Isolation and Characterisation of a Novel Angiotensin-Converting Enzyme Inhibitory Peptide from *Moringa oleifera* Leaves

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Abstract

Moringa oleifera is used in the treatment of various diseases, including hypertension. This study describes the isolation, purification, characterisation, amino acid composition and inhibition pattern of an angiotensin converting enzyme (ACE) inhibitory peptide from the leaves of *M. oleifera*. Crude proteins were extracted from the leaves using a protein extraction kit (Minute ™ Total Protein Extraction Kit for Plant Tissues) and purified by a three-step method: cold acetone precipitation, gel filtration and ion exchange chromatography. From the results, the ACE inhibitory activity of the peptide increased from 0.2534 to 0.0437 U, while specific inhibitory activity increased from 0.0066 to 0.1370 Umg^{-1}at a purification fold of 20.8 and yield of 17%. The optimum pH and temperature of the peptide were 9.0 and 40°C, respectively. The digestive enzymes, pepsin and trypsin significantly (P<0.05) decreased the activity of the peptide compared to the standard ACE inhibitor, enalapril. The peptide was found to exhibit a competitive pattern of inhibition. In conclusion, this study has shown that the ACE inhibitory peptide from *M. Oleifera* may be beneficial for the treatment of hypertension.

Keywords: Peptides, Purification, Angiotensin Converting Enzyme, *Moringa oleifera*

Introduction

Hypertension or high blood pressure is a common cardiovascular disorder which if not effectively treated results in an increased probability of coronary thrombosis, stroke and renal failure.[1] Treatment of hypertension reduces cardiovascular risk, and this has been a major focus of reducing cardiovascular mortality and morbidity.[2] A number of international guidelines suggest that blood pressure should be reduced at least to below 160/90mmHg to normalise cardiovascular disease. Despite the large armamentaria of drugs available for the treatment of hypertension, many more anti-hypertensive drugs have been introduced within the last decade. The guideline for the management of hypertension issued by World Health Organisation and the International Society of Hypertension classify ACE inhibitors as the first line treatment together with diuretics and β-blockers.[3]

An ACE inhibitor is a drug used primarily for the treatment of hypertension and congestive heart failure, as they cause the relaxation of blood vessels and decrease blood volume, which leads to lower blood pressure and decreased oxygen demand from the heart. They inhibit the angiotensin-converting enzyme, an important component of the renin angiotensin aldosterone system. Apart from hypertension, ACE inhibitors are useful in the treatment of other cardiovascular and renal diseases such as acute myocardial infarction, cardiac failure and diabetic nephropathy. Examples include perindopril, captopril, enalapril, lisinopril and ramipril. However, these synthetic ACE inhibitors have been shown to possess adverse side effects, hence, the search for safe and effective ACE inhibitors from natural sources.[4]

Moringa oleifera Lam (syn. *M. ptreygosperma* Gaertn.) is one of the best known and most widely distributed and naturalised species of a monogeneric

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family *Moringaceae*.[5] It is an important food commodity, which has had enormous attention as the 'natural nutrition of the tropics.' The leaves, fruit, flowers and immature pods are used as a highly nutritive vegetable in many countries.[6,7,8] The leaves are a rich source of natural antioxidants, like ascorbic acid, flavonoids, phenolics and carotenoids and thus enhance the shelf-life of fat-containing foods.[9,10] In the Philippines, it is known as 'mother's best friend' because it is used to increase breast milk production and sometimes prescribed for anaemia.[10,11] The leaves have been reported to possess anti-hypertensive, hypocholesterolemic, anti-ulcer and wound healing properties[12,13] Hydro alcoholic extract of the plant has been shown to possess significant antioxidant potential by increasing the activities of catalase, glutathione peroxidase and glutathione reductase and decreasing hepatic malondialdehyde level.[14] In an earlier study, proteins were partially-purified from the leaves and seeds of *M. oleifera*, but the yield and purification fold were low, [15] hence, this study aims to modify the purification steps to obtain ACE inhibitory peptides with higher specific activities, yield and purification fold from the leaves. The amino acid composition of the isolated peptide was also determined.

