACE-inhibitory peptides in functional foods. Many ACE-inhibitory peptides may give a sensory character of bitterness, which could limit their application in food industry. Enzymatic hydrolysis and physical methods have been developed to debitter peptides. Despite of this, there is limited research optimising these processes to achieve better debittering outcome while maintaining ACE inhibitory activity, thus warrants further investigation.

10. Acknowledgements

This review was supported by the School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne and College of Biotechnology and Food Science, Tianjin University of Commerce.

11. Authors contributions

Lu Xue and Rongxin Yin contributed to the manuscript concept and design, and conducted the literature review, drafted the first version, edited and proofread the manuscript. Kate Howell contributed to the editing and proofreading of the manuscript and provided critical feedback for revision. Pangzhen Zhang contributed to project management, manuscript concept and design, overall reviewing, editing and approval of the final submission.

12. Conflict of interest

The authors declare no conflict of interest.

13. References

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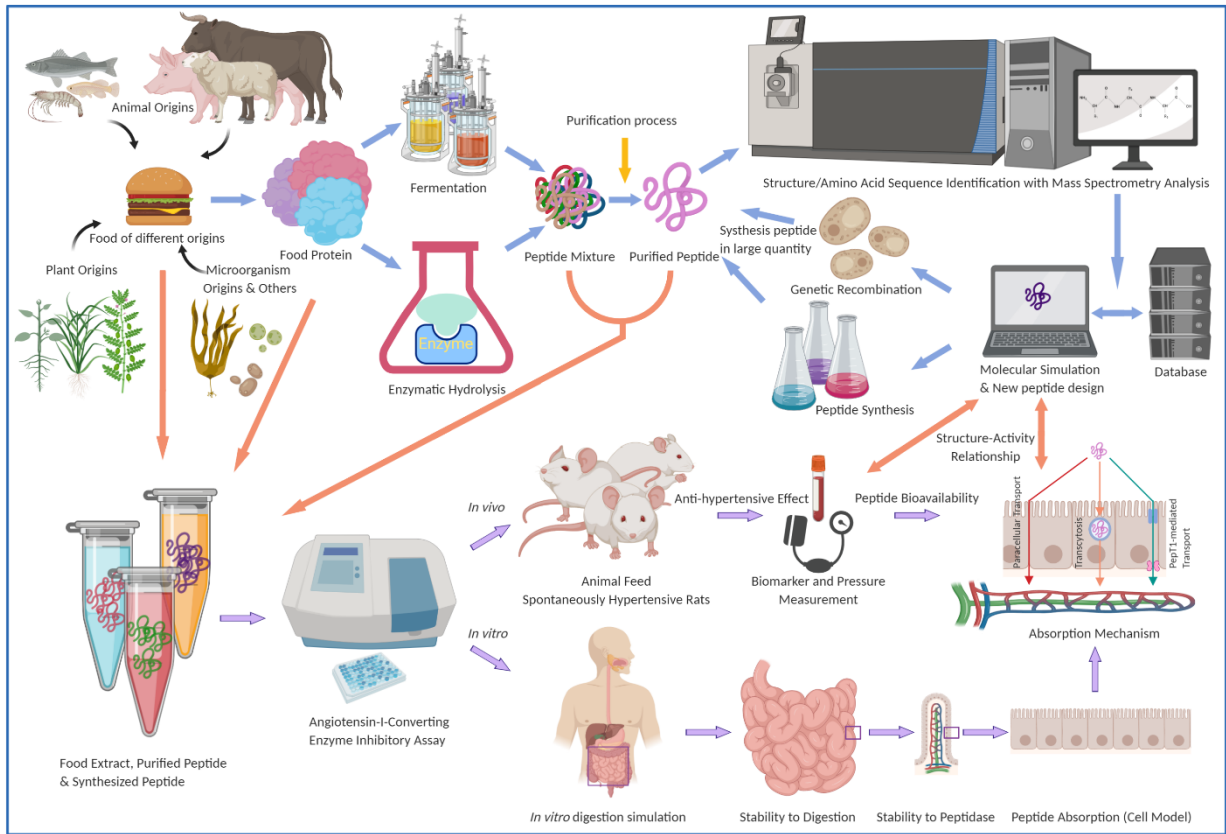


Figure 1- Research areas on Angiotensin-I-converting enzyme inhibitory peptides.

