Chapter V

L-PHE-D-HIS-L-LEU: A COMPETITIVE INHIBITOR OF ANGIOTENSIN CONVERTING ENZYME
INTRODUCTION

The inhibition of ACE activity has proven to be an effective treatment for the management of hypertension and cardiovascular diseases. The advantages of ACE inhibitors lie in their metabolic and cardioprotective benefits (McMurray, 2008). ACE inhibitors are originally synthesized from the peptides found in B. jararaca venom, which inhibit ACE, a component of the blood pressure-regulating RAS. Most of the commercialized ACE inhibitors have utilized peptides from the venom of B. jararaca, as model substances or prototype of ACE inhibitors (Cushman & Ondetti, 1991). Since the original discovery of ACE inhibitors in snake venom, synthetic ACE inhibitory drugs such as captopril, zofenopril, ramipril, enalapril, quinapril, perindopril, lisinopril, benazepril, imidapril and fosinopril have been developed and are currently in use as antihypertensive drugs (Michaud et al., 1997, Charles et al., 2009).

Despite their beneficial effects of lowering blood pressure, these drugs are associated with side effects including angioedema, dry cough, hyperkalemia, headache, dizziness, fatigue, nausea, renal dysfunction, skin rashes, taste disturbances, hypotension, impotence, allergic reactions, and increased inflammation-related pain (Israili & Hall, 1992; Agustí et al., 2003; Rossi, 2006; Fein, 2009). Therefore, scientists and researchers from different parts of the world are making efforts to develop new ACE inhibitors, either targeting chemical synthesis procedure or isolating from the natural sources, with improved safety and efficacy for the management of high blood pressure and cardiovascular diseases.

Several ACE inhibitory peptides have been isolated from natural resources including functional foods, marine organisms, plants and animals (Murray & FitzGerald, 2007; Hong et al., 2008; De Leo et al., 2009; Wijesekara & Kim, 2010; Wilson et al., 2011). Despite numerous reports regarding the ACE inhibitory peptides from various natural sources, chemical synthesis of ACE inhibitory peptides is an alternative. Using well-developed solid phase synthesis technology, large amounts of peptides can be obtained in a relatively short time (Ren et al., 2011). In this line, lot of efforts have also been made to chemically synthesize ACE inhibitory peptides and their analogues by utilizing the peptides from the functional foods, plant and animal sources (Cohen, 1985; Dive et al., 1999; Byun & Kim, 2002; Kuba et al., 2005;
ACE inhibitory peptides from natural sources generally contain L-amino acids but not D-amino acid(s). However, peptides containing D-peptides can be synthesized chemically in laboratory conditions. The peptides that contain D-amino acid residues can form specific and high-affinity interactions with natural protein targets and strengthen their promise as therapeutic agents (Welch et al., 2007). The peptides containing D-amino acids also have properties that make them attractive as drugs. The peptides containing D-amino acids are less susceptible to be degraded in stomach or inside cells by proteolysis. D-peptide drugs can therefore be taken orally and are effective for a longer period of time. D-peptides are easy to synthesize, when compared to many other drugs. In some cases, D-peptides can have a low immunogenic response (Welch et al., 2007; Welch et al., 2010).

The peptides containing D-amino acids have several theoretical advantages including resistance to proteolytic degradation in the digestive tract leading to substantial increase in serum half-life, and they can bind to targets with unique interface geometries, which are not available for L-peptides. Therefore, peptides containing D-amino acids are promising leads for therapeutic applications in the treatment of hypertension and cardiovascular complications (Pang et al., 2009). Furthermore, peptides containing one or more D-amino acids have also shown to inhibit enzyme activity strongly compared to the regular L-amino acid peptide (York et al., 1989), suggesting the significance of peptides containing D-amino acids as potent enzyme inhibitors.