Materials and Methods

Chemicals and Reagents

Total Protein Extraction Kit for Plant Tissues (Invent Technologies, U.S.A), ACE Inhibition Screening Kit (Kamiya Biomedical co. Seattle, U.S.A), Sephadex G-100, CM Sephadex, Pepsin (porcine stomach mucosa) and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the study were of analytical grade.

Collection and Identification of Plant Sample

Fresh leaves of *M. Oleifera* were collected from Kofar Ruwa, Dala Local Government Area, Kano State, Nigeria. The plant was authenticated at the herbarium unit of Botany Department, Bayero University, Kano, by a senior Botanist and issued an identification number BUKHAN 0011.

Purification of ACE Inhibitory Peptide from *Moringa oleifera*

The fresh leaves of *Moringa oleifera* were washed under running water and macerated separately with cold distilled water at a ratio of 1:10. It was then centrifuged at 4°C and 10000xg for 15 minutes to obtain the supernatants.

Isolation of Crude Protein

Fresh leaves of *Moringa oleifera* were washed clean under running water and crude protein was isolated with the Minute ™ total protein extraction kit for plant tissues. Briefly, about 50-100 mg fresh leaves of *Moringa oleifera* was placed in a pre-chilled protein extraction filter cartridge by folding and rolling into smaller volume. The leaves were punched repeatedly with a pipette tip before using a plastic rod with twisting force, until they were well macerated. About 50-100 µL buffer was added to the filter, capped and incubated at room temperature for 1-2 minutes, then centrifuged at top speed for 2-5 minutes. The supernatant obtained was transferred to a fresh tube (this is protein extract). This process was repeated using different protein extraction cartridges to obtain enough protein extract.

Acetone Precipitation

Supernatants obtained from leaves of *Moringa oleifera* were precipitated using cold acetone at a ratio of 1:4. The test tubes containing the precipitated proteins were vortexed and incubated at -20°C for 60 minutes before centrifuging for 10 minutes at 4°C and 10000xg. The supernatant was discarded leaving the pellet. Acetone was allowed to evaporate and pellet was reconstituted in 5 ml phosphate buffer (pH 7.4) to determine the ACE inhibitory activity and protein content.

Gel filtration using Sephadex G-100

The precipitated protein extract was then subjected to gel filtration chromatography. A column was packed with gel that had been soaked overnight in phosphate buffer (pH 7.8) and the protein poured into the column and eluted at a flow rate of 0.5cm^3/min. Thirteen (13) fractions of the sample were collected, 5cm^3 each. The protein content and ACE inhibitory activity of each fraction was determined.

Ion Exchange Chromatography

The fraction with the highest ACE inhibitory activity was subsequently loaded onto a CM Sephadex column pre-equilibrated with phosphate buffer solution (pH 7.8), and eluted at a linear gradient of

NaCl (0.1-0.5 M) in the same buffer. The protein content and ACE inhibitory activity of the eluates were assayed.

Determination of ACE Inhibitory Activity

The ACE inhibitory activity was determined with the K-ASSAY ACE inhibition screening kit. Briefly, 20 µL each of the sample, substrate and enzyme solution is added to a well (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 37^0C 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.

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.

One (1) unit inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions, which corresponds to 1 µmol of hippuric acid released by hydrolysis of HHL per minute per milliliter of inhibitor.

Determination of Protein Content

The protein concentration of the fractions collected was determined according to Bradford method using Serum Bovine Albumin (BSA) as standard and the concentration was expressed in milligram per milliliter (mg/cm^3).

Characterisation of ACE Inhibitory Proteins

Effect of temperature and pH on ACE inhibitory activity.

The influence of temperature on inhibitor activity was analysed by varying the incubation temperature from 10 to 70°C.The peptide was dissolved in milli-Q water and subsequently incubated with ACE. The effect of pH on the peptide was determined by varying the pH of reaction mixture from 3 to 10. The activity of inhibitor was analysed by the previously described method.

