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ORIGINAL ARTICLE

Isolation and Characterization of a Potential Angiotensin-Converting Enzyme Inhibitory Peptide from the Leaves of *Leptadenia hastata* (Asclepiadaceae)

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Abstract

This paper describes the isolation, purification and characterization of a potential Angiotensin-Converting Enzyme (ACE) inhibitory peptide from the leaves of *Leptadenia hastata* (Asclepiadaceae). The leaves were collected from Kano state, Nigeria. Crude proteins were extracted from the leaves using a protein extraction kit. The proteins were purified by a three-step method: cold acetone precipitation, gel filtration using chromatography sephadex G-100 and ion exchange chromatography using CM-sephadex. The ACE inhibitory activity, protein content, effect of pH, temperature and digestive enzymes on the activity of the isolated and purified peptide were determined. In addition, the inhibition pattern, amino acid composition and sequence of the purified peptide were investigated. The specific inhibitory activity of the peptide increased from 0.0018 to 0.0085 U.mg-1 at a purification fold of 4.72 and yield of 9.57%. The optimum temperature and pH of the peptide ACE inhibitory activity was found to be 40ºC and 7.0 respectively. The digestive enzymes, pepsin and trypsin significantly ($P<0.05$) reduced the activity of the peptide compared to antihypertensive drug enalapril. The amino acid composition of the peptide was found to be aspartate (Asp), glutamate (Glu), glycine (Gly), valine (Val), leucine (Leu) and phenylalanine (Phe). The purified peptide showed a mixed pattern type of ACE inhibition. In conclusion, the results of this work suggest that *Leptadenia hastata* (Asclepiadaceae) leaves could be a potential source of peptides with high ACE inhibitory activities.

Keywords: *Leptadenia hastata* (Asclepiadaceae); angiotensin converting enzyme; hypertension; peptide.

Introduction

Angiotensin converting enzyme (ACE, dipeptidyl carboxypeptidase I, kinase II, EC. 3.4.15.1) is a multifunctional zinc metallopeptidase found in various tissues (Ondetti and Cushman, 1982). ACE has four functional amino acid residues (tyrosine, Tyr; arginine, Arg; glutamate, Glu and lysine, Lys) at the active site, and three hydrophobic binding sub-sites. It plays an important role in the regulation of blood pressure via the renin-angiotensin system (RAS) and kallikrein-kinin system (KKS) (Li et al., 2004). RAS plays crucial role in the blood pressure

regulation and in the pathophysiology of cardiovascular diseases. Within the RAS, angiotensin is converted by plasma renin into angiotensin I (decapeptide), which undergoes proteolytic cleavage in the presence of ACE, to form angiotensin II (octapeptide), a potent vasoconstrictor. Angiotensin II raises blood pressure by acting directly on blood vessels, sympathetic nerves and adrenal glands (Matsui et al., 2000). In addition, ACE degrades bradykinin, which has vasodilatation properties in KKS (Li et al., 2004). Thus, to prevent the pathogenesis of hypertension, production of angiotensin II has to be suppressed via inhibition of ACE activity. Several ACE inhibitors, such as captopril, enalapril, and lisinopril used as antihypertensive drugs in clinical practice possess adverse effects (Vercruysse and Smagghe, 2005). Hence, the need for search and development of safe and natural ACE inhibitors from dietary sources.

Adequate evidence from epidemiological and laboratory studies link several nutrients, minerals, food groups and dietary patterns with a decreased risk of hypertension (Reddy and Katan, 2004). In recent years, food-derived ACE inhibitory peptides have been extensively studied as an alternative for prevention or management of high blood pressure. The occurrence of ACE inhibitors in maize, sunflower, wheat germ, mushrooms, and lentils has been reported. Also, food protein sources such as soybean, egg, milk and fish have been found to be rich sources of ACE inhibitory peptides (Chia-Ling et al., 2012). Indigenous plants, such as Moringa oleifera also contain ACE inhibitory peptides (Abdulazeez et al., 2015a).

Leptadenia hastata belongs to the Asclepiadaceae family. It is wild, edible non-domesticated vegetable found throughout Africa. It is used as food and for the treatment of various ailments (Aliero et al., 2001; Barbana and Boye, 2011). The plant is commonly used in the Hausa speaking communities in Nigeria as a spice (Ibrahim et al., 2012), for the treatment of hypertension, catarrh and skin diseases (Dambatta and Aliyu, 2011). In Senegal, the leaves are used for to improve lactation and as a purgative (Arbonnier, 2000; Kerarho and Adam, 1974). The antimicrobial effects of *L. hastata* have been reported (Aliero et al., 2001) and the result of its toxicity studies showed that the plant is relatively safe (Tamboura et al., 2005). However, there is paucity of information on its ACE inhibitory activity, hence this study aims to isolate and characterize ACE inhibitory peptide from *L. hastata*, determine the amino acid composition, and investigate its inhibition pattern.