In the present investigation, a tripeptide containing D-amino acid; L-Phe-D-His-L-Leu has been designed based on the amino acid sequence at C-terminal of Ang I, the physiological substrate of ACE. L-Phe-D-His-L-Leu will be evaluated for its ACE inhibitory activity. Further, the type of enzyme inhibition, biophysical mechanism of interaction will be established. In addition, molecular docking is also carried out in silico in order to establish the potency and usefulness of D-peptide as antihypertensive drug.
RESULTS AND DISCUSSION

ACE, the key regulator of blood pressure has got great attention due to its involvement in chronic elevation of blood pressure. It is well known that hypertension is major risk for cerebral stroke, heart failure, acute myocardial infarction and diabetic neuropathy. Hence, it is critically important to control this dangerous circulatory disease. Since the discovery of an ACE inhibitory peptide in snake venom, several peptides have been identified from different sources. ACE inhibitory peptides are indeed mainly studied due to their beneficial effect on hypertension (Pihlanto et al., 2008).

Till date, a number of food-derived peptides and peptides from snake venom, microorganisms, plants and animals have shown to be antihypertensive through their inhibitory action on ACE (Hong et al., 2008; Kobayashi et al., 2008; De Leo et al., 2009; Wilson et al., 2011). Although the peptides are excellent source of biologically active agents, their importance has long been ignored, due to the lack of oral availability. It is now clear that a significant portion (30%-50%) of dietary nitrogen is absorbed in the form of small peptides (Leibach & Ganapathy, 1996; Kompella & Lee, 2001).

The studies conducted in vivo have shown the significant reduction in blood pressure by ACE inhibitory peptides. However, the peptides had little or no effect on blood pressure of normotensive subjects, suggesting that they exert no acute hypotensive effect (Sekiya et al., 1992; Nurminen et al., 2000). An additional advantage of peptides lies in their safety, since they are not associated with harmful side effects, which are reported for synthetic ACE inhibitors such as dry cough, skin rashes and angioedema (FitzGerald & Meisel, 2000; FitzGerald et al., 2004). Furthermore, the peptides would also signify a low-cost alternative and therefore, ACE inhibitory peptides might be an alternative treatment for hypertension.

In the present investigation, we have used a structure-based approach to design a novel inhibitor of ACE, a tripeptide containing D-amino acid; L-Phe-D-His-L-Leu. Amino acid sequence at C-terminal of Ang I, the site of action of ACE has been considered and modified the stereochemistry of histidine residue in order to check whether the change in stereochemistry of amino acid residue and peptide bond formed between L-phenylalanine and D-histidine is helpful in designing of potent ACE inhibitory peptides. Accordingly, L-Phe-D-His-L-Leu (MW 416.10 Da) was custom-
synthesized from Genemed Synthesis, Inc., (San Antonio, TX 78244, USA). The purity (94.77%) of the peptide was measured by HPLC and its structure was verified by mass spectroscopy (Genemed Synthesis, Inc.). The chemical structures of L-Phe-D-His-L-Leu and L-Phe-L-His-L-Leu, differing in their stereochemistry are depicted in Figure 5.01A and 5.01B respectively.

L-Phe-D-His-L-Leu was dissolved in 50 mM HEPES buffer (pH 8.3) containing 300 mM NaCl and evaluated for its ACE inhibitory activity in vitro. The inhibition of ACE from porcine kidney carried out with L-Phe-D-His-L-Leu exhibited significant ACE inhibitory activity at its maximum concentration tested (250 nM). On the other hand, standard ACE inhibitory drug, lisinopril exhibited a potent ACE inhibition compared to L-Phe-D-His-L-Leu, even at the lowest concentration of 25 nM (Figure 5.02). Although, efficiency of L-Phe-D-His-L-Leu in inhibiting ACE activity was lower when compared to lisinopril, the enzyme inhibition by L-Phe-D-His-L-Leu was found to be promising.

Further, concentration-dependent ACE inhibition was studied using varied concentrations of L-Phe-D-His-L-Leu. Figure 5.03 shows the concentration-dependent enzyme inhibition of L-Phe-D-His-L-Leu with up to 90% decrease in enzyme activity obtained at a concentration of 200 nM. The IC$_{50}$ value of L-Phe-D-His-L-Leu was calculated by Boltzmann’s dose response analysis using Origin 8 software. Lisinopril, a standard ACE inhibitor was used as the positive control for all the inhibitory assays in the study.