Effect of Digestive Enzymes on Ace Inhibitory Activity.

The stability of ACE inhibitory peptide against some digestive enzymes was assessed *in vitro*in order to simulate the human gastrointestinal digestion process. Briefly, digestive enzymes (pepsin and trypsin) were added to the ACE inhibitory peptide. The reaction mixture was incubated at 37^oC for 1hr and the reaction stopped by standing in boiling water bath for 10mins. After centrifugation at 2800xg, the supernatant was assayed for ACE inhibitory activity.[16]

Determination of Amino Acid Composition

The Amino Acid profile of the purified peptide was determined using methods described by Benitez.[17] The known sample was dried to constant weight, hydrolysed and evaporated in a rotary evaporator before loading into the Technicon sequential Multi-Sample Amino Acid Analyser (TSM).

Determination of the inhibition pattern of the peptide Various substrate (3HB-GGG) concentrations (0 to 5 mM) were co-incubated with the ACE solution in the absence and presence of 0.5 and1.0 mg/mL of purified peptideat 37°C, and each reaction mixture was assayed for ACE inhibition. The kinetic constants (K_m and V_{max}) values for the reaction at different concentrations of purified peptide were determined using Line weaver-Burk plots.

Results

Purification Steps of Peptide from *M. oleifera* leaves

The steps involved in the purification of ACE inhibitory proteins are summarised on Table 1. From the gel filtration chromatographic separation profile (Figure 1), there was a decrease in percentage inhibition of ACE increased from fraction 7 to 10, after which it decreased until no activity was observed in fraction 15. Fractions 1, 10, 11 and 12 having the highest inhibitory activities of 95%, 89.8%, 87.8% and 82.7%, respectively were pooled together and further purified by ion exchange chromatography.

Figure 2 shows the ion exchange chromatographic profile of ACE inhibitory proteins from *M. oleifera*. There was an initial decrease in activity of the

peptide from 0.1 to 0.2 mM NaCl concentration, but a steady increase in activity was observed from 0.3 to 0.5 mM NaCl. The fraction with the highest ACE inhibitory activity was eluted at 0.5mM NaCl gradient.

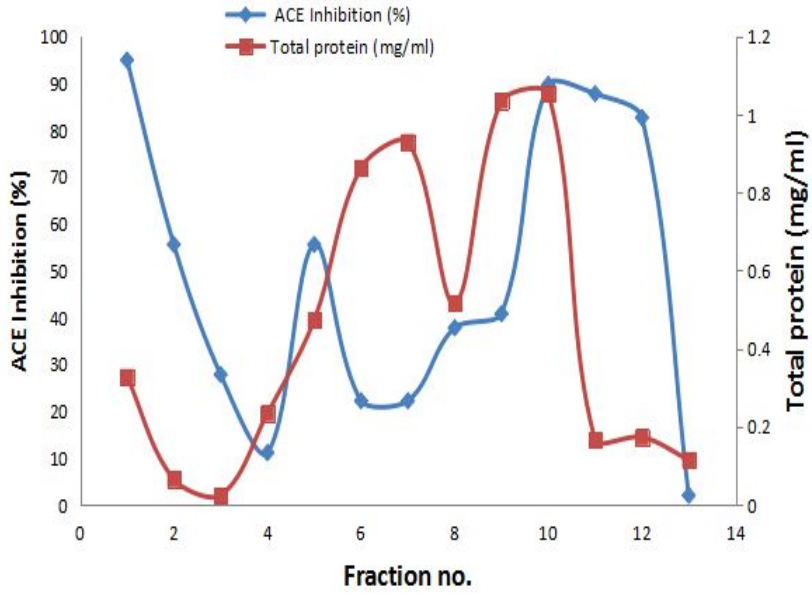

Figure 1: Elution profile of the purified ACE inhibitory peptide from Sephadex G-100 gel filtration chromatography