Materials and Methods

Chemicals and Reagents

Angiotensin Converting Enzyme Inhibition Screening Kit (Kamiya Biomedical Company, Seattle, U.S.A), Pepsin (porcine stomach mucosa) and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), sodium chloride, sodium monophosphate, sodium diphosphate, hydrochloric acid, sodium hydroxide, acetone, Bradford reagent, Bovine Serum Albumin, potassium diphosphate and all other reagents used in the study are of analytical grade.

Collection and Identification of Plant Sample

Fresh leaves of L. hastate (Asclepiadaceae) were collected from Rijiyar Zaki, Gwale Local Government Area, Kano state, Nigeria. The plant was authenticated at the herbarium of the Department of Botany, Bayero University, Kano with a voucher number BUKHAN 0248.

Purification of ACE Inhibitory Proteins from Leptadenia hastata

Isolation of Crude Protein

Fresh leaves of *Leptadenia hastata* were washed under running tap water until clean and crude protein was isolated using protein extraction kit (Minute TM Total Protein Extraction Kit for Plant Tissues, Invent Biotechnologies, Inc., USA). Briefly, 50-100 mg fresh leaves of *L. hastata* was placed in the protein extraction filter cartridge that had been pre-chilled in collection tube on ice, by folding and rolling into smaller volume. About 50 - 100 µL buffer was added to the filter and the leaves ground using a plastic rod for 3 minutes with twisting force. The filter was capped and incubated at room temperature for 1 - 2 minutes, then centrifuged at 3000 rpm for 5 minutes. The protein extract was collected as supernatant into a fresh test tube. This process was repeated using new protein extraction cartridges to obtain enough protein extract.

Protein Precipitation

Protein extracted (supernatants) obtained from leaves of *L. hastata* were precipitated using cold acetone at a ratio of 1:4. The precipitated proteins were vortexed and incubated at -20°C for 60 minutes and then centrifuged for 10 minutes at 4°C and 3000 rpm. Acetone was allowed to evaporate from the uncapped test-tube. The pellet was reconstituted in 5 mL phosphate buffer (pH 7.4) to determine the ACE inhibitory activity and protein content.

Dialysis

A 10,000 Da membrane was prepared and loaded with 50 mL of the extracted protein, placed in a container containing 200 ml of phosphate buffer (pH 8.4), and dialyzed at 4ºC for 2 hours. The buffer was changed and volume increased to 500 mL. This was left to dialyze for another 28 hours.

Gel Filtration Using Sephadex G-100

The precipitated protein was dissolved in phosphate buffer and subjected to gel filtration chromatography. The gel was soaked overnight in phosphate buffer (pH 7.8). The column was packed with the gel and washed with the same buffer. The protein was poured into the column and allowed to elute at a flow rate of 0.5 mL.min-1. Twelve (12) fractions of the sample were collected, 5 mL each. The protein content and ACE inhibitory activity of each fraction was determined and fractions that showed high ACE inhibitory activity were further purified by ion exchange chromatography.

Ion Exchange Chromatography

The fractions with strong ACE inhibitory activities obtained after gel filtration was pulled together and loaded onto a CM-Sephadex column which was pre-equilibrated with phosphate buffer solution (pH 7.8), then eluted at a linear gradient of NaCl (0.1 - 0.5 M) in the same buffer. Protein content and ACE inhibitory activity of the eluents were assayed.

Determination of ACE Inhibitory Activity

This was done using the K - Assay ACE inhibition screening kit, Kamiya Biomedical Company, USA, which utilizes 3-hydroxybutyl gly-gly-gly (3HB-GGG) as a substrate for ACE, and the amount of cleaved 3-hydroxybutyric acid (3HB) from 3HB-GGG measured by an enzymatic method. Briefly, 20 µL each of the samples, substrate and enzyme solution was added to a sample well, while 20 µL each of deionized water, substrate and enzyme solution added to another well (blank 1). For blank 2, 40 µL of deionized water and 20 µL of substrate

were added. The plate was incubated at 37ºC for 60 minutes, before adding 200 µL of indicator working solution to each well. This was further incubated for 10 minutes. The absorbance was read at 450 nm with a microplate reader.

$$\text{ACE inhibitory activity (inhibition rate \%)} = \frac{\left(A_{blank1} - A_{sample}\right) \times 100}{\left(A_{blank1} - A_{blank2}\right)}$$

Inhibition was expressed as the concentration of inhibitor that inhibits 50% of ACE activity (IC_{50}), and was calculated using a non-linear regression from a plot of activity versus inhibitory concentration of at least five separate determinations. Each assay was performed in triplicate.

Determination of Protein Content

The protein concentration of all fractions collected was determined by Bradford method using Serum Bovine Albumin (BSA) as standard and the concentration was expressed in milligram per milliliter.