L-Phe-D-His-L-Leu exhibited a potent ACE inhibitory activity with significantly lower IC$_{50}$ value of 53.32±0.13 nM ($p$ value: 1.4656) when compared to most of the earlier reports on ACE inhibitory peptides with low IC$_{50}$ values including VAP; 2 μM (Maruyama et al., 1987), VRP; 2.2 μM (Kohmura et al., 1990), LRP; 0.27 μM (Miyoshi et al., 1991), GPV; 1.2 μM (Nakamura et al., 1995), IVY; 0.48 μM, VFPS; 0.46 μM (Matsui et al., 1999), IKW; 0.21 μM, LKP; 0.32 μM (Fujita et al., 2000), YQY; 4 μM (Li et al., 2002), DLP; 4.8 μM (Wu & Ding, 2002), TVY; 15 μM (Tauzin et al., 2002), MRW; 0.6 μM (Yang et al., 2003), VPP; 9 μM, IPP; 5 μM (FitzGerald et al., 2004) and LRW; 0.15 μM (Wu et al., 2006a).

However, L-Phe-D-His-L-Leu was found to be less potent when compared to lisinopril and captopril (IC$_{50}$ of 4 and 41 nM respectively), the synthetic ACE inhibitory drugs (Femia et al., 2008; Megias et al., 2004). Further, to understand the
detailed mechanism of inhibition of ACE by L-Phe-D-His-L-Leu, we examined its inhibition by varying the substrate with indicated inhibitor concentrations. With results obtained from this experiment, the mechanism underlying inhibition at kinetic level was determined, by plotting Lineweaver-Burk (LB) plot for ACE with or without L-Phe-D-His-L-Leu (40 nM and 80 nM concentrations). Thus obtained LB plot with an intersection on the 1/v axis indicated that L-Phe-D-His-L-Leu is competitive inhibitor of ACE (Figure 5.04), which is similar to that of competitive ACE inhibitor, lisinopril (Rimar & Gillis, 1992).

Similar enzyme inhibitory kinetic studies carried out by other researchers have also shown a number of peptides that are exhibiting competitive inhibition of ACE. Some of the examples of competitive ACE inhibitory peptides include LRW (Wu et al., 2006a), VLIVP (Gouda et al., 2006), FCVLRP, IFVPAF and KPPETV (Hai-Lun et al., 2006), KRQKYDI (Katayama et al., 2008), DDTGHDFTGEAM (Lee et al., 2009), IKP (Jimsheena & Gowda, 2010), AQGERHR (Lee et al., 2010), IPP, VPP and LPP (Lehtinen et al., 2010).

Moreover, it has been reported that most of ACE inhibitory peptides are relatively short sequences with low molecular mass. This is in agreement with the results of crystallography studies, which demonstrated that the active site of ACE cannot accommodate larger peptide molecules (Natesh et al., 2003). The decrease in ACE inhibitory activity was shown by elongating a potent peptide at the N-terminus, i.e., VRP (IC<sub>50</sub>: 2.20 μM), VVRP (IC<sub>50</sub>: 81.00 μM) and AVVRP (IC<sub>50</sub>: 74.00 μM) (Kohmura et al., 1990). Further, the binding of inhibitor to ACE is strongly influenced by its C-terminal tripeptide sequence. ACE prefers inhibitor containing hydrophobic (aromatic or branched side chains) amino acid residues at the C-terminal positions (Vermeirssen et al., 2004). In majority of the cases, it appears that tripeptides are more potent than dipeptides as ACE inhibitors, i.e., VP (IC<sub>50</sub>: 570.00 μM) and VPP (IC<sub>50</sub>: 9.00 μM) (Ichimura et al., 2003; Nakamura et al., 1996).

Furthermore, many naturally occurring ACE inhibitory peptides contain Tyr, Phe, Trp, Pro or Lys at C-terminal end especially the dipeptide and tripeptide inhibitors. Furthermore, Gómez-Ruiz et al., (2004) have suggested that a C-terminal leucine residue may contribute significantly to increasing ACE inhibitory potential. Further, it has also been demonstrated that ACE has a requirement for L-configuration of the amino acid at position three from C-terminal. This was shown clearly by...
Maruyama et al., (1987) where D-VAP had an IC$_{50}$ of 550.00 μM while peptides VAP and D-FVAP exhibited IC$_{50}$ values of 2.00 and 17.00 μM respectively.