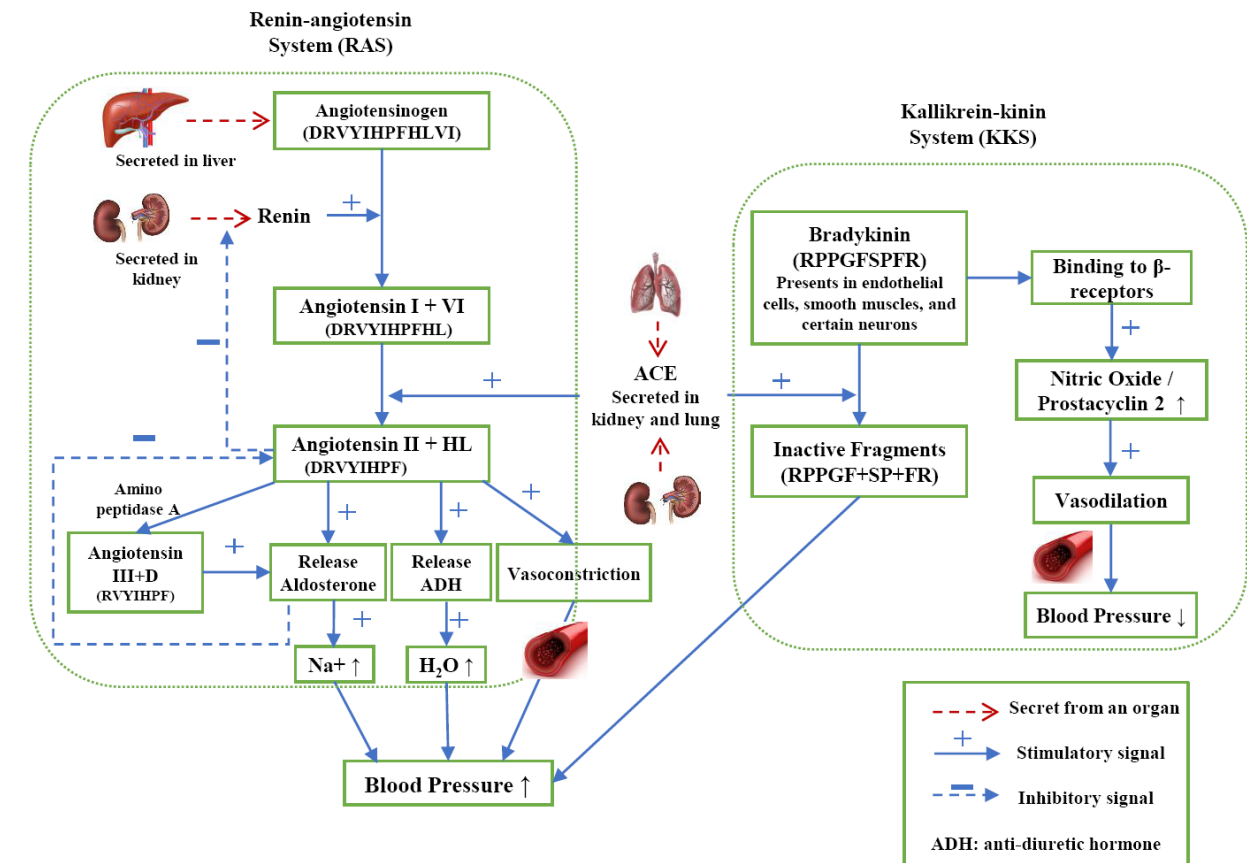


Figure 2 - The role of angiotensin converting enzyme (ACE) in the renin-angiotensin system (RAS) and the kallikrein-kinin system (KKS).