Characterization of ACE Inhibitory Peptide from Leaves of L. hastata

Effect of Temperature on ACE Inhibitory Activity

The extracted peptide was dissolved in milli - Q water and subsequently incubated with ACE. The effect of temperature on the activity of the purified peptide was analyzed after incubation at 20, 30, 40, 50, 60 and 70°C for 1 h.

Effect of pH on ACE Inhibitory Activity

The effect of pH on the peptide was determined by varying the pH of reaction mixture from 3 to 10, and the ACE inhibitory activity determined at the different pH.

Effect of some Metal Ions on ACE Inhibitory Activity

ACE inhibitory activity was determined (method described in the previous section on Determination of ACE Inhibitory Activity) in the presence of the monovalent and divalent metal ions in their chloride forms; Sodium, Potassium, Magnesium, Calcium as well as EDTA; each at a concentration of 2 mM.

Effect of Digestive Enzymes on ACE Inhibitory Activity

The stability of ACE inhibitory peptide against some digestive enzymes was assessed *in vitro*. The purified peptide solution was successively digested with pepsin and trypsin as previously described and the digests were performed for the determination of ACE inhibitory activity.

Determination of the Inhibition Pattern of the Peptide.

The substrate (3HB-GGG) at various concentrations (0 to 5 mM) was incubated with the ACE solution in the absence and presence of 0.5, 1.0 mg/mL of inhibitor solution (purified peptides) at 37°C, and each reaction mixture assayed for ACE inhibition. The kinetic constants (K_m and V_{max}) values for the reaction at different concentrations of purified peptides were calculated using the Line weaver-Burk plots.

Determination of Amino Acid Composition

The amino acid profile of the peptide was determined using methods described by Benitez (1989). The sample was dried to constant weight, hydrolyzed and evaporated in a rotary evaporator before loading into the Technicon sequential Multi-Sample Amino Acid Analyzer (TSM), Technicon Industrial Systems, USA.

Results

Purification of Leaves of L. hastata

Table 1. summarises the purification steps used in the isolation of proteins from the leaves of *L. hastata*. The results showed that both the crude proteins obtained from the leaves and the purified peptide after acetone precipitation, gel filtration and ion exchange chromatography possess ACE inhibitory activity. The total protein content obtained decreased from 52.86 mg of crude to 1.05 mg after ion exchange chromatography. The inhibitory activity of the crude protein was estimated to be 0.0940 U with a specific inhibitory activity of 0.0018 U.mg-1. After undergoing acetone precipitation, gel filtration and ion exchange chromatography, the specific inhibitory activity increased to 0.0085 U.mg-1 at a purification fold of 4.72 and a percentage yield of 9.57 (Table 1).

Table 1. Purification table of the purified ACE inhibitory peptide from *L. hastata* leaves

Purification step	Protein content (mg/mL)	Total protein (mg)	Inhibitory activity (µmol/min)	Specific inhibitory activity (µmol/min/mg)	Purification fold	Yield %
Crude	1.762	52.86	0.0940	0.0018	1.00	100
Acetone Precipitation	1.532	22.98	0.0521	0.0023	1.260	55.43
Gel filtration	1.394	6.97	0.0245	0.0035	1.953	26.06
Ion exchange Chromatography	0.175	1.05	0.0090	0.0085	4.722	9.57

The percentage ACE inhibition (Fig. 1) increased from fraction 1 to 3, decreased sharply from fraction 3 to 4 and then increased from fraction 4 to 5. The percentage ACE inhibition of the proteins decreased gradually from fraction 9 to 12. Fractions 3, 5, and 9 having higher ACE inhibitory activity and a proportionate protein concentration were then pulled together for ion exchange chromatography. Each fraction from ion exchange chromatography was collected for each gradient (0.1 to 0.5M NaCl) eluted (Fig. 2). Fraction 1 (0.1M NaCl) having the highest ACE inhibitory activity of 78% was then characterized.

Effect of pH and Temperature on Purified Peptide from L. hastata Leaves

The inhibitory activity of the purified peptide on ACE was optimal at pH 7.0, with the activity decreasing as the pH tends to 10 (Fig. 3), while the activity increase with increase in temperature until a peak was obtained at 40^{o}C, after which further increase in temperature caused a decrease in inhibitory activity (Fig. 4).

