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STRUCTURAL CHARACTERISTICS AND ANTIHYPERTENSIVE EFFECTS OF ANGIOTENSIN-I-
CONVERTING ENZYME INHIBITORY PEPTIDES IN THE RENIN-ANGIOTENSIN AND
KALLIKREIN KININ SYSTEMS

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Abstract

Background: The commercially available synthetic angiotensin-I-converting enzyme (ACE) inhibitors are known to exert negative side effects which have driven many research groups globally to discover the novel ACE inhibitors.

Method: Literature search was performed within the PubMed, ScienceDirect.com and Google Scholar.

Results: The presence of proline at the C-terminal tripeptide of ACE inhibitor can competitively inhibit the ACE activity. The effects of other amino acids are less studied leading to difficulties in predicting potent peptide sequences. The broad specificity of the enzyme may be due to the dual active sites observed on the somatic ACE. The inhibitors may not necessarily competitively inhibit the enzyme which explains why some reported inhibitors do not have the common ACE inhibitor characteristics. Finally, the in vivo assay has to be carried out before the peptides as the antihypertensive agents can be claimed. The peptides must be absorbed into circulation without being degraded, which will affect their bioavailability and potency. Thus, peptides with strong in vitro IC50 values do not necessarily have the same effect in vivo and vice versa.

Conclusion: The relationship between peptide amino acid sequence and inhibitory activity, in vivo studies of the active peptides and bioavailability must be studied before the peptides as antihypertensive agents can be claimed.

Keywords: Angiotensin-I-converting enzyme, Antihypertensive activity, Bioactive peptides, ACE inhibitor

Introduction

According to Chen et al. (2012), in the year 2000, it was roughly estimated that about 972 million adults had hypertension. This number is expected to swell to a total number of 1560 million in 2025. Hypertension is associated with a minimum of 7.6 million deaths annually worldwide (13.5% of all deaths), making it the primary risk factor for cardiovascular diseases (Chow et al., 2013). Currently, approximately 35% of the Latin American population, 20-30% of the Chinese and Indian populations and approximately 14% of the Sub-Saharan African population suffer from hypertension (Mittal & Singh, 2010). In a national study of 16,440 subjects in Malaysia, the prevalence of hypertension in subjects aged 15 and above was 27.8%. The prevalence of hypertension among individuals aged 30 and above rose to 40.5% in 2004 compared to 32.9% in 1996. Among subjects aged 60 and above, the prevalence of hypertension was noted to be 69.3% in both sexes (Rampal et al., 2008).

Different biochemical pathways are involved in regulating blood pressure such as the renin-angiotensin system (RAS), kallikrein-kinin system (KKS), and chymase that convert angiotensin I to angiotensin II (Li et al., 2004; Kanemitsu et al., 2006). Typically, RAS is widely associated with the regulation of the blood pressure since it plays a significant role in regulating arterial pressure. In this pathway, angiotensinogen is converted to angiotensin I by renin and later this angiotensin I is further cleaved to angiotensin II by angiotensin-converting-enzyme (ACE), which leads to the vasoconstriction of the artery and then to the elevation of blood pressure (Hong et al., 2008).

ACE, a dipeptidyl carboxypeptidase (EC.3.4.15.1), plays an important role in the regulation of blood pressure. Apart from cleaving angiotensin I to induce vasoconstriction, it also inactivates bradykinin which functions as a vasodilator in KKS (Wijesekara & Kim, 2010; Jung et al., 2006).

Two forms of ACE are present in humans; a somatic ACE of approximately 170 kDa found in endothelial, epithelial and neuronal cells and an approximately 100 kDa size of a smaller testicular isof orm that exists in the germinal cells (Michaud et al., 2014). The somatic ACE is necessitated in cardiovascular regulation while germinal ACE is involved in male reproduction. According to Andujar-Sanchez et al. (2003), ACE is also present in the plasma in addition to the somatic and germinal forms. The plasma flowing ACE resulted by cleavage from the somatic form. The ACE inhibitors, calcium channel antagonists, beta and alpha blockers, natriuretic agents and endothelin receptor antagonists are groups of drugs which fall into the antihypertensive drug categories. For the treatments of high blood pressure and heart failure, the ACE inhibitors are the well-known drugs prescribed to patients. These drugs have also been shown to protect the organs from being damaged by diseases. For example, when administered alone or in
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combination, Benazepril produces a renoprotective effect. Besides, when elevated blood pressure can be reduced within a normal range, Benazepril will prevent the cardiovascular disease (Saïto, 2008; Barrios & Escobar, 2010).

Despite the effectiveness, these drugs have undesirable effects like coughing, taste disturbance, kidney problem and angioeurotic oedema (Marczak et al., 2003). Hydrochlorothiazide and chlorothalidone can lessen potassium, magnesium, phosphorus, sodium, chloride, folate, B6, zinc, iodine, and CoQ10 while augmenting homocysteine, calcium, creatinine, glucose, insulin resistance, and type-2 diabetes mellitus at a rate of 5% annually (Alexander, 2014).

Higher plasma homocysteine has been suggested as a risk factor for cardiovascular morbidity and mortality (Marcus et al., 2007), while the elevation in serum creatinine concentration within the normal range is an indicator for the augmented risk of cerebrovascular disease in both subjects of normotensive and hypertensive (Wannamethee et al., 1997). The drugs also increase the occurrence of renal deficiency by more than 35% after 10 years of treatment. The administration of the first- and second-generation beta blockers to patients will lower CoQ10 while the ACE inhibitors and angiotensin-receptor blockers will lower zinc (Alexander, 2014). Besides that, the lichenoid reactions, white lesions categorized by linear striations on the buccal mucosa are sometimes noticed in the hypertensive patients resulting from the side effects of the ACE inhibitor drugs, particularly captopril (Kumar et al., 2012). Apart from the above mentioned problems, there is also a need to search for new antihypertensive drugs to overcome drug-resistant hypertension which occurs in approximately one in eight patients with elevated blood pressure (Townsend, 2011).

Unlike these synthetic drugs, antihypertensive peptides do not show any adverse effect (Puchalska et al., 2015) although not all were tested for its toxicity. The inhibition of ACE is without a doubt the utmost studied mechanism to control the high blood pressure with respect to food derived biologically from active peptides. Most peptides have been noticed to inhibit ACE to a certain degree. The food industry has acknowledged the possibility of these natural antihypertensive agents as potential functional ingredients that can assist in the primary prevention and/or management of high blood pressure (Norris & FitzGerald, 2013). In this article, some of the ACE-inhibitory peptides derived from different protein sources, the ACE inhibition type, relationship between peptide amino acid sequence and inhibitory activity, in vivo studies of the active peptides, bioavailability and in silico bioavailability of the peptides are reviewed.