However, there are no reports on ACE inhibitory tripeptides containing D-amino acid. Herein, for the first time, we are reporting a potent ACE inhibitory tripeptide containing D-Histidine, with all the necessary conditions that are required to become an effective ACE inhibitory drug, as described above. The rationale behind designing this peptide was to change stereochemistry of the peptide bond, so that it will not be hydrolyzed by ACE and thereby exerting better antihypertensive effect. This kind of approach may be valuable in future, to develop new antihypertensive drugs with better efficacy and safety for the treatment of hypertension.

The studies of inhibitor-enzyme interactions are often supported by fluorimetric interaction studies. Many inhibitors of enzymes bring about changes in intensity of the spectra or the characteristic native fluorescence of enzyme (Nataraju et al., 2007). The change in intrinsic fluorescence reflects a conformational change in the enzyme due to substrate or ligand interaction (Nataraju et al., 2007). Similarly, ACE inhibitors like lisinopril and captopril have been shown to result in characteristic quenching in absorbance of ACE in circular dichroism studies (Jones & Clarke, 2004).

Therefore, in order to study the direct interaction of L-Phe-D-His-L-Leu with ACE, fluorescence studies were carried out with purified ACE using fluorescence spectroscopy. The interaction study of L-Phe-D-His-L-Leu with ACE depicted in this chapter indicated quenching of enzyme fluorescence upon increased concentration of inhibitor. The characteristic fluorescence absorption spectra of native enzyme and enhanced relative fluorescence of ACE by L-Phe-D-His-L-Leu in a concentration-dependent manner have been shown in Figure 5.05. As evident from the enhancement of relative fluorescence of ACE with increased concentrations of L-Phe-D-His-L-Leu, it can be concluded that inhibition by L-Phe-D-His-L-Leu is due to direct interaction with the ACE.

To substantiate the fluorescence data, interaction of L-Phe-D-His-L-Leu with ACE was determined using circular dichroism (CD). It is known that, interaction of some linear or aromatic molecules with the amino acid residues of a protein induce large chirality changes and consequent significant changes in the CD spectra of native protein. The appearance of CD spectrum reflects the binding geometry and binding
mode of ligands as well as changes in the secondary and tertiary arrangement of protein molecule upon interacting with ligand (Nataraju et al., 2007). In the present study, the far UV-CD spectrum of ACE in the reaction mixture without inhibitor showed a prominent single large negative band, which had maximum absorbance at 206 and 225 nm. The negative band of absorbance was diminished and the negative peak height was reduced compared to the native protein band in the presence of IC$_{50}$ concentration of L-Phe-D-His-L-Leu (Figure 5.06). There was no significant shifting and broadening of the protein band upon interaction with ligand, L-Phe-D-His-L-Leu. This observation substantiated the fluorescence data that the L-Phe-D-His-L-Leu interacts directly with enzyme. However, the secondary structure of ACE did not change significantly, which is in parallel with CD spectral analysis results of lisinopril and captopril with ACE (Jones & Clarke, 2004). The change in percentage of secondary structure of ACE upon interaction with L-Phe-D-His-L-Leu is summarized in Table 5.01.

Further, in silico molecular modeling studies were performed to suggest putative binding sites for the interaction between ligands (L-Phe-D-His-L-Leu, lisinopril) and crystal structure of human ACE (PDB: 108A). Molecular modeling was carried out using molecular docking platform Molegro Virtual Docker based on MolDock molecular docking algorithm (Thomsen & Christensen, 2006). Both L-Phe-D-His-L-Leu and lisinopril directly interacted with ACE by forming hydrogen bonds with amino acid residues resided at active site of ACE having a distance range within 3.5 Å (Table 5.02). The electrostatic molecular surface of ACE showed that both L-Phe-D-His-L-Leu and lisinopril are deeply buried into the catalytic site. L-Phe-D-His-L-Leu formed hydrogen bonds with Tyr$^{520}$, Tyr$^{523}$, Asp$^{415}$ and Gln$^{281}$ while lisinopril formed hydrogen bonds with Glu$^{384}$, Tyr$^{523}$ and Glu$^{162}$. The interaction of L-Phe-D-His-L-Leu and lisinopril with ACE is represented in Figure 5.07 and 5.08. L-Phe-D-His-L-Leu when compared to lisinopril, displayed high negative values of MolDock score (energy score), Rerank score and binding energy, indicating its higher binding affinity and interactions with ACE (Table 5.03).