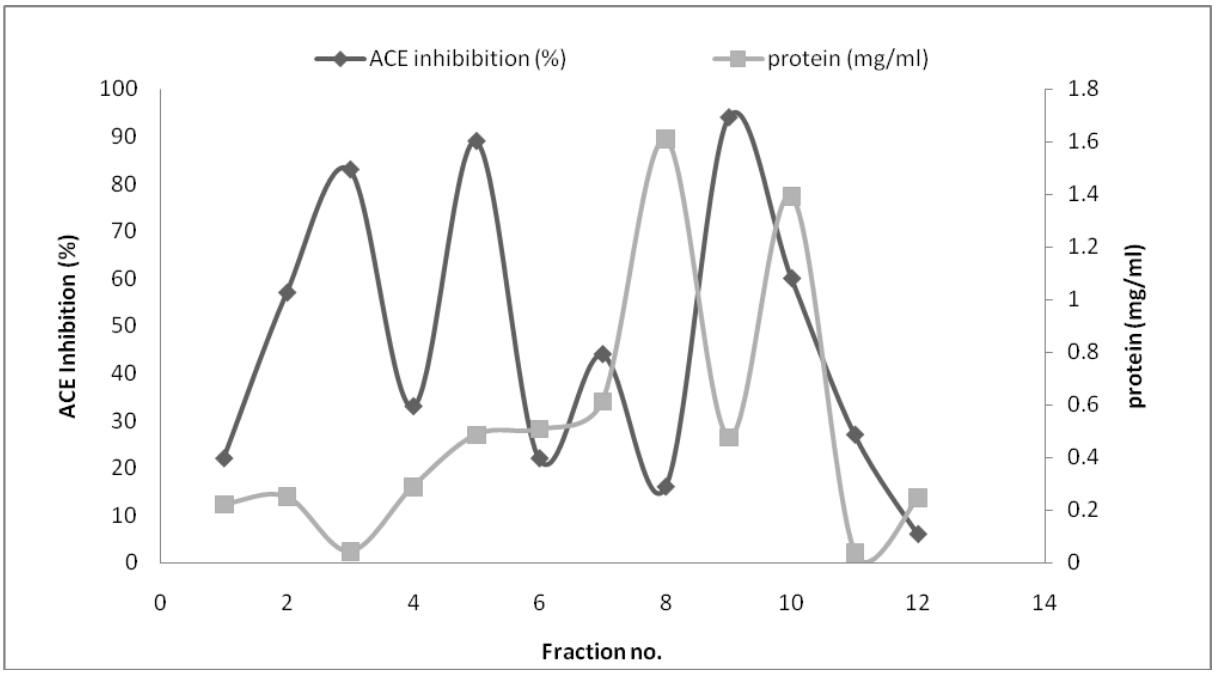

Figure 1. Elution profile of the purified ACE inhibitory peptide from sephadex G-100 gel filtration chromatography

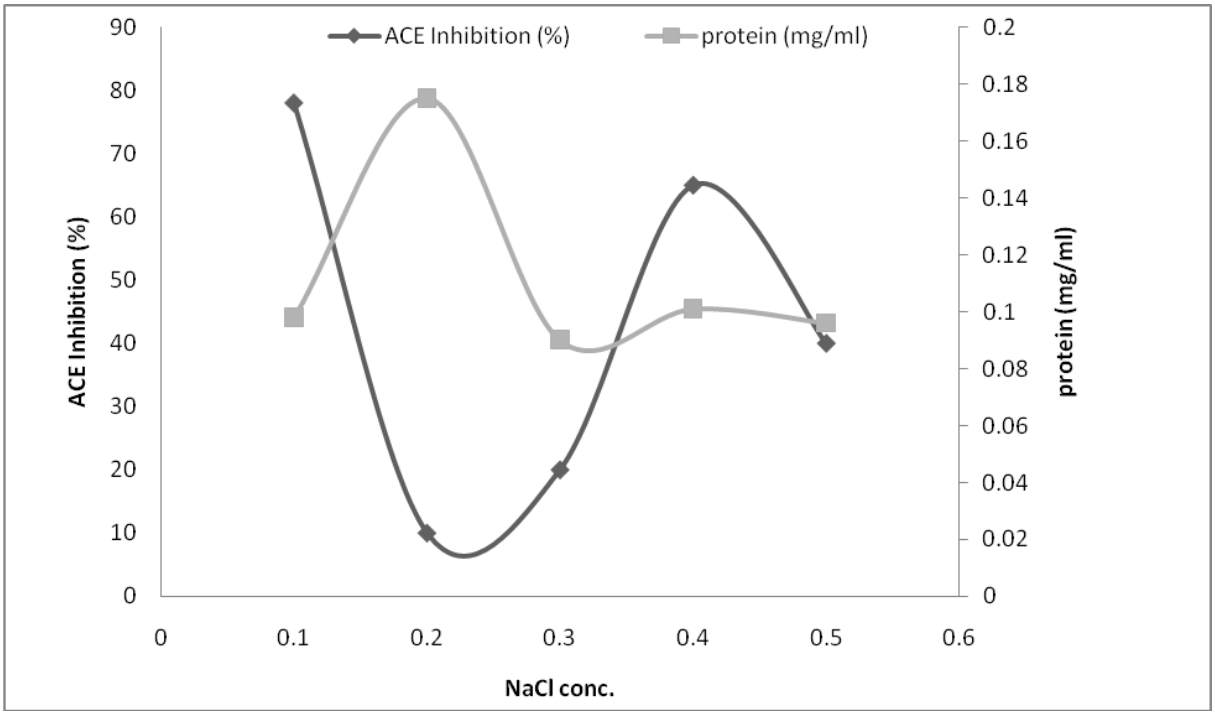

Figure 2. Elution profile of the purified ACE inhibitory peptide after ion exchange chromatography using CM-sephadex