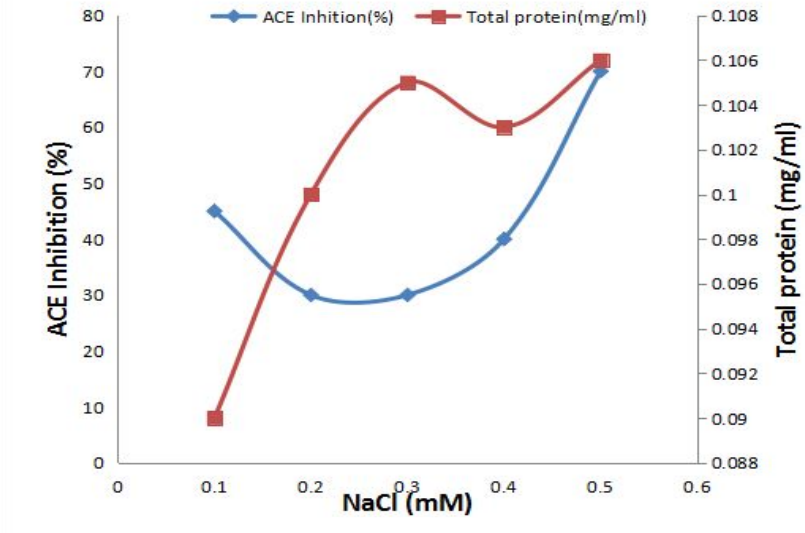

Figure 2: Ion exchange chromatography elution profile of the purified ACE inhibitory peptide from *Moringa oleifera*

The crude proteins from the leaves of *M. oleifera* were found to possess ACE inhibitory activity. The crude protein was estimated to be 1.926 mg/cm^3 with a specific inhibitory activity of 0.0066 U/mg,

which increased to 0.0067 U/mg at a recovery rate of 43% after precipitation with cold acetone. Passing the protein through Sephadex-G100 further increased the specific activity to 0.0132 U/mg, which increased to 0.137 U/mg after ion exchange chromatography with a purification fold of 20.8 (Table 1).

Table 1: Purification Table of the Purified ACE Inhibitory Peptide from *M. oleifera* leaves.

Purification Steps	Protein content (mg/ml)	Total Protein Content (mg)	Inhibitory Activity (µmol/min/ml)	Specific Inhibitory Activity (U/mg)	Purification fold	Yield (%)
Crude	1.926	38.52	0.2534	0.0066	1	100
Acetone precipitation	1.633	16.33	0.1086	0.0067	1.02	43
Gel filtration	0.4305	6.458	0.0850	0.0132	20	34
Ion exchange	0.1063	0.3189	0.0437	0.1370	20.8	17

1 U of inhibitory activity was defined as the amount of the inhibitor that decreased the ACE activity by 50%.

Characterisation of purified peptide from *M. oleifera*
Effect of temperature and pH on the purified peptide
The ACE inhibitory activity of the peptide increased as the temperature was raised from 20 and the highest inhibitory percentage (70%) was observed at 40°C (Figure 3). Also, the inhibitory activity of the peptide increased as the pH was raised from 5 and the highest activity was seen at pH 9 after which there was subsequent decrease (Figure 4).