The mechanism of action of angiotensin

In order to induce constriction, angiotensin I has to be cleaved by the ACE to produce angiotensin II. This angiotensin II has the ability to bind to the angiotensin receptors AT1 and AT2, which are two principal angiotensin II receptor subtypes heterogeneously distributed in the peripheral tissues and brain (Chung et al., 1998). In the cardiovascular system, the AT1 receptor is ubiquitously expressed and facilitates most of the physiological and pathophysiological actions of Angiotensin II. The AT2 receptor is vastly expressed in developing foetus, and its expression is very little in the normal adult cardiovascular system. According to Lemarié and Schiffrin (2010), the angiotensin II physiological actions induced through the AT2 receptor opposes that of mediated by the AT1 receptor. While activation of the AT1 receptor by angiotensin II promotes the constriction of vascular beds, activation of the AT2 receptors causes vasodilation.

Relationship between the structure and activity of the ACE inhibitory peptide

An understanding of the correlation between the peptide sequence and its bioactivity as an inhibitor is crucial in generating the potentially potent peptide ACE inhibitor. With the knowledge, it would reduce the need for long conventional ACE inhibitor peptide discovery approach. Unfortunately, there is inadequate information on the structure-activity relationship of antihypertensive peptides because current researches in bioactive peptide field are mainly focusing on the generation and characterization of the antihypertensive peptides.

The first structural characteristic of the ACE-inhibitory peptides is the chain length. Out of many peptide sequences reported, potent ACE inhibitor peptides are typically short with 2-12 amino acids long (Norris & FitzGerald, 2013). The short peptide sequences are easily absorbed into the blood circulation and retain their activity. This is most probably because oligopeptides may not be further digested into shorter fragments in the gastrointestinal tract and are readily absorbed into the circulation, and thus having good bioavailability. It is the best that the ACE inhibitory peptides should not have any enzymatic cleavage site for a gastrointestinal enzyme. However, depending on factors affecting the catalysis ability of these enzymes, it is possible that the peptides can escape from being degraded in the gastrointestinal tract. For example, proline and hydroxyproline comprising peptides are unaffected by the action of digestive proteases especially tripeptides with C-terminal Pro-Pro, which are resistant towards the proline-specific peptidases (Jao et al., 2012).

Some oligopeptides (parent chain) may be cleaved into several smaller fragments. The outcome of the cleavage depends on the fragments produced where it may increase or reduce the overall ACE inhibition activity. The ACE inhibitory activity may increase if suitable amino acid such as proline is present at the C-terminal of the fragments. Thus, the degradation in the gastrointestinal tract may be beneficial in enhancing the activity of the inhibitor and similarly will cause the loss in the activity if the degradation causes the loss of a suitable structure needed to inhibit ACE activity.
Yet, several inhibitory peptides with longer sequences have been reported (Norris & FitzGerald, 2013). However, the biological efficacy of long peptide sequences cannot be determined through in vitro tests. In some cases, the good inhibitory activity observed in vitro is lost when in vivo assays are carried out and vice versa (Iwaniak et al., 2014). Long peptides which are able to produce good ACE inhibitory effects most probably carry potent amino acids in the sequence, particularly within the four amino acids at the C-terminal of the sequence (Norris & FitzGerald, 2013). Depending on the nature of the peptide whether it is water or lipid soluble, these peptides may pass through the tight junction between cells of the paracellular or transcellular route, which explains the bioavailability of long peptides (Vermeirssen et al., 2004).

Studies have shown that the C-terminal tripeptide sequence of an inhibitory peptide highly influences its binding by ACE. The published ACE inhibitory peptide sequences are listed in Table 1. Looking at the sequence, it seems there is a correlation between the IC_{50} values with a sequence of the peptides, where it could be predicted if a given sequence will be a good ACE inhibitor or not. Potent ACE inhibitors comprised of hydrophobic (aromatic or branched side chains) amino acid residues at the C-terminus where the utmost preferred is the presence of proline (Norris & FitzGerald, 2013). The presence of proline at C-terminal and antepenultimate position results in a good binding to the ACE, while the enzyme binds weakly if proline is present at the penultimate position of the peptide (Miguel et al., 2012). In many potent ACE inhibitor peptide sequences, tyrosine, phenylalanine, and tryptophan residues are also present at the C-terminus particularly for the di- and tripeptide inhibitors. The presence of hydrophobic amino acid leucine at the C-terminus also suggests that it may contribute to the ACE inhibition. In addition, the presence of positive charges on the side chains of arginine and lysine residues at the C-terminus have been identified to contribute to the peptide ACE inhibitory effectiveness (Norris & FitzGerald, 2013).
## Table 1: Peptide sequences of ACE inhibitor from different sources and their IC₅₀ value