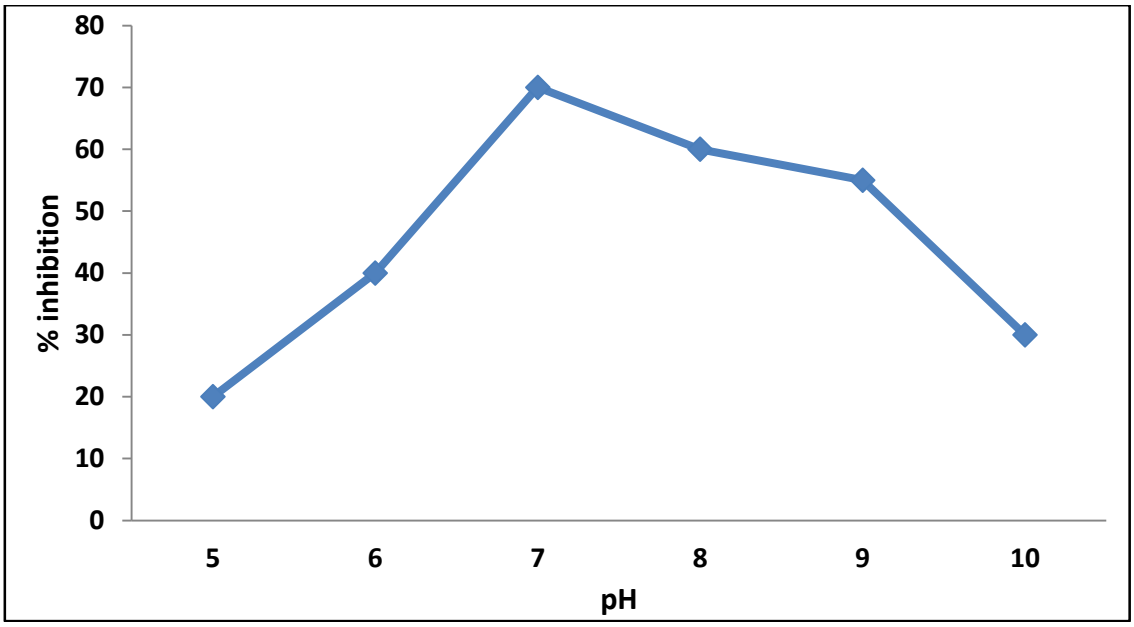

Figure 3. Effect of pH on the activity of purified peptide obtained from the leaves of *L hastata*

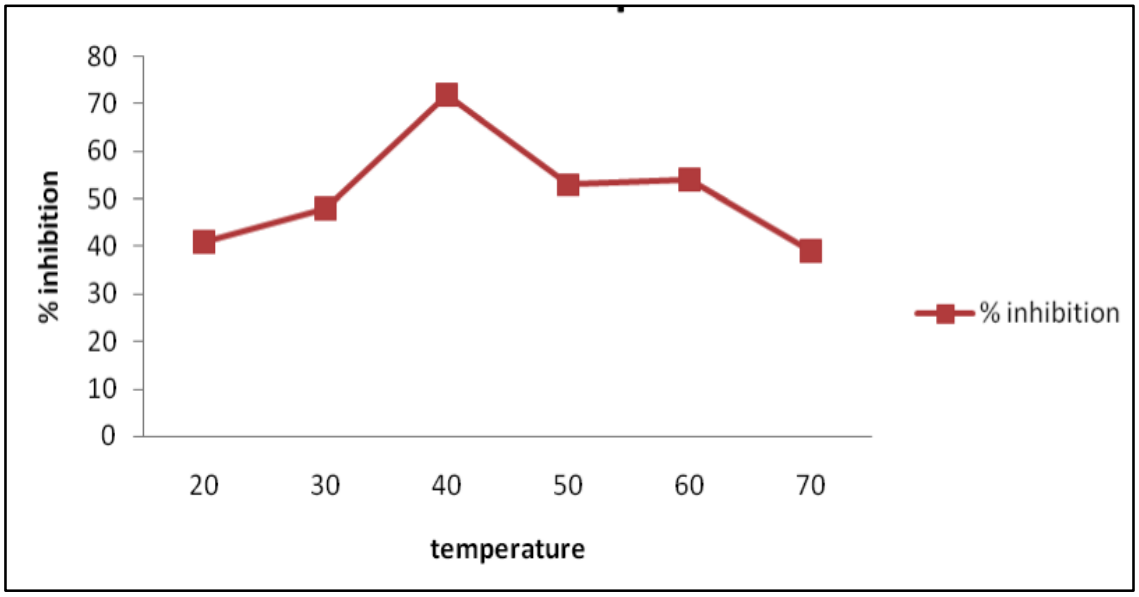

Figure 4. Effect of temperature on the activity of purified peptide obtained from the leaves of *L. hastata*