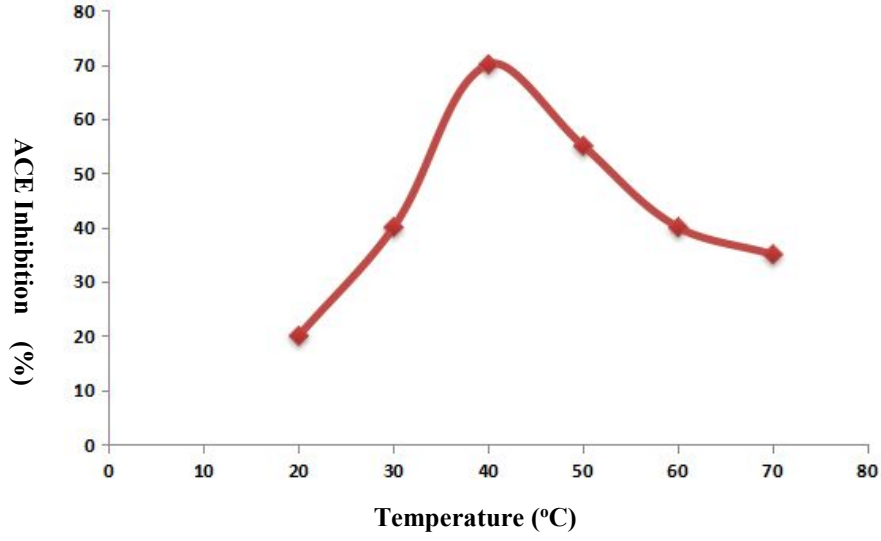

Figure 3: Effect of temperature on the ACE Inhibitory activity of purified Peptide from the leaves of *Moringa oleifera*

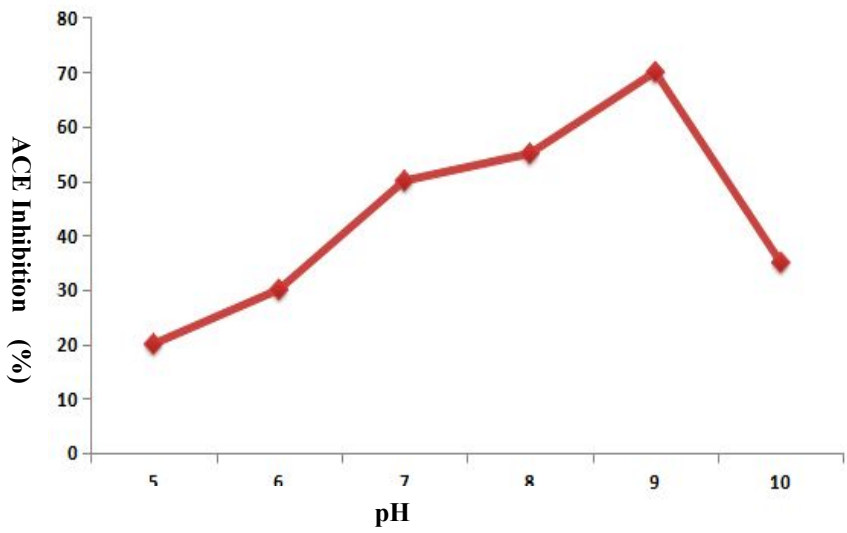

Figure 4: Effect of pH on the ACE inhibitory activity of the purified peptide from the leaves of *Moringa oleifera*

Effect of digestive enzymes on the purified inhibitor

The result shows that there was no significant ($P<0.05$) difference in the ACE inhibition percentage of the purified peptide (80 ± 11.60 %) and enalapriltreated peptide (93 ± 12.50%). However, treatment of the peptide with pepsin (70 ± 12.17 %) and trypsin (44± 12.39%), significantly ($P<0.05$) decreased the inhibition percentage when compared to the standard drug, enalapril and control sample (Figure 5).

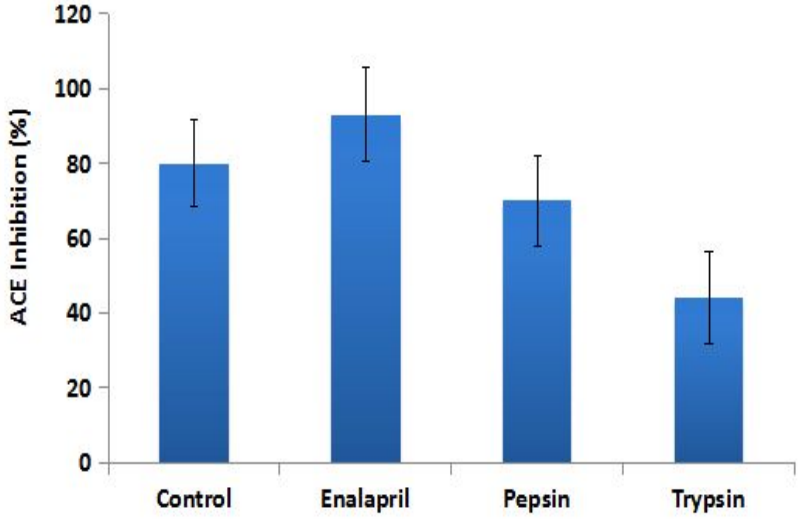

Figure 5: Effect of some gastrointestinal enzymes on the ACE inhibitory activity of the purified peptide from the leaves of *Moringaoleifera.*