| Source             | Preparation                                                                 | IC₅₀  | Peptide     | Reference                        |
|--------------------|------------------------------------------------------------------------------|-------|-------------|----------------------------------|
| *Agaricus bisporus*| Ammonium sulphate precipitation                                              | 63    | AHEPVK      | Lau et al., 2013                 |
| Alaskan pollack    | Alcalase, pronase E, and collagenase                                          | 2.6   | GPL         | Byun and Kim, 2001               |
| Alaskan pollack    | Pepsin                                                                        | 14.7  | FGASTRGA     | Je et al., 2004                  |
| Alaskan pollack    | Alcalase → pronase E                                                         | 0.72  | LGP          | Byun and Kim, 2002               |
| Algae              | Pepsin                                                                        | 29.6  | VECYGPNRPQF  | Sheih et al., 2011               |
| Beef               | Thermolysin + proteinase A                                                   | 23.2⁵ | VLAQYK      | Jang and Lee, 2005               |
| Blue mussel        | Fermentation with 25% NaCl                                                    | 19.3⁴ | EVMAGNLYPG   | Je et al., 2005                  |
| Bonito             | Thermolysin; boiling water extract                                           | 5.1   | IWHHT       | Yokoyama et al., 1992            |
|                   |                                                                              | 3.7   | IY          |                                  |
| Bonito             | Thermolysin; ACE                                                              | 2.4   | LKPNM       | Fujita and Yoshikawa, 1999       |
|                   |                                                                              | 0.32  | LKP         |                                  |
| Bonito             | Autolysis                                                                    | 1.0   | LRP         | Matsumura et al., 1993           |
| Bovine             | Alcalase → Pronase E                                                         | 2.55  | GPL         | Kim et al., 2001                 |
| Bovine lactoferrin | Pepsin                                                                       | 0.47  | LIWKL       | Ruiz-Gimenez et al., 2012        |
| Broccoli           | Water extraction                                                             | 10.5⁵ | YPK         | Lee et al., 2006a                |
| Buckwheat          | Water extraction                                                             | 6.2⁵  | GPP         | Ma et al., 2006                  |
| Bullfrog           | Alcalase                                                                     | 0.95  | GAAELPCSADWW | Qian et al., 2007b              |
| Canola meal        | Alcalase                                                                     | 0.15  | VSV         | Wu et al., 2008b                 |
| Catfish            | Thermolysin                                                                  | 0.86  | GPPP        | Ghassem et al., 2012             |
| Chicken            | Aspergillus oryzae protease                                                  | 29    | GAXGLXGP    | Saiga et al., 2008               |
| Common fig         | Latex collection from tree                                                    | 4.5   | LYPVK       | Maruyama et al., 1989            |
| Corn               | Pescalase                                                                    | 0.1⁰  | PSQQYY      | Suh et al., 1999                 |
| Corn               | Alcalase                                                                     | 14.2  | AY          | Yang et al., 2007                |
| Cotton leaf worm larvae | Subsequent hydrolysis using pepsin, trypsin and chymotrypsin     | 2123  | AVF         | Vercruysse et al., 2008          |
| Cuttlefish         | Digestive proteases                                                           | 6.1   | VYAP        | Balti et al., 2010a              |
| Cuttlefish         | Bacterial proteases                                                          | 11.6  | AHSY        | Balti et al., 2010b              |
| Dwarf gulper shark | Thermoase                                                                    | 3.3⁴  | VW          | Ikeda et al., 2015               |
| Egg white          | Alcalase                                                                     | 20    | RVPSL       | Liu et al., 2010                 |
| Egg white lysozyme | Combination of pepsin, α-chymotrypsin and trypsin                            | 2.86  | VAW         | Rao et al., 2012                 |
| Organism            | Enzyme (or method) | Peptide Sequences | References                        |
|---------------------|--------------------|-------------------|-----------------------------------|
| Garlic              | Aqueous extract    | 3.74 FY           | Suetsuna, 1998.                   |
| Grass carp          | Alcalase           | 5.34 FYVAP        | Chen et al., 2012                 |
| Grifola frondosa    | Water extract      | 2.6 KYTFAVTTVKTWV | Ohtsuru et al., 2000              |
| Hard clam           | Protamex           | 51 YN             | Tsai et al., 2008                 |
| Hypsizygus marmoreus| Water extract      | 190 LSGASASLSP    | Kang et al., 2013                 |
| Larva               | Alcalase           | 17 YAN            | Dai et al., 2013                  |
| Lizard fish         | Neutral protease   | 41 SPRCR          | Wu et al., 2012                   |
| Lysozyme            | Papain–trypsin     | 30 FESNFNTQATNR   | Asoodeh et al., 2012              |
| Microalgae          | Alcalase           | 383.4 VEGY        | Ko et al., 2012                   |
| Milk                | Proteinase, aminopeptidase, x-prolyl-dipeptidyl aminopeptidase | 9.13 VPP, 5.15 IPP | Pan et al., 2005                  |
| Mung bean           | Alcalase           | 13.4 KLPAGTLF     | Li et al., 2006                   |
| Ostrich egg white   | Trypsin            | 80.2 AKFDEDTEEVPR | Tanzadehpanah et al., 2013        |
| Oyster              | Pepsin             | 66 VYPWTQRF       | Wang et al., 2008a                |
| Pleurotus cornucopia| Water extraction   | 460 RLSEPFDLSAFLRA | Jang et al., 2011                |
| Pleurotus cystidiosus| Ammonium sulphate precipitation | 62.8 AHEPVK | Lau et al., 2013                  |
| Porcine             | Thermolysin        | 549.0 ITTNP       | Arihara et al., 2001              |
| Porcine             | Pepsin             | 6.1 KRVIQX        | Muguruma et al., 2009             |
| Porcine haemoglobin | Pepsin             | 4.92 LGFPTTGTKYFP | Yu et al., 2006                   |
| Porcine skeletal troponin | Pepsin          | 26.2 KRQKYDI     | Katayama et al., 2008             |
| Porcine troponin C  | Pepsin             | 34 RMLGQTPTK     | Katayama et al., 2003             |
| Rainbow trout muscle| Pepsin             | 63.9 KVNGPAMSPNAN | Kim and Byun, 2012               |
| Rice                | Hydrolysate        | 4.5 VWP           | Chen et al., 2013                 |
| Rice                | Alcalase           | 18.2 TQVY        | Li et al., 2007                   |
| Rice wine           | Concentrates       | 340 QFYAV         | Kang et al., 2012                 |
| Salmon              | Alcalase           | 7.72 FNVPLYE      | Ahn et al., 2012                  |
| Salmon skin         | Alcalase → papain  | 60 AP             | Gu et al., 2011                   |
| Sardine             | Alkaline protease  | 1.63 KW           | Matsufuji et al., 1994            |
| Sea bream           | Protease           | 7.5 VIY          | Fahmi et al., 2004                |
| Sea cucumber        | Bromelain and alcalase | 15.9 MEGAQEAQGD  | Zhao et al., 2009                 |
| Food Source          | Enzyme/Method                          | Activity | Ref.                  |
|----------------------|----------------------------------------|----------|-----------------------|
| Sea cucumber         | Bromelain, alcalase                    | 14.2a    | Zhao et al., 2007     |
| Seaweed pipefish     | Alcalase                               | 620b     | Wijesekara et al., 2011|
| Sesame               | Thermolysin                            | 0.78     | Nakano et al., 2006   |
| Shark                | Protease                               | 1.45     | Wu et al., 2008a      |
| Shrimp               | Fermentation by *Lactobacillus fermentum* SM 605 | 2.15     | Wang et al., 2008b    |
| Silkworm albumin     | Acid protease (*Aspergillus usami NO. 537*) | 47a      | Wang et al., 2011     |
| Sour milk            | Fermentation by *Lactobacillus helveticus*, *Saccharomyces cerevisiae* | 9, 5     | Nakamura et al., 1995 |
| Soy bean             | Acid proteinase                        | 70, 65   | Kuba et al., 2005     |
| Soy bean             | Homogenization with dH2O               | 29.9     | Kuba et al., 2003     |
| Soy bean             | Fermentation                           | 2.2a     | Shin et al., 2001     |
| Soy bean             | Rapid fermentation                     | 43.7     | Rho et al., 2009      |
| Soybean had          | Protease P                             | 1.69     | Gouda et al., 2006    |
| Spinach rubisco      | Pepsin-pancreatin                      | 0.38     | Yang et al., 2003     |
| *Spirulina platensis* | Alcalase                              | 5.77     | Lu et al., 2010       |
| Squid                | Esperase                               | 47.78    | Alemán et al., 2013   |
| Styela plicata       | Protamex                               | 24.7     | Ko et al., 2011       |
| Sunflower            | Pepsin and pancreatin                  | 6.9      | Megias et al., 2004   |
| *Tricholoma giganteum* | Water extraction                     | 40a      | Lee et al., 2004      |
| Tuna                 | Pepsin                                 | 11.28    | Lee et al., 2010a     |
| Tuna                 | Pepsin                                 | 21.6     | Qian et al., 2007a    |
| Wakame               | Pepsin                                 | 21       | Suetsuna and Nakano, 2000 |
| Wakame               | Protease S “Amano”                     | 1.5      | Sato et al., 2002     |
| Walnut               | Hydrolysate                            | 25.67a   | Liu et al., 2013      |
| Walnut               | Hydrolysate                            | 128.98a  | Wang et al., 2014     |
| Yellowfin sole       | α-chymotrypsin                         | 28.7a    | Jung et al., 2006     |