Effect of Digestive Enzymes on the Purified Peptide from L. hastata Leaves

The ACE inhibitory activities of the purified peptide from the leaves of *L*. *hastata* were expressed based on the inhibition percentage values. From the results, the activity of the peptide decreased significantly (P<0.05) when treated with gastrointestinal enzymes, pepsin and trypsin compared to the standard antihypertensive drug, enalapril (93 ± 2.5 $U.ml^{-1}$) and untreated peptide (Fig. 5). The percentage inhibition of the untreated peptide obtained from the leaves (80 ± 1.3 %) decreased significantly (P<0.05) when treated with pepsin (75 ± 2.18 %) and trypsin (43 ± 2.34 %) (Fig. 5).

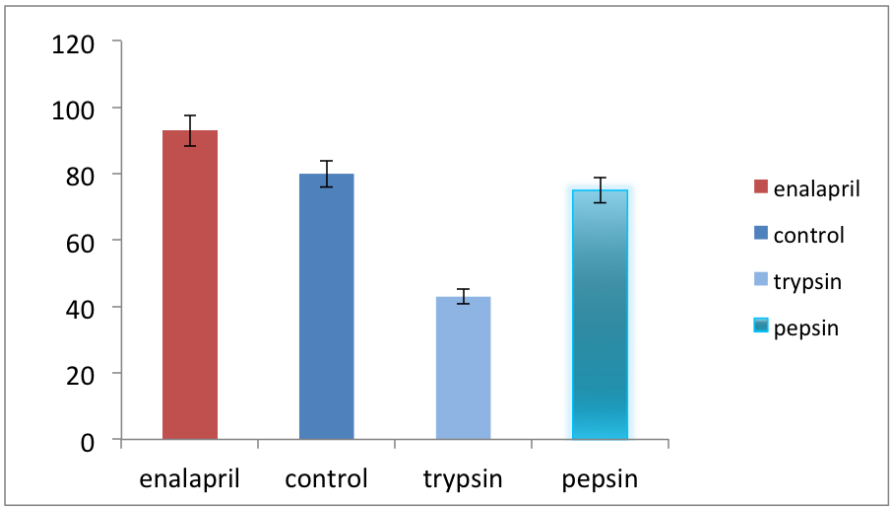

Figure 5. Effect of digestive enzymes on the purified peptide obtained from the leaves of *L. hastata*

Amino Acid Composition of the Purified Peptide from L. hastata Leaves

The crude protein contained seventeen amino acids, with Glutamic acid concentration (10.59 g/100g protein) being the highest, followed by Aspartic acid (7.95 g/100g protein), while Cysteine (0.69 g/100g protein) was lowest (Table 2). On the other hand, the purified peptide recovered was a hexapeptide containing six amino acids Asp, Glu, Gly, Val, Leu, Phe. And just as in the crude protein, Glutamic acid was present in the highest concentration (1.21g/100g), followed by aspartic acid with 0.79g/100g protein (Table 3). The peptide had an aspartic acid at the N terminal end and phenylalanine at the C terminal end.

Table 2. Amino acid analysis of crude protein obtained from *L. hastata* leaves

Amino Acid	Protein Concentration (g/100g protein)
Lysine	3.79
Histidine	2.16
Arginine	4.76
Aspartic acid	7.95
Threonine	2.98
Serine	2.99
Glutamic acid	10.59
Proline	2.08
Glycine	3.35
Alanine	3.97
Cystine	0.69
Valine	4.25
Methionine	0.86
Isoleucine	3.06
Leucine	6.69
Tyrosine	2.85
Phenylalanine	3.78

Table 3. Amino acid analysis of the purified peptide obtained from the leaves of *L. hastate*

Amino Acid	Concentration (g/100 g protein)
Aspartic acid	0.79
Glutamic acid	1.21
Glycine	0.46
Valine	0.54
Leucine	0.55
Phenylalanine	0.35

Determination of Inhibitory Pattern of Purified Peptide from L. hastata Leaves

The ACE inhibition pattern of the purified peptide showed a mixed type of inhibition, with a decrease in V_{max} and an increase in K_m as concentration of inhibitor increased. The V_{max} of the peptide decreased from 0. 04U.ml^{-1} (no inhibitor) to 0.034 U.ml^{-1} (0.5 mg.ml^{-1} inhibitor) and finally 0.03 U.ml^{-1}(1.0 mg.ml^{-1} inhibitor). On the other hand, the K_m value increased from 3.3 mM (no inhibitor) to 4.1 mM and 6.3mM at 0.5 mg.ml^{-1}and 1.0 mg.ml^{-1}of the inhibitor respectively (Fig. 6).