Amino acid composition of the leaves of *M. oleifera*

The amino acid composition of the crude protein and purified ACE inhibitory peptide isolated from the leaves of *Moringa oleifera* are presented on Tables 2 and 3 below. The crude protein contained seventeen amino acids with high glutamic acid (11.23g/100g) content followed by Aspartic acid (8.29g/100g), Leucine (6.28g/100g), Arginine (5.27g/100g), Lysine (4.34g/100g), Phenylalanine (4.22g/100g) and Glycine (3.60g/100g). Other amino acids, such as valine, alanine, threonine, serine, proline, isoleucine, tyrosine, histidine, cysteine and methionine, were present in very low concentrations (Table 2). After purification, an octapeptide with an amino acid composition: Asp, Glu, Ser, Gly, Val, Ile, Leu and Phe was obtained. The glutamic acid concentration (4.26g/100g) of the octapeptide was higher than other amino acids (Table 3).

Table 2: Amino Acid Composition of Crude Protein Obtained from *M. Oleifera* Leaves

Amino Acid	Concentration (g/100g protein)
Lysine	4.34
Histidine	2.25
Arginine	5.27
Aspartic Acid	8.29
Threonine	3.29
Serine	3.11
Glutamic Acid	11.23
Proline	3.01
Glycine	3.99
Alanine	3.60
Cysteine	0.83
Valine	3.22
Methionine	1.07
Isoleucine	3.32
Leucine	6.28
Tyrosine	2.98
Phenylalanine	4.22

Table 3: Amino Acid Composition of Purified Peptide Obtained from *M. Oleifera* Leaves

Amino Acid	Concentration (g/100g protein)
Aspartic Acid	0.54
Glutamic Acid	4.26
Serine	0.32
Gycine	0.41
Valine	0.48
Isoleucine	0.64
Leucine	0.64
Phenylalanine	0.40

Inhibitory Pattern of Purified ACE Inhibitory Peptides from M. oleifera

The ACE inhibitory pattern of the purified peptide from *M. Oleifera* was investigated using Lineweaver-Burk's plot. The peptide exhibited a competitive type of inhibition, showing that it binds competitively with the substrate at the active site of the enzyme, ACE. The peptide showed a dose-dependent inhibitory effect on ACE, as it's K_m increased from 0.6 mM without inhibitor, to 2.17 and 5.0 mM at 0.5 mg/cm^3 and 1 mg/cm^3 inhibitor, respectively, while the V_{max} remained unchanged at 0.07µmol/min/cm^3.

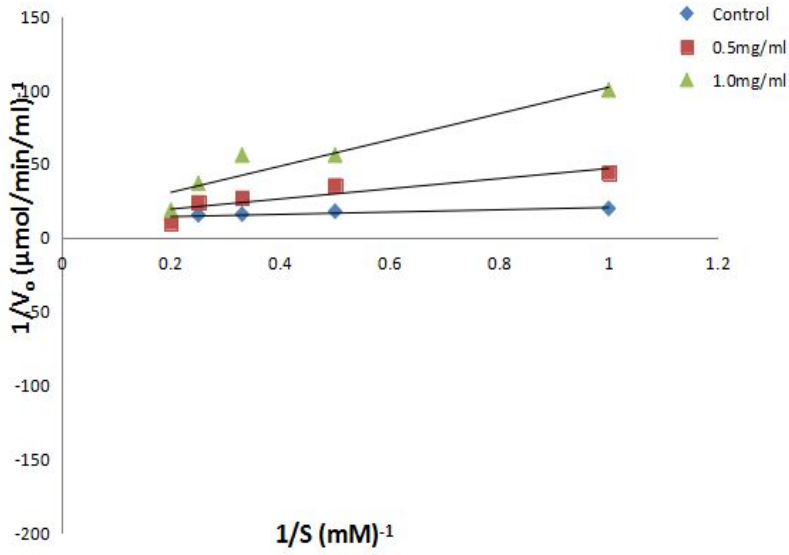

Figure 6: Lineweaver-Burk's Plot for the Inhibition of angiotensin-converting enzyme by the purified peptide from the leaves of *M. oleifera*