a = µg/mL; b = mM; while others are expressed in µM.
In the case of tetrapeptides, the best promising amino acids (beginning from C-terminus) are tyrosine and cysteine (Iwaniak et al., 2014). At the tetrapeptide second position, residues like histidine, tryptophan, and methionine are commonly found. In the peptide chain third position, residues such as isoleucine, leucine, valine, or methionine are positioned and tryptophan is located as the fourth residue (Iwaniak et al., 2014). However, this is not the only structure required for the inhibition. For example, the peptide GPPP with IC$_{50}$ of 0.86 µM, apart from its shorter sequence which allows easy absorption, also shows significant inhibition due to the presence of proline at C-terminal and antepenultimate position. It can be concluded that the presence of proline can also play a significant role for the tetrapeptide inhibitor. The location of proline in this sequence best describes why it can significantly inhibit ACE.

Two peptides (TKVIP and AYFYP) with proline residue present at the C-terminus studied by Maeno et al., (1996) did not produce strong ACE inhibitory activity. Although the proline residue is important in inhibiting ACE, this study has shown that the presence of proline alone at the C-terminal does not promise a good inhibition of the enzyme. In the same research, when KVLPVPQ was digested to a shorter KVLPVP, IC$_{50}$ was improved from 1000 µM to 5 µM. This is possibly due to the presence of proline at C-terminal and also at the antepenultimate position of the peptide, which causes the peptide to bind stronger to the ACE active site as compared to the presence of proline at the penultimate position. The deletion of glutamine produced a better inhibition by placing proline at C-terminal and also the antepenultimate position of the peptide.

When YKVPQL was partially hydrolysed by pancreatic YKVP, IC$_{50}$ of the peptide increased from 22 µM to more than 1000 µM (Maeno et al., 1996). The YKVP sequence was not a significant sequence for the ACE inhibition based on the amino acids arrangement. For a tetrapeptide sequence, tyrosine and cysteine are the best promising amino acids at the C-terminal and the sequence arrangement for tetrapeptide was explained by Iwaniak et al., (2014). As mentioned above, the GPPP sequence does not fit in this explanation and it is hypothesized that the presence of few prolines in tetrapeptides especially at the antepenultimate and C-terminal, will yield a good ACE inhibition.

Generally, proline and hydroxyproline comprising peptides are unaffected by the digestive proteases (Jao et al., 2012). Compared to the original sequence of YKVPQL, the presence of proline at the antepenultimate position and leucine at the C-terminal are considered as a good sequence for increased potency. For a longer or oligopeptide sequence, the arrangement of the C-terminal amino acids is important so that the peptide can bind strongly to the ACE active site (Norris & FitzGerald, 2013).

In another study of the long peptide, once the phenylalanine at the C-terminus of the GFXGTXGLXGF peptide was removed, IC$_{50}$ was altered to 25000 µM from the original IC$_{50}$ of 46 µM. This indicates that the presence of phenylalanine at C-terminus is also an utmost importance for the ACE inhibitory activity (Saiga et al., 2006). According to Ni et al., (2012), the peptides with phenylalanine at the C-terminal have high ACE inhibitory activity. Similar characteristic was observed for the peptide sequence of VECYGPNRPQF, with proline at the antepenultimate and phenylalanine at the C-terminal.

Compared to the peptides described above, the ACE inhibitory activity of peptide LPGRPP1KPWPL was reported with lower IC$_{50}$ value of 128.98 µg/mL. Although leucine is located at the C-terminal of the peptide, it does not necessarily make peptide a good inhibitor. Similarly, even though peptide has several proline residues, the position of this residue at the end of the C-terminal is really crucial. The presence of proline at the penultimate position will cause the peptide to bind weakly to the ACE active site (Miguel et al., 2012).

The proline penultimate and antepenultimate positions are not really applicable for the tripeptide and dipeptide. Since the sequences are already short, the presence of proline may be exceptional. As mentioned above, the proline and hydroxyproline comprising peptides are unaffected to the action by the digestive proteases. Referring to Table 1, the results of tripeptides with proline at various positions such as VPP, IPP, GPP, GPL, LKP, and YPK give a good IC$_{50}$ for ACE inhibition. Likewise, VSV produced a good IC$_{50}$ of 0.15 µM. This probably suggests that the presence of valine at C-terminal for a tripeptide produces significant ACE activity reduction. To support this statement, Loponen, (2004) demonstrated that the VSP peptide sequence produced higher IC$_{50}$ of 10 µM. Another characteristic of a good ACE inhibitor is the presence of glutamic acid at the C-terminal of the peptide. The good activity of such peptide can be explained by the ability of the peptide to chelate zinc by glutamic acid, which is a segment of the ACE active centre (Iwaniak et al., 2014). For example, the peptide FNVPLYE with a significant IC$_{50}$ value consists of glutamic acid at the C-terminal.

**Kinetic study of the peptides**

In explaining the competitive inhibition of the inhibitors, the C-terminal tripeptide amino acids possibly will interact with the subsites S1, S1’, and S2’ at the active site of ACE. The zinc ion of ACE is correctly positioned between S1 and S1’ to contribute in the hydrolytic cleavage of the substrate peptide bond causing the release of the dipeptide product. ACE active sites S1, S1’ and S2’ have high attractions for the side chains of tryptophan, alanine, and proline, respectively (Byun & Kim, 2002). Therefore, it can be concluded that ACE seems to favour substrates or competitive inhibitors that comprise hydrophobic amino acid residues at the three locations of the C-terminal which will interact with these subsites (Hong et al., 2008). Thus, Tovar-Pérez et al. (2009) recommended that active naturally-occurring ACE inhibitory peptides should comprise proline or aromatic amino acids. They also suggested that the
broad substrates or inhibitor specificity of the enzyme may be due to the two active enzymatic sites in ACE. The somatic ACE comprises two homologous domains, both bearing a functional active site.

To prove the in vivo involvement of each active site in releasing angiotensin II and the inactivation of bradykinin, radiolabeled angiotensin I and bradykinin act as the ACE physiological substrates were deployed in in vitro and in vivo studies to expand understandings into the functional roles of these dual active sites of somatic ACE. In vitro experiments showed that a complete inhibition of the angiotensin I and bradykinin cleavage needs a blockade of the dual ACE active sites. Oppositely, the in vivo studies in mice revealed that the selective inhibition of either the N-domain or the C-domain of ACE by these inhibitors reduced the conversion of angiotensin I to angiotensin II; however, bradykinin protection involves the inhibition of the dual ACE active sites (Georgiadis et al., 2003).