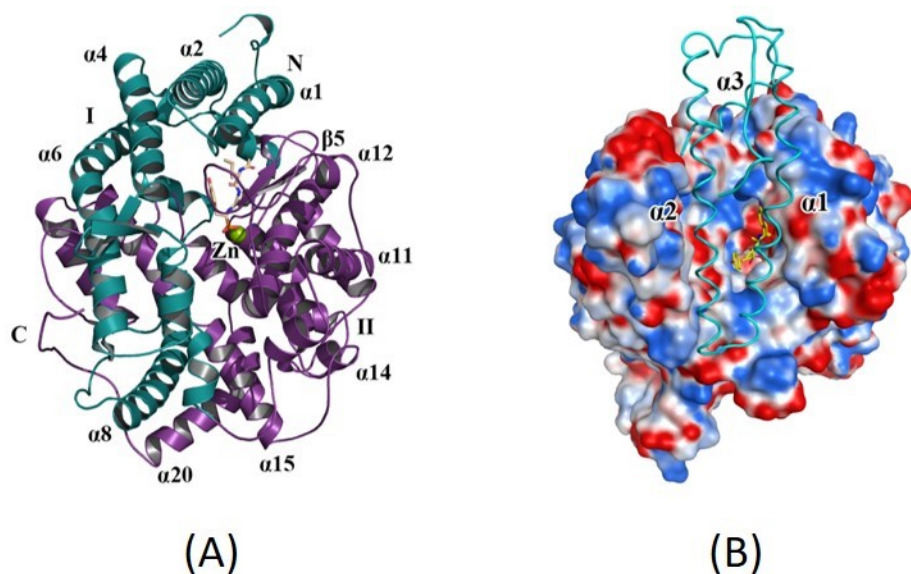


Figure 3 - Three-dimensional structure of angiotensin converting enzyme (ACE). (A) The crystal structure of ACE. Green is subdomain I: 1-269, purple is subdomain II: 270-612, and Zn^{2+} is shown as sphere. α represents α -helix, β represent β -turn. YQKFPQYLQY was depicted as sticks in green. (B) Surface of ACE. Red represents negatively charged atoms, blue represents positive charge atom, white represents neutral atoms.

Table 1 - Similarities and Differences between somatic (sACE) and testicular (tACE) angiotensin converting enzyme

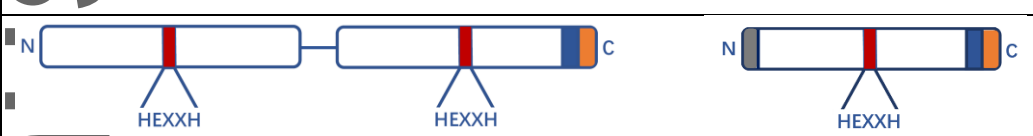
	sACE	tACE
Location	Distributed in all tissues of the body, especially endothelial cells of lung, kidney, small intestine and epididymis	Only in spermatids and spermatozoa, testosterone dependent (Riordan, 2003)
Number of amino acids	1306	732 (Riordan, 2003)
Active site	2	1
Schematic	 Extracellular domain is in white, transmembrane domain is in blue, carboxy-terminal cytosolic domain is in orange	
N-terminal	sACE doesn't have the first 36 residues of N-sequence of tACE (Conrad et al., 2016)	
C-terminal	Human tACE and sACE have the same carboxy-terminal transmembrane and cytosolic sequences (Riordan, 2003)	
Association with cell membrane	Bound to plasma membranes by its C-terminal hydrophobic transmembrane anchor (Coates, 2003)	
Specific Substrate	Low specificity Angiotensin I is not the only substrate (Riordan, 2003)	Unknown (Atanassova et al., 2009)

Table 2 - Angiotensin converting enzyme-inhibitory Peptides Derived from Natural Resources

Peptide sequence	Origin	Production (enzymes)	IC50 (μM)	Antihypertensive activity(Δ mmHg)	Stability to digestion or peptidase	Transport Pathway	Reference
1. Animal Food Origin							
AAATP	dry-cured ham	Water soluble peptide extracts	100	-25.6±4.5 ^a	unstable	-	(Escudero et al., 2013), (Gallego et al., 2016)
AAPLAP			14.38	-			
KPVAAP			12.37	-			
VKKVLGNP	Porcine Skeletal Muscle Myosin	pepsin	28.5	-24 ^b	-	-	(Katayama et al., 2007)
VLAGTL	beef sarcoplasmic protein	thermolysin, proteinase	35	-	-	-	(Jang & Lee, 2005)
GPL	bovine skin gelatin	collagenase	2.55	-	-	-	(Kim et al., 2001)
GPV			4.67	-	-	-	
VGPV	bovine collagen	alcalase	405.12	-	-	paracellular	(Fu, Young, et al., 2016)
GPRGF		papain	200.91				