Although, there are few limitations, in silico approach can be used in combination with experimental evidence to supplement our understanding of complex and dynamic molecular interactions between ligand and ligate. The docking simulations were used to analyze the interactions between ACE and L-Phe-D-His-L-
Leu as well as ACE and lisinopril, in order to explore the structural factors of peptide that might contribute significantly to their binding in response to ACE inhibition. The data analysis revealed that L-Phe-D-His-L-Leu exhibited slightly higher affinity towards ACE, when compared to lisinopril. The molecular docking study of L-Phe-D-His-L-Leu with ACE substantiated the results obtained from *in vitro* ACE inhibitory studies and suggested L-Phe-D-His-L-Leu as a potent ACE inhibitor.

In conclusion, L-Phe-D-His-L-Leu exhibited concentration-dependent ACE inhibitory activity with lower IC$_{50}$ value of 53.32±0.13 nM. It was found to be competitive inhibitor of ACE, as revealed by LB plot and further, the docking results indicated the slightly higher binding affinity of L-Phe-D-His-L-Leu towards ACE with higher negative energy score and binding energy when compared to lisinopril. Thus, the strategy of incorporation of D-amino acids in ACE inhibitory peptides could be used for the development of potent drug molecules for the management of hypertension.
FIGURES

**Figure 5.01A:** Chemical structure of L-Phe-D-His-L-Leu

**Figure 5.01B:** Chemical structure of L-Phe-L-His-L-Leu
Figure 5.02: Comparative ACE inhibitory activities of L-Phe-D-His-L-Leu and lisinopril: Porcine kidney ACE (5 mU) was pre-incubated with different concentrations of L-Phe-D-His-L-Leu and lisinopril for 10 min. The substrate (12.5 mM HHL) was added and incubated for 30 min at 37°C after adjusting the reaction volume to 125 μL with 50 mM HEPES buffer, pH 8.3 containing 300 mM NaCl. The absorbance of hippuric acid released by the action of ACE was measured at 228 nm. The decreased concentration of hippuric acid in the test reaction compared to control and enzyme activity reaction was expressed as percentage of inhibition. Data represents the mean ± SD (n=3).

Figure 5.03: Concentration-dependent ACE inhibitory activity of L-Phe-D-His-L-Leu: Porcine kidney ACE (5 mU) was pre-incubated with different concentrations of L-Phe-D-His-L-Leu for 10 min. The substrate (12.5 mM HHL) was added and incubated for 30 min at 37°C after adjusting the reaction volume to 125 μL with 50 mM HEPES buffer, pH 8.3 containing 300 mM NaCl. The absorbance of hippuric acid released by the action of ACE was measured at 228 nm. The decreased concentration of hippuric acid in the test reaction compared to control and enzyme activity reaction was expressed as percentage of inhibition. Data represents the mean ± SD (n=3).
Figure 5.04: Lineweaver-Burk plot of porcine kidney ACE inhibition by L-Phe-D-His-L-Leu: Porcine kidney ACE (5 mU) was pre-incubated with 40 and 80 nM L-Phe-D-His-L-Leu for 10 min. The substrate (0.5-5 mM HHL) was added and incubated for 30 min at 37°C after adjusting the reaction volume to 125 μL with 50 mM HEPES buffer, pH 8.3 containing 300 mM NaCl. The absorbance of hippuric acid released by the action of ACE was measured at 228 nm. The decreased concentration of hippuric acid in the test reaction compared to control and enzyme activity reaction was used to calculate the extent of inhibition. The initial reaction velocities were calculated from hippuric acid released.