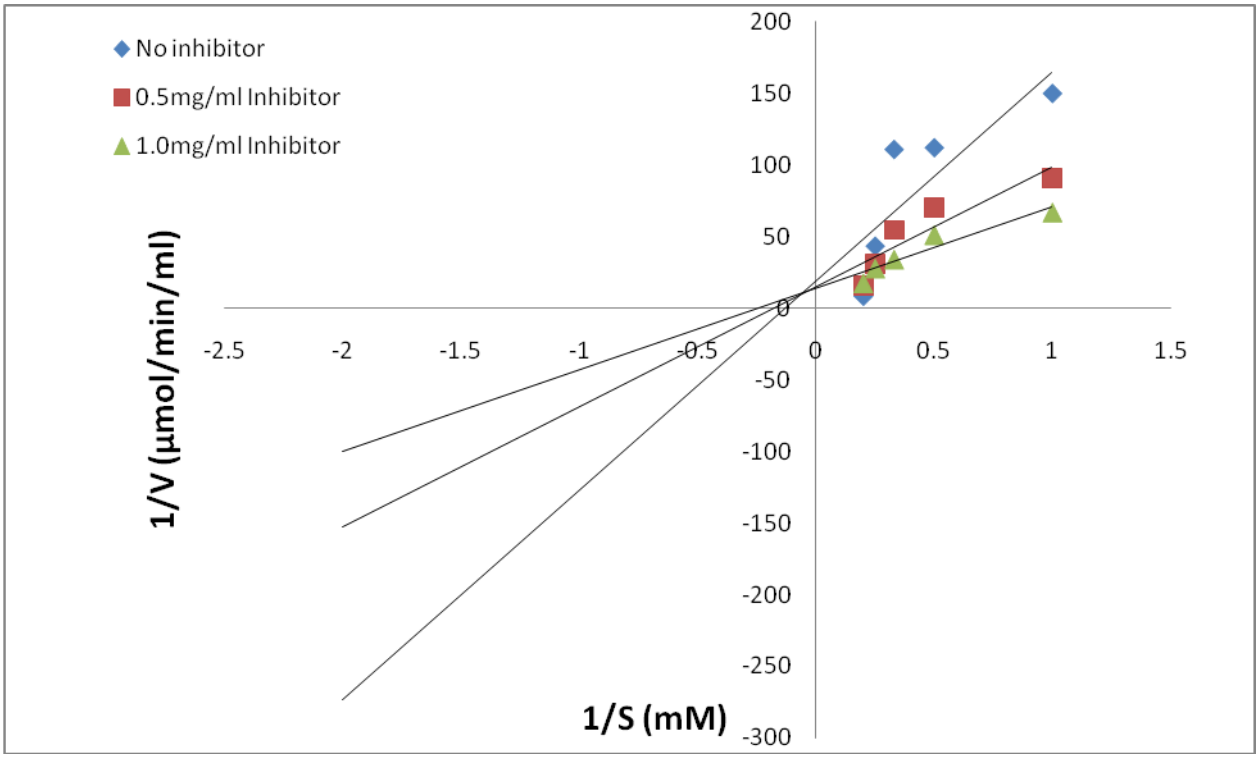

Figure 6. Lineweaver-Burk's plot for the inhibition of angiotensin converting enzyme by the purified peptide from the leaves of *Leptadenia hastata*

Discussion

In Nigeria and most African countries, herbs have been used as constituents of traditional medicines because they are relatively inexpensive, easily available with few adverse effects (Bako et al., 2005). Many antihypertensive ACE inhibitors have been isolated from natural sources, including algae protein waste (Sheih et al., 2009), oyster (Jiapei et al., 2008) and

buckwheat (Min-Suk et al., 2006). These inhibitors have also been isolated from *Moringa oleifera* (Abdulazeez et al., 2015a; Abdulazeez et al., 2016) and *Peristrophebi calyculata* (Abdulazeez et al., 2015b) herbs used in the treatment of hypertension in Nigeria. In the present study, preliminary evaluation revealed that the peptide obtained from *L*. *hastata* actively inhibited ACE *in vitro* with an IC_{50} value of 0.0090 ± 0.0007 U/mL. This is lower than that of peptides from *Hibiscus rosasinensis* (0.43 ± 0.04 U/mL) and *Vinica* leaves (1.37±0.06 U/mL) (Aprilita et al., 2013), and may be attributed to differences in assay conditions substrate used and source of ACE.