According to Chen et al. (2010), the somatic angiotensin-I-converting enzyme (sACE) isolated from the pig lung had a molecular weight of 180 kDa. Upon the proteolytic cleavage, two fragments with the size of about 90 kDa were attained and identified by amino-terminal sequence analysis and later known as N- and C-domains of sACE where each domain comprises a functional Zn²⁺ binding active centre (Danilov et al., 2014). The germinal ACE which is smaller than sACE composes only a single catalytically active domain alike to the C-domain. To some extent, N- and C-domains of sACE have a different enzymatic activity where the C-domain is better in converting Ang I into Ang II but having similar efficiency in cleaving bradykinin (Michaud et al., 2014).

Some peptides do not have clear amino acid sequences for example GDLGKTTTVSNWSPPKXKDTP from the tuna frame and yet had a significant ACE inhibitory activity with IC₅₀ of 11.28 µM and noncompetitively inhibited ACE (Lee et al., 2010a). It is possible that these peptides are non-competitive inhibitors where they did not bind to the active site of ACE. Several research groups have determined the type of inhibition of their peptides. To our knowledge, the reported ACE inhibitors are either competitive or non-competitive inhibitor based on the Lineweaver-Burk graph plotted (Table 2).

Interestingly, Ni et al., (2012) have shown that the yeast-derived hexapeptide, TPTQQS, non-competitively inhibited ACE by binding N-terminal helices of ACE while its serine residue attracts the zinc residue in the active site, resulting in the conformational change of the active site. Thus, even though substrates can bind to the active site catalysis cannot be carried out.
Table 2: ACE inhibitory peptides and its inhibition mode

| Source                      | Peptide          | Type of inhibitor         | References         |
|-----------------------------|------------------|---------------------------|--------------------|
| Alaska pollack              | FGASTRGA         | Non-competitive           | Je et al., 2004    |
| Algae                       | VECYGPRQPF       | Non-competitive           | Sheih et al., 2009 |
| Bigeye tuna dark muscle     | WPEAAELMEVDP     | Non-competitive           | Qian et al., 2007a |
| Bovine lactoferrin          | LRPVAA           | Non-competitive           | Lee et al., 2006b  |
| Bullfrog muscle protein     | GAAELPCSADWW     | Non-competitive           | Qian et al., 2007b |
| Cuttlefish                  | AHSY             | Non-competitive           | Balti et al., 2010b|
| Dwarf gulper shark          | VW               | Competitive               | Ikeda et al., 2015 |
| Freshwater zooplankton      | AQGERHR          | Competitive               | Lee et al., 2010b  |
| Hen egg white lysozyme      | MKR, RGY, VAW    | Competitive               | Rao et al., 2012   |
| Manchego cheese             | VRYL             | Competitive               | Ruiz et al., 2004  |
| Marine Chlorella ellipsoidea| VEGY             | Competitive               | Ko et al., 2012    |
| Mushroom                    | GEP              | Competitive               | Lee et al., 2004   |
| Mushroom                    | AHEPVK           | Competitive               | Lau et al., 2013   |
| Mushroom                    | RIGLF, PSSNK     | Competitive               | Lau et al., 2014   |
| Oyster                      | VVYPWTQRF        | Non-competitive           | Wang et al., 2008a |
| Porcine                     | VVYPWT LGFTTKTFYFPF | Competitive            | Yu et al., 2006   |
| Rainbow trout muscle        | KVNGPAMSPNAN     | Competitive               | Kim and Byun., 2012|
| Rice                        | VNP, VWP         | Competitive               | Chen et al., 2013  |
| Salmon                      | VWDPPKFD         | Non-competitive           | Ahn et al., 2012   |
| Soybean                     | VLIVP            | Competitive               | Gouda et al., 2006 |
| Spirulina platensis         | IQP              | Non-competitive           | Lu et al., 2010    |
| Tofuyo                      | IFL, WL          | Non-competitive           | Kuba et al., 2003  |
| Tuna frame                  | GDLGKTTTVSNWSPPKXKDT | Non-competitive | Lee et al., 2010a  |
| Turtle egg white            | IVRDPNMGGAW      | Competitive               | Rawendra et al., 2013|
| Wakame                      | VY, VW           | Competitive               | Sato et al., 2002  |
|                            | IY, LW           | Non-competitive           |                   |
|                            | AW, FY, IW       | Un-competitive           |                   |
| Yeast                       | TPTQQS           | Non-competitive           | Ni et al., 2012    |
The ACE inhibitory percentages observed in vitro by a peptide do not always correlate with the in vivo effect when administered in spontaneously hypertensive rats (SHR). There are many factors contributing to the effectiveness of the peptides once they are administrated in vivo. The digestion by gastrointestinal enzymes would be the first factor causing the loss of the peptide availability where the degraded peptides may no longer have a structure suitable to inhibit ACE. Secondly, as mentioned previously, the size of peptides may affect the absorption and thus, their availability. On top of that, in vitro analysis typically only focuses on one inhibition of ACE and yet there are many pathways involved in regulating blood pressure. Once administered into the body, there is a possibility that the peptide may also interact with enzymes other than ACE or that the inhibition of ACE alone would not be sufficient to reduce blood pressure. These activities will lead to the incomparable outcome between the in vivo and in vitro study. The blood pressure lowering effects by the ACE inhibitory peptide on SHR are listed in Table 3.
Table 3: Blood pressure lowering effect by ACE inhibitory peptide in SHR