Discussion

Nutrition science has evolved towards obtaining optimal health contributing to disease prevention.[18] One of such areas is the focus on the production and isolation of ACE inhibitory peptides from different food proteins.[19,20,21,22] Captopril, enalapril, alacepril, lisinopril and many other ACE inhibitors are extensively used medications in the treatment and prevention of hypertension. However, these drugs often cause side effects, hence the search for cheap, natural plant-derived ACE inhibitors with little or no harmful side-effects,[23] that may be used as drugs or functional foods. In the present study, the purification of ACE inhibitory proteins by acetone precipitation and gel filtration increased the specific activity from 0.0067 to 0.0132 U/mg and after passing through ion exchange chromatography, increased to 0.1370 U/mg with a purification fold of 20.8% (Table 1).The high purification fold (20.8%) obtained from this study compared to 8.41% by Abdulazeez et al. [15]can be attributed to the modification of the purification steps, resulting in the removal of components that may affect the activity of the peptide.[24]

Having an optimum pH (9) and temperature (40^{o}C) shows that the activity of the peptide may be affected by high pH and temperature variations, although it maintained some level of inhibition. However, the low inhibition percentages at high temperatures indicate it may undergo thermal denaturation. This result disagrees with reports by Bernstein et al. [25]that the optimum pH and temperature of most ACE peptides falls within a pH range of 7 to 8 and temperature range of 37 to 40^{o}C.

The effect of gastrointestinal enzymes on ACE inhibitory peptides is important because after ingestion, they may be broken down and their activity affected. Thus, it is expected that hydrolysates of inhibitors obtained after treating with gastrointestinal enzymes would resist further *in vivo* gastrointestinal hydrolysis after oral intake and consequently increase the *in vivo* ACE inhibitory activity. The present result shows that treatment of the peptide with pepsin and trypsin significantly ($P<0.05$) decreased the ACE inhibitory activity. This may be due to variations in the type of ACE-inhibitory peptides liberated after hydrolysis with the enzymes.[26]Studies have shown that a decrease in ACE inhibitory activity of a peptide after treatment with gastrointestinal enzymes does not make it ineffective,[27]as other factors such as routine of administration; food matrix and intestinal absorption play a significant role in the activity of

the peptide. [28]Also, it has been demonstrated that some ACE inhibitory peptides with weak *in vitro* activity produce a strong antihypertensive effect *in vivo*. Therefore, even if the enzymes affected the activity of the peptide obtained from *Moringa oleifera*, it may still be effective in reducing blood pressure.

Results from the Lineweaver-Burk's plot (Figure 6) showed that the purified peptide exhibited a competitive type of inhibition, suggesting that it binds competitively with the substrate at the active site of ACE. This finding conforms to ACE inhibitors obtained from *T. giganteum*[29] and *Grifolafrondosa,*[27] and agrees with the findings of Gouda et al. [30] that most ACE inhibitory peptides and constituents are competitive inhibitors. It has been well established for competitive inhibitorsthat the structure-activity relationship of ACE inhibitory peptides is affected by the C and N terminal tripeptide sequence, as ACE shows preference to substrates or competitive inhibitors containing hydrophobic amino acid residues at C-terminal and aliphatic amino acids at N-terminal.[31]

Conclusion

In conclusion, this study has demonstrated that the purified ACE inhibitory peptide obtained from *Moringa oleifera* may be beneficial as nutraceutical or drug for treatment of hypertension and other cardiovascular-related diseases. However, further studies are necessary to establish its efficacy *in vivo* for application in the medicinal industry.

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