Figure 5.05: Intrinsic fluorescence spectra of porcine kidney ACE with L-Phe-D-His-L-Leu: The reaction mixture contained 25 μg of porcine kidney ACE in 10 mM potassium phosphate buffer, (pH 7.4), with increasing concentrations of L-Phe-D-His-L-Leu (from bottom to top direction). Fluorescence spectra were recorded after excitation at 280 nm. Emission spectral intensity was recorded from 300 to 400 nm. Brown-ACE alone; pink-ACE+10 nM; blue-ACE+25 nM; red-ACE+50 nM; green-ACE+100 nM and black-ACE+250 nM.
Figure 5.06: Far-UV-Circular dichroism spectra of ACE with L-Phe-D-His-L-Leu: Circular dichroism spectra were recorded for porcine kidney ACE in absence and presence of IC\textsubscript{50} concentration of L-Phe-D-His-L-Leu between 200 and 260 nm on Jasco J715 spectropolarimeter. The samples were dissolved in 10 mM potassium phosphate buffer, (pH 7.4). Quartz cuvette with path length of 2 mm was used. Measurements were made with bandwidth of 1 nm and response time of 2 s.
Figure 5.07: In silico molecular docking of L-Phe-D-His-L-Leu with crystal structure of human ACE (PDB: 108A): (A) and (B) images are showing interactions between L-Phe-D-His-L-Leu and active site amino acid residues with polypeptide backbone of ACE. The interactions between L-Phe-D-His-L-Leu and functional groups of active site amino acid residues with putative H-bonding (green dashed lines) are displayed. L-Phe-D-His-L-Leu is represented in purple coloured stick model. The amino acids within the distance of 3.5 Å are represented in ball and stick model in different color, zinc and chlorine in red and green ball color respectively. (C) Electrostatic molecular surface of ACE showing that L-Phe-D-His-L-Leu deeply buried into the catalytic site depicted. The images were created using Molegro molecular docker (version 2008.3.0.0).
**Figure 5.08:** *In silico* molecular docking of lisinopril with crystal structure of human ACE (PDB: 108A): (A) and (B) images are showing interactions between lisinopril and active site amino acid residues with polypeptide backbone of ACE. The interactions between lisinopril and functional groups of active site amino acid residues with putative H-bonding (green dashed lines) are displayed. Lisinopril is represented in purple coloured stick model. The amino acids within the distance of 3.5 Å are represented in ball and stick model in different color, zinc and chlorine in red and green ball color respectively. (C) Electrostatic molecular surface of ACE showing that lisinopril deeply buried into the catalytic site depicted. The images were created using Molegro molecular docker (version 2008.3.0.0).
## Chapter V

*L-Phe-D-His-L-Leu*........

### TABLES

**Table 5.01:** Effect of L-Phe-D-His-L-Leu and lisinopril on secondary structure of porcine kidney ACE

<table>
<thead>
<tr>
<th></th>
<th>ACE alone</th>
<th>ACE + L-Phe-D-His-L-Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-helix</td>
<td>40.18%</td>
<td>44.4%</td>
</tr>
<tr>
<td>β-sheet</td>
<td>10.65%</td>
<td>13.51%</td>
</tr>
<tr>
<td>Others</td>
<td>49.17%</td>
<td>42.09%</td>
</tr>
</tbody>
</table>

Note: Secondary structure contents were calculated using K2D2 software.

**Table 5.02:** Putative binding amino acids identified from crystal structure of human ACE (PDB code: 108A) interface with L-Phe-D-His-L-Leu and lisinopril

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Amino acid residues interacted with ligand (within distance of 3.5Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Phe-D-His-L-Leu</td>
<td>Tyr&lt;sup&gt;520&lt;/sup&gt;, Tyr&lt;sup&gt;523&lt;/sup&gt;, Asp&lt;sup&gt;415&lt;/sup&gt;, Gln&lt;sup&gt;281&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>Glu&lt;sup&gt;583&lt;/sup&gt;, Tyr&lt;sup&gt;523&lt;/sup&gt;, Glu&lt;sup&gt;162&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 5.03:** Docking results of L-Phe-D-His-L-Leu and lisinopril in complex with crystal structure of human ACE (PDB code: 108A)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>MolDock score</th>
<th>Rerank Score</th>
<th>Binding energy (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Phe-D-His-L-Leu</td>
<td>-160.128</td>
<td>-120.867</td>
<td>-17.8517</td>
</tr>
<tr>
<td>Lisinopril</td>
<td>-140.224</td>
<td>-103.377</td>
<td>-13.2400</td>
</tr>
</tbody>
</table>