| Source                        | Peptide          | Dose       | mmHg reduction | Reference                  |
|-------------------------------|------------------|------------|----------------|---------------------------|
| Bigeye tuna dark muscle       | WPEAAELMEVDP     | 10 mg/kg   | ~17 mmHg       | Qian et al., 2007a        |
| Blue mussel                   | EVMAGNLYPG       | 10 mg/kg   | 19 mmHg        | Je et al., 2005           |
| Bullfrog                      | GAELPCSADWW      | 10 mg/kg   | 10 mmHg        | Qian et al., 2007b        |
| Chlorella ellipsoidea         | VEGY             | 10 g/kg    | 22.8 mmHg      | Ko et al., 2012           |
| Corn gluten meal              | AY               | 50 mg/kg   | 9.5 mmHg       | Yang et al., 2007         |
| Dried bonito                  | LKPNM            | 100 µg/kg  | 30 mmHg        | Fujita and Yoshikawa, 1999|
| Dried bonito                  | IKP              | 10 mg/kg   | 70 mmHg (IV route) | Fujita et al., 2000 |
| Egg white                     | RVPSL            | 50 mg/kg   | 24 mmHg (compared to negative control) | Yu et al., 2014 |
| Garlic                        | SY               | 200 mg/kg  | ~50 mmHg       | Suetsuna, 1998            |
|                              | GY               |            | ~52 mmHg       |                           |
|                              | FY               |            | ~32 mmHg       |                           |
|                              | NY               |            | ~35 mmHg       |                           |
|                              | SF               |            | ~39 mmHg       |                           |
|                              | GF               |            | ~25 mmHg       |                           |
|                              | NF               |            | ~54 mmHg       |                           |
| Oyster                        | VVYPWTQRF        | 20 mg/kg   | ~32 mmHg       | Wang et al., 2008a        |
| Porcine myosin B              | KRVIQX           | 10 mg/kg   | 23 mmHg        | Muguruma et al., 2009     |
| Porcine skeletal muscle troponin | KRQKYDI         | 10 mg/kg   | 9.9 mmHg       | Katayama et al., 2008     |
| Rice                          | VNP              | 5 mg/kg    | 29 mmHg        | Chen et al., 2013         |
|                              | VWP              |            | 38 mmHg        |                           |
| Rice                          | TQVY             | 30 mg/kg   | ~40 mmHg       | Li et al., 2007           |
| Sea cucumber                  | MEGAQEAQGD       | 3 µM/kg    | ~17 mmHg       | Zhao et al., 2007         |
| Skimmed milk                  | VPP              | 1.80 mg/kg | ~17 mmHg       | Pan et al., 2005          |
|                              | 3.60 mg/kg       |            | ~25 mmHg       |                           |
| Soybean                       | HHL              | 5 mg/kg    | 32 mmHg (single injection) | Shin et al., 2001 |
|                              |                 |            | 61 mmHg (triple injection) |                   |
|                                   |             |             |             |             |
|-----------------------------------|-------------|-------------|-------------|-------------|
| **Spinach rubisco**               | MRW         | 20 mg/kg    | 20 mmHg     |             |
|                                   | MRWRD       | 30 mg/kg    | 13.5 mmHg   |             |
|                                   | IAYKPG      | 100 mg/kg   | 15 mmHg     |             |
|                                   | LRIPVA      | 100 mg/kg   | 0 mmHg      |             |
| **Tenebrio molitor (L.) larvae**  | YAN         | 400 mg/kg   | 27 mmHg     |             |
| **Tuna frame**                    | GDLGKT'TTVSNWSPPKXKDTP | 10 mg/kg | 21 mmHg     |             |
| **Wakame**                        | AIYK        | 50 mg/kg    | ~38 mmHg    |             |
|                                   | YKYY        |             | ~48 mmHg    |             |
|                                   | KFYG        |             | ~42 mmHg    |             |
|                                   | YNKL        |             | ~52 mmHg    |             |
| **Yellowfin sole**                | MIFPGAGGPEL | 10 mg/kg    | 22 mmHg     |             |

Yang et al., 2003
Dai et al., 2013
Lee et al., 2010a
Suetsuna and Nakano, 2000
Jung et al., 2006
Digestion of ACE inhibitory peptides

In the digestive tract, proteinases and peptidases may degrade or reduce the activity of peptide and thus, it is utmost importance that the peptide needs to be resistant to the enzymes if an intact peptide is required to inhibit ACE. In the stomach, the acidic pH favours pepsin to digest proteins and peptides. Later, the action of pancreatic proteases, trypsin, α-chymotrypsin, elastase, and carboxypeptidases A and B further shorten polypeptides at more alkaline pH. In the in vitro model, the bioavailability of bioactive peptides is commonly confirmed by successive hydrolysis with pepsin and pancreatic enzymes so as to mimic the gastrointestinal condition.

It has been reported that during the simulated gastrointestinal digestion, the peptide inhibitory activity reduces (Norris & FitzGerald, 2013; Jao et al., 2012). After the simulated digestion on a potent ACE inhibitory peptide YLVPQL, a peptide YLVP with low ACE inhibitory activity was produced which resulted in a non-significant antihypertensive effect in SHR (Jao et al., 2012). Casein-derived ACE inhibitory peptide KVLVPQ was cleaved to KVLVPV in the simulated gastrointestinal digestion and produced similar ACE inhibitory activity (Maeno et al., 1996). When these peptides were administered orally to SHR at the dose of 2 mg/kg, a reduction in the systolic blood pressure was recorded at 32.2 and 31.5 mmHg, respectively. Thus, a similar pattern could be observed between the in vitro and in vivo analysis.

Therefore, a prediction on how stable a peptide is against gastrointestinal digestion may be helpful in deducing the availability of peptide sequence after oral administration. In Table 4, with the aid of BIOPEP database, the stability of peptides listed in Table 1 was tested against the gastrointestinal enzymes trypsin, chymotrypsin, and pepsin. The BIOPEP application comprises a database of biologically active peptide sequences and a program facilitating the creation of profiles of the potential biological activity of protein fragments, calculation of quantitative descriptors as measures of the value of proteins as the potential precursors of bioactive peptides, and the prediction of bonds susceptible to the hydrolysis by endopeptidases in a protein chain (Minkiewicz et al., 2008). Out of 75 ACE inhibitory peptide sequences, only 12 were predicted to be stable against the action of these three enzymes. There are many more enzymes present in the gastrointestinal tract and the fate of these 12 peptides are not known although several peptides like IPP and VPP can be absorbed into circulation without being cleaved (Ryan et al., 2011). From Table 4, the reason why several peptides lost their activity in vivo although yielded significant result in vitro or vice versa could be hypothesized. For example, the peptide TQVY from rice produced an IC₅₀ value of 18.2 µM but based on the prediction, pepsin can cleave this sequence and release TQ-VY. From the BIOPEP database, it was suggested that TQ and VY are two dipeptides with ACE inhibitory characteristic. As predicted, when TQVY was administered to SHR, a significant reduction in blood pressure of 40 mmHg was observed (Li et al., 2007). It is possible the reduction of blood pressure was due to these two dipeptides, rather than the intact peptide. In another study, a peptide AVF yielded the IC₅₀ value of 2123 μM and from the IC₅₀ value (Vercruysse et al., 2008), it was predicted that this peptide will not give a significant reduction of elevated blood pressure in vivo. Interestingly, AVF was predicted to be cleaved to A-VF by pepsin. The peptide VF was suggested as the ACE inhibitor by the database. Although the inhibitory activity of the intact peptide was weak in vitro, it is possible that peptide could have a better activity in vivo due to the digestion.
Table 4: Hydrolysis of ACE inhibitor peptides by trypsin, pepsin and chymotrypsin as predicted by BIOPEP database