^aDose: 1 mg/kg body weight^b 10 mg/kg body weight

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IWHHT	Spent hen	thermolysin	9.93	-	-	Paracellular	(Fan et al., 2018; Gu et al., 2019)
IWH			-				
IW			2.0			PepT1	
IKW	chicken	thermolysin	0.21	-50 ^b	-	-	(Fujita et al., 2000)
IY	bonito		2.1	-45 ^b			
IKP	bonito		1.6	-70 ^b			
IWHHT	bonito		5.8	-60 ^b			
IWH	bonito		3.5	-70 ^b			
LKPNM	bonito		2.4	-80 ^b			
LKP	bonito, chicken		0.32	-75 ^b			
LKP	chicken/fish muscle	thermolysin	0.32	-	stable	PepT1, paracellular	(Gleeson et al., 2017; Gleeson et al., 2015)
KPLCS	chicken	pepsin–pancreatin	0.37	-	unstable	-	(Sangsawad et al., 2017),
KPLL			11.98	-	stable	-	(Sangsawad, Chooiwongkomo
ELFTT			6.35	-	stable	-	n, et al., 2018)
APP	chicken myosin	pepsin–pancreatin	4.6	-	-	-	(Sangsawad, Roytrakul, et al., 2018)
AHLL	loach	bromelain	40.3	-	-	transcellular	(Li et al., 2017; Li et al., 2012)
						paracellular	
MVGSAPGVL	skate skin	Alcalase/protease	3.09	-	-	-	(Ngo et al., 2015)
LGPLGHQ	gelatin		4.22				

GASSGMPG	pacific cod	pepsin	6.9	-	-	-	(Ngo, Vo, Ryu, & K., 2016)
LAYA	skin gelatin		14.5				
GPL	Alaska	alcalase,	2.6	-	-	-	(Byun & Kim, 2001)
GPM	pollack skin gelatin	pronase E, collagenase	17.13				
PGPLGLTGP	skate skin	α-chymotrypsin	95	-	-	-	(Lee et al., 2011)
ELGFLGPR			148				
VAP	grass carp	alcalase	16.62	-	stable	-	(Chen, Wang, Zhong, Wu, & Xia, 2012)
VPAAPPK	Haruan	thermolysin,	0.45	-	-	-	(Ghassem et al., 2011)
NGTWFEPP	myofibrillar	proteinase K	0.63				
VELYP	cuttlefish muscle	cuttlefish proteases	5.22	-20 ^b	stable	-	(Balti et al., 2015)
GIHETTY			25.66	-	-		
EKSVELP			14.41	-	-		
SFHPYFSY			82.71	-	-		
AFVGVVLP		A21 proteases	18.02	-	-		
KNGDGY			51.63	-	-		
STHGVW			19.30	-	-		
RSIKGF			32.74	-	-		
SSSKAKKMP	shrimp waste protein	Savinase	-	-	-	-	(Krichen et al., 2018)
HGEGGRSTHE							
WLGHGGRPDHE							
WRMDIDGDIMISEQ							
EAHQQR							
SV	fermented shrimp	fermentation	60.68	-	-	-	(Kleekayai et al., 2018)

IF	pastes			70.03				2015)
NAPHMR	sea cucumber	protamex		260.22	-	stable	-	(Zhong et al., 2018)
QPGPT	<i>Rhopilema esculentum</i>	pepsin and papain		80.67	-9.82 ^b	-	-	(Liu et al., 2013)
GDIGY				32.56	-15.48 ^b			
AWLHPGAPKVF	<i>Phascolosoma esculenta</i>	pepsin		130.9	-30 ^b	-	-	(Du et al., 2013)
ASL	silkworm pupa protein	pepsin, trypsin, α-chymotrypsin		102.15	-	-	-	(Q. Wu et al., 2015)
YV	ostrich ovalbumin	alkaline hydrolysis		214.42	-	stable	-	(Khueychai et al., 2018)
IVRDPNGMGAW	soft-shelled turtle egg white protein	thermolysin		4.39	-	-	-	(Rawendra et al., 2013)
QIGLF	egg white protein	alcalase		75	-	stable	-	(Yu, Zhao, et al., 2011)
TNGIIR	ovalbumin	alcalase		70	-	-	paracellular	(Ding et al., 2016; Yu et al., 2012)
RVPSL	ovotransferri	alcalase		20	-24 ^c	unstable	paracellular	(Yu, Yin, et al., 2011), (Ding et al., 2015; Yu, Yin, Zhao, Chen, & Liu, 2014)
YAEERYPIL	ovalbumin	pepsin		4.16	-31.6± 2.6 ^d	unstable	-	(Miguel et al., 2006)
RADHPFL				5.5	-34.0± 1.6	unstable	-	(Miguel et al., 2006)
				229.49±3				