The removal of components capable of interacting with the peptide, and thus affecting its activity; during purification via acetone precipitation, gel filtration and ion exchange chromatography may have increased the specific activity from 0.0940 to 0.0090 U/mg (Table 1). This conforms to studies by Sallau et al. (2008), who attributed the increase in activity of proteins during purification to the removal of other synergistically interacting components of the protein. The percentage yield of the purified peptide (9.57%) was low, though the purification folds increased, suggesting that the modified purification steps may increase peptide yield (Sallau et al., 2008).

The isolated peptide exhibited optimal ACE inhibitory activity at pH 7.0, showing that acidic or alkaline conditions away from neutrality may affect it. The optimal temperature of the peptide was found to be 40°C hence, higher and lower temperatures affect its activity, possibly due to thermal denaturation at high temperatures (Qu et al., 2010). Hence, the peptide may be regarded as unstable even though, it still possessed some degree of activity (>10%) at temperatures between 20 and 70°C and pH 5 and 9. The optimum temperature (40°C) conforms to that of ACE inhibitors obtained from *M. oleifera* (Abdulazeez et al., 2015a), but higher than that of *P. adiposa* (30°C) (Koo et al., 2006) and lower than *P. cornucopiae* (50°C) (Jang et al., 2011). This peptide is unlike ACE inhibitors obtained from *Porphyra yezoensis* (Qu et al., 2010) and algae protein waste (Sheih et al., 2009) found to be stable at different temperatures and pH, it is therefore important to investigate its effect under gastrointestinal conditions.

In order to produce antihypertensive activity *in vivo*, peptides must be absorbed intact through the intestine and reach the target organ (Wilson et al., 2011). The *in vitro* gastrointestinal enzyme incubation provides an easy process to imitate the fate of peptides after oral administration, because some ACE inhibitors may fail to show their hypotensive activity after oral administration *in vivo*, due to the possible hydrolysis of these peptides by ACE or gastrointestinal proteases (Fujita et al., 2000; Li et al., 2004). To evaluate the stability of the purified peptide under gastrointestinal enzymes digestion, the purified peptide was first incubated with two gastrointestinal enzymes; pepsin and trypsin, and then subjected to ACE inhibitory activity assays. The results showed an apparent change after *in vitro* incubation with gastrointestinal enzymes ($p > 0.05$), suggesting little resistance of the peptide to digestion in the gastrointestinal tract, and that the active sequence of the peptide may be affected by these enzymes., but it remained active. This report agrees with studies by Choi et al. (2001) who reported that a decrease in ACE activity after treatment with gastrointestinal enzymes does not mean they may be ineffective after oral administration because they still maintained their inhibitory activity. Also, Fujita and Yoshikawa (1999) demonstrated that degradation of ACE inhibitory peptides by gastrointestinal enzymes might not affect the hypotensive activity as several ACE inhibitory peptides with weak *in vitro* activity produce a strong antihypertensive effect in vivo. They attributed this to the fact that *in vivo* activity also depends on other factors such as routine of administration, food matrix and intestinal absorption. Therefore, purified peptide obtained from the leaves of *L. hastata* may still be effective in reducing blood pressure.

The difference observed from the number and types of amino acids found in the crude protein compared to that in the purified peptide might be attributed to the purification steps (Table 2 and 3). The purified peptide contained the following amino acids: Asp, Glu, Gly, Val, Leu, Phe. Though most of the reported peptides exhibiting ACE inhibitory activity contained 5-13 amino acids (Li et al., 2004), many more ACE inhibitory proteins contained less than five, e.g. those obtained from human plasma (Nakagomi et al., 2000), and fermented

soybean food (Kuba et al., 2003). Other proteins include that from *Limanda aspera* frame (Jung et al., 2006) and human serum albumin (Nakagomi et al., 2000).

Studies have demonstrated that most reported peptides are competitive inhibitors against ACE, and a few inhibit ACE in a non-competitive manner (Li et al., 2004). From the Lineweaver-Burks' plot (Fig. 6), the inhibition pattern of the purified peptide from *L. hastata* showed a mixed type of inhibition. This means that the inhibitor binds to the enzyme at a different site away from the enzymes active site or it binds to the enzyme-substrate complex, thereby inhibiting the enzyme by causing a conformational change, which prevents the enzyme from converting substrate to product. The V_{max} decreased and K_m may either be increased or decreased. ACE inhibitors isolated from *Grifola frondosa* (Choi et al., 2001), *T. giganteum* (Lee et al., 2004) and *Peristrophe bicalyculata* (Abdulazeez et al., 2013) were reported to exhibit a competitive pattern of inhibition, while those isolated from algae protein waste (Sheih et al., 2009) and lentils (Burkill, 1985) were found to be non-competitive inhibitors.

Conclusion

In conclusion, this study has established the potential antihypertensive health benefit of a novel peptide from *Leptadania hastata*, and thus its potential for use as functional food component. This also explains its traditional use in the treatment of hypertension..

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