| Original sequence | Trypsin | Pepsin (pH > 2) | Chymotrypsin (A) |
|-------------------|---------|-----------------|------------------|
| AHEPVK            | AHEPVK  | A - HE - PVK    | AHEPVK           |
| AHSY              | AHSY    | A - HSY         | AHSY             |
| AP                | AP      | A-P             | AP               |
| AY                | AY      | A-Y             | AY               |
| DP                | DP      | DP              | DP               |
| EDPGA             | EDPGA   | E - DPGA        | EDPGA            |
| EVMAGNLYPG        | EVMAGNLYPG | E - VMA - GNL - Y - PG | EVMAGNLYPG |
| EVSQGRP           | EVSQGR - P | E - VSQ - GRP  | EVSQGRP          |
| FNVPLYE           | FNVPLYE | F - NVPL - Y - E | F - NVPL - Y - E |
| FVNPQAGS          | FVNPQAGS | F - VNPQ - A - GS | F - VNPQAGS     |
| FY                | FY      | F-Y             | F-Y              |
| GEP               | GEP     | GE - P          | GEP              |
| GPL               | GPL     | GPL             | GPL              |
| GPP               | GPP     | GPP             | GPP              |
| GPPP              | GPPP    | GPP             | GPPP             |
| HHL               | HHL     | HHL             | HHL              |
| IPP               | IPP     | IPP             | IPP              |
| IW                | IW      | IW              | IW               |
| IVY               | IVY     | IVY             | IVY              |
| KRQKYDI           | KRQ - QK - YDI | KRQ - KY - DI  | KRQKYDI          |
| KVNGPAMSPNAN      | KVNGPAMSPNAN | KVNGPA - MSPNA - N | KVNGPAMSPNAN    |
| LAIPVNKP          | LAIPVNKP | L - A - IPVNKP | L - AIPVNKP     |
| LGFPTTKTYFPHF     | LGFPTTK - TYFPHF | L - GF - PTTKTY - F - PHF | L - GF - PTTKTY - F - PHF |
| LIWK              | LIWK - L | L - IW - KL    | L - IW - KL     |
| LKPNM             | LK - PNM | L - KPNM       | L - KPNM        |
| LKY               | LKY     | LKY             | LKY              |
| LRIPVA            | LR - IPVA | L - RIPVA     | L - RIPVA       |
| LVQGS             | LVQGS   | L - VQ - GS    | L - VQGS        |
| LW                | LW      | L - W           | L - W           |
| doi:10.21010/ajtcam.v14i2.39 |
|-------------------------------|
| LY | L - Y | L - Y |
| LYP | LYP | L - Y - PPP | L - Y - PPP |
| MEGAQAQQGD | MEGAQAQQGD | ME - GA - Q - E - A - Q - GD | MEGAQAQQGD |
| MIFPGAGGPEL | MIFPGAGGPEL | MIF - PGA - GGPE - L | MIF - PGAGGPEL |
| MLLCS | MLLCS | ML - L - CS | ML - L - CS |
| MVGSAPGVL | MVGSAPGVL | MVGSA - PGVL | MVGSAPGVL |
| RVPSL | RVPSL | R - VPSL | RVPSL |
| SPRCR | SPRCR | SPRCR | SPRCR |
| TQVY | TQVY | TQ - VY | TQVY |
| VAP | VAP | VA - P | VAP |
| VAW | VAW | VA - W | VAW |
| VECYGPNRQP | VECYGPNR - PQF | VE - CY - GPNRPQ - F | VECY - GPNRPQF |
| VELYP | VELYP | VE - L - Y - P | VEL - Y - P |
| VLIYV | VLIYV | VL - IVP | VL - IVP |
| VPP | VPP | VPP | VPP |
| VSV | VSV | VSV | VSV |
| VVYWPWQRF | VVYWPWQRF - F | VVY - PW - TQ - RF | VVY - PW - TQRF |
| VW | VW | VW | VW |
| VWP | VWP | VW - P | VW - P |
| VYAP | VYAP | VY - A - P | VY - AP |
| WL | WL | W - L | W - L |
| WPERPPQIP | WPER - PPQIP | W - PE - RPPQ - IP | W - PERPPQIP |
| YAN | YAN | Y - A - N | Y - AN |
| YI | YI | Y - I | Y - I |
| YN | YN | Y - N | Y - N |
| YNKL | YNKL | Y - NKL | Y - NKL |
| YPK | YPK | Y - PK | Y - PK |
| LKP | LKP | L - KP | L - KP |
| FE | FE | F - E | F - E |
| GAAELPCSADWW | GAAELPCSADWW | GA - A - E - L - PCSA - DW - W | GAAEL - PCSADW - W |
| APPPK | APPPK - K | A - APPPK | APPPK |
| IQP | IQP | IQ - P | IQP |
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|                |             | Moderate IC<sub>50</sub> |                |             |
|----------------|-------------|--------------------------|                |             |
|                | FESNFNTQATNR | F - E - SNF - NTQ - A - TNR | F - ESNF - NTQATNR |             |
| AFKDEDTEEVPFR  | AFK - DEDTEEVPFR | A - F - KDE - DTE - E - VPF - R | AF - KDEDTEEVPF - R |             |
| AYLHPGAPKVF    | AYLHPGAPK - VF | A - W - L - HPGA - PKVF | AW - L - HPGAPKVF |             |
| GMNNLTP        | GMNNLTP | GMNNL - TP | GMNNL - TP |             |
| HLFGPPGKKDPV   | HLFGPPGK - K - DPV | HL - F - GPPGKKDPV | HL - F - GPPGKKDPV |             |
| LPGRPPPKPWPL   | LPGR - PPK - PWPL | L - PGRPPPKPW - PL | L - PGRPPPKPW - PL |             |
| LSMGSASLSP     | LSMGSASLSP | L - SMGSA - SL - SP | L - SMGSASL - SP |             |
| VEGY           | VEGY | VE - GY | VEGY |             |

|                |             | High IC<sub>50</sub> |                |             |
|----------------|-------------|------------------------|                |             |
| AVF            | AVF | A-VF | AVF |             |
| ITTPN          | ITTPN | ITTPN | ITTPN |             |
| QFYAV          | QFYAV | Q - F - Y - A - V | QF - Y - AV |             |
| RLPSEFDLSAFLRA | RLPSEFDL - A | RL - PSE - F - DL - SA - F - L - RA | RL - PSEF - DL - SAF - L - RA |             |
| TFPHGYP        | TFPHGYP | TF - PHGP | TF - PHGP |             |
| YAHYSYA        | YAHYSYA | Y - A - HY - SY - A | Y - AHY - SY - A |             |

Peptide sequences in green colour are the peptides which can exert ACE inhibitory activity as predicted by BIOPEP database.
Peptide sequences in red colour are the peptides which can exert ACE inhibitory activity based on its characteristics as predicted by the authors. Some of the sequences in red have been reported earlier.
BIOPEP database does not obey the Keil rule. Generally, Keil rule is defined as trypsin cleaves next to amino acid arginine (R) or lysine (K), but not before proline (P) (Rodriguez et al., 2008).
Due to the complexity of the blood pressure regulation system the in silico digestion should not be taken as a definitive outcome; there were in vivo data which cannot simply be explained by the problem of digestion. For example, YGGY did not produce any reduction in the elevated blood pressure when administered orally to SHR even at the dose of 100 mg/kg despite its IC₅₀ value of 3.4 µM (Saito et al., 1994). Its digested product also has a good inhibitory activity. YGGY can be cleaved by pepsin pH >2 to Y-GGY. The tripeptide, GGY was suggested as an ACE inhibitor by BIOPEP database where this tripeptide was reported to have the IC₅₀ value of 1.3 µM (Shamloo et al., 2015). To a lesser extent, the released amino acid tyrosine can also exert ACE inhibitory activity on its own as shown by Sved et al. (1979). When tyrosine was given intraperitoneally at the dose of 50 mg/kg and 200 mg/kg, about 12 mm Hg and 40 mm Hg reductions were observed, respectively. Thus, other factors may explain the loss of its ACE inhibitory activity (Section 5.4).