^c 50 mg/kg body weight

^d 2 mg/kg body weight

RADHP			0.74	N/A	e	-	2008)
					stable		
IRW	ovotransferri	thermolysin-pepsin	0.6	-20.97 ^e	stable	PepT1	(Bejjani & Wu, 2013; Liao, Fan, Davidge, & Wu, 2019; Majumder & Wu, 2010)
VLPVPQK	casein	pepsin, trypsin	15	-	stable	PepT1	(Vij et al., 2016)
RYLGY	casein	pepsin	0.71	- 25 ± 3 ^f	-	-	(Mar Contreras et al., 2009)
AYFYPEL			6.58	N/A			
YQKFPQY			20.08	N/A			
YQKFPQYLQY	casein	pepsin, trypsin	11.1	-40±4.2 ^g	-	-	(Xue et al., 2018)
INNQFLPYPY			166.3	-			
MKP	casein	-	0.43	Lowered pressure from 180 to 166 ^h	-	-	(Yamada et al., 2015)
VYPFPGPIHN	β-casein	pepsin, pancreatin	656	-	stable	-	(Petrat-Melin, Le, Møller, Larsen, & Young, 2017)
VYPFPGPIP			123	-	unstable	-	
VPP	β-casein	<i>Lactobacillus helveticus</i> , <i>L. bulgaricus</i> J24, <i>S. thermophilus</i>	9	-	stable	Paracellular	(Nakamura et al., 1995), (Satake et al., 2002) (Foltz et al., 2008), (Ohsawa et al., 2008), (Kawaguchi et

^e 15 mg/kg body weight

^f 5 mg/kg body weight

^g 9 mg/kg body weight

			TN1,				al., 2012),
			fermentation				(Panayotova et al., 2018)
IPP	β -casein	<i>Lactobacillus helveticus</i> , <i>L. bulgaricus</i> J24, <i>S. thermophilus</i> TN1,	5	-18 ^f	stable	PepT1 paracellular	(Nakamura et al., 1995), (Foltz et al., 2008), (Ohsawa et al., 2008), (Kawaguchi et al., 2012), (Gleeson et al., 2015), (Gleeson et al., 2017), (Panayotova et al., 2018)
NMAINPSKENLCSTFC K	casein	trypsin	129.07		-	-	(Tu et al., 2018)
PPEIN	yak milk casein	alcalase	0.52	-	-	-	(Mao, Ni, Sun, Hao, & Fan, 2007)
PLPLL			0.44				
RLSFNP	sour milk	<i>Lactobacillus heveticus</i> LB10	177.39	-	unstable	paracellular	(Pan & Guo, 2010), (Guo et al., 2018)
LRPVA	lactoferrin		0.7	-	-	-	(Yoshikawa, 2015)
LRP			0.21				
LIWKL	lactoferrin	Pepsin	0.47	-25.3 $\pm$ 3.5 ^b	unstable	-	(Ruiz-Giménez et al., 2012)
RPYL			56.5	-18.9 $\pm$ 2.3 ^b	unstable	-	
LNNSRAP			105.3	-15.3 $\pm$ 3.7 ^b	unstable	-	
RRWQWR	lactoferricin	-	32	N/A	unstable	no-transporter	(Ruiz Gimenez et al., 2010);
		Digestion					

RWQ		enzyme	-		stable	d	(Fernández-
WQ			-		stable	paracellular	Musoles et al., 2013)
						paracellular	
ALPMHIR	β -lactoglobulin	-	42.6	-	stable	hardly transported	(Vermeirssen et al., 2002)
2. Plant Food Origin							
LSW	soybean	thermolysin	2.7	-	stable	PepT1, paracellular	(Gu & Wu, 2013), (Lin et al., 2017)
LVQGS	soybean	<i>Aspergillus oryzae</i> fermentation	43.7	-	-	-	(Rho et al., 2009)
AF	soybean	<i>Aspergillus oryzae</i> fermentation	165.3	-	-	-	(Zhu et al., 2008)
IF			65.8				
EAQRLLF	green soybean	alcalase	878	-	-	-	(Hanafi et al., 2018)
PSLRSYLAE			532				
RGQVLS			993				
ACEKP	pistachio	pepsin, trypsin	126	-	-	-	(Li et al., 2014)
WG	potato	ficain	231.22	-	-	-	(Fu et al., 2017)
PRY	patatin		97.59				
VNP	rice	alcalase	6.4	-29 ^f	-	-	(Chen et al., 2013)
VWP			4.5	-38 ^f			
YSK	rice bran	trypsin	76	-	-	-	(Wang et al., 2017)