Another interesting example is the peptides YPI and RADHP with the IC₅₀ value of more than 1000 µg/mL and 153 µg/mL respectively before and after the digestion. These peptides were derived from YAERYPIL and RADHPFL respectively, where the parent peptides possess the in vitro ACE inhibitory effect. However, these peptides (YPI and RADHP) can significantly reduce the elevated blood pressure in SHR at the dose of 2 mg/kg, even though both peptides are considered as weak inhibitors based on the in vitro studies. The authors concluded that the peptides possibly did not exert the antihypertensive effect via ACE inhibitory mechanism (Miguel et al., 2006).

Absorption of ACE inhibitory peptides

When given orally, peptides should be absorbed in the intestine and induce the effect once they reach the target site. Several proteins or peptide structures are noted to be resistant to the action of gastrointestinal digestion, depending whether they have sequences recognized by the digestive enzymes. Peptides with proline and hydroxyproline residues have been noted being resistant towards hydrolysis. Similarly, modified peptides such as glycopeptides and Maillard reaction products were shown to be resistant towards the GI tract enzyme cleavage (Norris & FitzGerald, 2013).

Peptides must also be transported through the brush border membrane intact and be resistant towards the serum peptidases. By mean of a specific peptide transport system for example peptide transporter PepT1, shorter peptides with two or three amino acids are absorbed undamaged through the brush border membrane (Vermeirssen et al., 2004). Peptide IPP and VPP are two well-documented peptides. They are derived from milk which possesses the ACE inhibitory activity. Using various models, it was found that these two peptides were readily transported through the intestinal epithelium. It has been described that IPP escapes gastrointestinal digestion and can pass in the human circulatory system undamaged (Ryan et al., 2011).

As for longer intact peptides, paracellular and transcellular routes were suggested although debates are still going on regarding the importance of the routes. Via the paracellular route, large water-soluble peptides pass through the tight junctions between cells. On the other hand, highly lipid soluble peptides seem capable of diffusing through the transcellular route. Apart from these, peptides also possibly enter the enterocytes through endocytosis. Biologically active peptides produced in food can be absorbed intact via the intestine and yield biological effects at the tissue level. Yet, the effectiveness of the given peptides drops as the chain-length increases. In the case of infants, the gastrointestinal barrier is not yet fully developed, thus, intact peptides and proteins are much well absorbed in infants compared to adults (Vermeirssen et al., 2004).

An interesting study was carried out by Ryan et al. (2011) to study the peptide resistance towards peptidases and its transportation at the brush boarder where the ACE inhibitory peptide LHPLP from β-casein was used. Due to partial hydrolysis by brush border peptidases, HLPLP was formed from the parental sequence. With a concentration dependent manner, the hydrolysed peptide was promptly absorbed through the Caco-2 cell monolayer. By the in vitro approach, the hydrolysed peptide was also shown to be resistant towards serum proteases and remained intact after one-hour incubation in the human plasma.

Mode of administration in vivo

Other than oral route, peptides can be administered intravenously (IV). Compared to the oral route, injection of the peptide through IV will produce 100% bioavailability. Large peptide easily entered the circulation but comparing the easiness to administer the drug in patients, the oral route is much preferred. The antihypertensive activity of peptide LRPVAA with an IC₅₀ value of 4.14 µM was studied using SHR where the peptide was administered via IV. The most significant reduction of the elevated blood pressure was recorded at the rate of 1 nmol/ml/kg. The reduction of the blood pressure by this peptide was calculated as 210% of captopril (10 pmol/ml/kg) where this drug was used as a positive control (Lee et al., 2006b).

Other blood lowering pathways

Apart from ACE, chymase can convert angiotensin I to angiotensin II. Both ACE and chymase are present in the RAS and kallikrein-kinin system and thus, inhibiting ACE alone may not be sufficient to reduce blood pressure. Thus, even though some peptides are very potent for ACE inhibition, due to the complex system of regulating blood
pressure, their ACE inhibition activity cannot be observed in vivo. According to Iwaniak et al., (2014), they also agreed that sometimes the results of in vitro and in vivo produced by peptides do not correlate with each other possibly due to the regulation of blood pressure by factors other than ACE.

RAS is not the only pathway that regulates blood pressure. It is possible that these peptides inhibit other mechanisms in vivo and cause a significant reduction in blood pressure. Apart from the ACE inhibition, a few other antihypertensive mechanisms induced by a peptide derived from food have been reported as endothelin converting enzyme (ECE) and endothelin 1 (ET-1) release inhibition, arginine-nitric oxide pathway inhibition, renin inhibition, calcium channel blocking and angiotensin receptor blocking (Udenigwe & Mohan, 2014). Thus, this may explain why some peptides with high IC50 value for ACE inhibition in vitro have good blood pressure lowering property in vivo.

Another reason why in vivo analyses have to be carried out is that the pattern of blood pressure lowering could be monitored, which may be unsuitable for patients even if the peptide is very potent. For example, after administered orally in SHR, dipeptide with tyrosine at the C-terminal will produce a slow but prolonged systolic blood pressure reduction. However, when phenylalanine is present in dipeptides at the C-terminal, it resulted in a more rapid reduction and the duration of action becomes shorter (Vermeirssen et al., 2004). Since phenylalanine is hydrophobic and aromatic while tyrosine is aromatic amino acid, the interaction of these peptides to the ACE’s S1, S1’, and S2’ subunits may differ according to the nature of the amino acids.

**Conclusion**

Knowledge of the structure-activity relationship of the ACE-inhibitory peptides is still limited. Apart from the proline residue positions in the peptide sequence, other residues have not been completely studied which lead to the difficulties in predicting the potent sequence. Compared to the N-terminal of the peptide, C-terminal of the peptide amino acids sequence is utmost importance in order for the peptide to bind to the ACE active site. Although the ACE inhibitory activity of a peptide can be predicted from the amino acids residue arrangement, it does not necessarily give a positive outcome, especially when in vivo test is carried out. To retain its activity, it is important for the peptide not to be hydrolysed by gastrointestinal system and readily absorbed into the blood circulation. Since some of the peptides will be hydrolysed by the action of trypsin, pepsin, pancreatin based on the cleavage site in the sequences, their activity in vivo is questionable. Peptide administered through IV route produced good ACE inhibition. However, although this route can provide 100% bioavailability, this is not a practical way of a nutraceutical administration if care is to be taken on the easiness of nutraceutical administration in patients for treatment purposes.

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