LLSGTQNQPSFLSGF	lentil	Savinase	119.75	-	-	-	(Garcia-Mora et al., 2017)
NSLTLPILRYL			77.14				
TLEPNSVFLPVLLH			117.81				
AVKVL	hazelnut	alcalase	73.06	-	-	-	(Liu et al., 2018)
YLVR			15.42				
TLVGR			249.3				
EPNGLLLPQY	walnut	pepsin	233.17 8	-	-	-	(Wang et al., 2018)
NAQRP	peanut arachin	pepsin	32 ± 2	-	-	-	(Jimsheena & Gowda, 2011)
DGVVYY	tomato seed	<i>Bacillus subtilis</i> fermentation	2	-	-	-	(Moayed et al., 2018)
GVLY	hemp seed	alkaline solubilization	16	-	-	-	(Orio et al., 2017)
LGV			145				
RVR			526				
FQLPKF	barley	papain	28.2	-	-	-	(Gangopadhyay et al., 2016)
GFPTLKIF			41.2				
LY	rapeseed	alcalase	110	-26.73 ± 4.24 ^h	stable	-	(R. He et al., 2013)
RALP		alcalase	970		unstable	-	(Yang et al., 2017)
RIY		subtilisin	28	-16.66 ± 8.57 ^h		-	(Marczak et al., 2003)
				-10 ⁱ			
WNI	flaxseed	pepsin, pancreatin	-	-	-	-	(Marczak et al., 2003)

^h 30 mg/kg body weight

ⁱ 5.8 mg/kg body weight

LNA						-	
NI/LDTDI/L						-	
VSV	canola	alcalase	0.15		-	-	(Wu et al., 2008)
FL			1.33		-	-	
IVY	brewers' spent grain	alcalase, Corolase and Flavourzyme	80.4	-	-	-	(Connolly et al., 2015)
ILDL		PP	96.4				
LRP	zein	thermolysin	0.27	-15 ^h	-	-	(Miyoshi et al., 1991)
MRWRD	Spinach	pepsin,	2.1	-13.5 ^h	-	-	(Yang et al., 2003)
MRW	Rubisco	pancreatin	0.6	-20 ^h	-	-	
3. Microorganism Origin and Others							
IQP	<i>Spirulina platensis</i>	alcalase	5.77	N/A	stable	paracellul	(Lu, Ren, Xue, et al., 2010), (Lu, Ren, Wang, & Tanokura, 2010), (He et al., 2018)
VEP			27.36	-	stable	ar paracellul ar	
IVVE	<i>Chlorella vulgaris</i>	pepsin	315.3	-	-	-	(Suetsuna & Chen, 2001)
AFL			63.8				
FAL			26.3				
AEL			57.1				
VVPPA			79.5				
IAE			34.7				
FAL	<i>S. platensis</i>		26.3				
AEL			57.1				
IAPG			11.4				

NAF			35.8				
GMNNLTP	<i>Nannochloropsis oculata</i>	pepsin	123	-	-	-	(Samarakoon et al., 2013)
LEQ			173	-	-	-	
YMGLDLK	<i>Isochrysis galbana</i>	trypsin	36.1	-	-	-	(H. Wu et al., 2015)
LVTVM	<i>Nannochloropsis Oculata</i>	alcalse	18				(Qian et al., 2013)
QVEY	<i>Gracilariopsis lemaneiformis</i>	trypsin	474.36	-	-	-	(Cao et al., 2017)
VEGY	<i>Chlorella ellipsoidea</i>	alcalase	128.4	-22.8 ^b	stable	-	(Ko et al., 2012)
VECYGPNRPOF	<i>Chlorella vulgaris</i>	pepsin	29.6	-	stable	-	(Sheih et al., 2009)
LPESVHLDK	<i>Kluyveromyces marxianus</i>	trypsin & chymotrypsin	22.88	-	-	-	(Mirzaei et al., 2018)
VLSTSFPPK			15.2				
YGKPVAVPAR	<i>Saccharomyces cerevisiae</i>	Sonication-trypsin	397.2	-	-	-	(Mirzaei et al., 2015)
KVLPVP	<i>Escherichia coli</i>	DNA recombinant	4.6	-21.4 ± 7.2 ^j	-	-	(Liu et al., 2007)
VLPVP	<i>Escherichia coli</i>	DNA recombinant	1.7	-37.7 ± 17.5 ^k	-	Paracellular & MRP2	(Lei et al., 2008)

SBP: systolic blood pressure

^j 0.3 mg/kg body weight

^k 0.4 mg/kg body weight