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Amino Acid Composition and Angiotensin-I Converting Enzyme Inhibitory Activity of Cowpea (*Vigna unguiculata*) Pepsin-Pancreatin Protein Hydrolysate Fractions

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Abstract
Legumes are high in protein and have been linked to the management and prevention of chronic diseases due to their bioactive components. This study investigated the amino acid composition of cowpea protein hydrolysate and its inhibitory impact on angiotensin-I converting enzyme (ACE-I). Cowpea (Vigna unguiculata) protein hydrolysate (CPH) was produced through sequential digestion with pepsin and pancreatin. CPH was then separated using G-50 gel filtration chromatography and reverse phase high performance liquid chromatography (RP-HPLC). The fractions' ACE-I inhibitory activity was then assessed. The IC_{50} value for the RP-HPLC fraction with the highest ACE-I inhibitory activity ($P<0.05$) was 6.23 µg/mL. The amino acid profile revealed that a large proportion of hydrophobic amino acids were present in the RP-HPLC fraction containing the peptide with the highest ACE-I inhibitory activity. These findings suggest that CPH has the potential to be employed in the development of functional foods that can aid in the prevention and treatment of hypertension.

Keywords: *Vigna unguiculata*, Pepsin, Pancreatin, Hydrolysate, Angiotensin, Angiotensin-I converting enzyme.

INTRODUCTION
Hypertension is a global cardiovascular disease that affects 15 to 20% of all individuals and it can cause issues like stroke, myocardial infarction, and end-stage kidney failure (Ulasi *et al*., 2011). Blood pressure is regulated by the renin–angiotensin system (RAS) (Erdmann *et al*., 2008; Chen *et al*., 2009). The angiotensin I-converting enzyme (ACE-I), a RAS enzyme,

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hydrolyzes angiotensin I (a decapeptide) into angiotensin II (an octapeptide), a powerful vasoconstrictor that causes arterial tightness and increases blood pressure (Riordan, 2003). ACE also inhibits the production of bradykinin, a vasodilator that lowers blood pressure (Chen *et al*., 2009). As a result, ACE inhibition is critical for the reduction of blood pressure (Erdmann *et al*., 2008; Chen *et al*., 2009).

There are several effective ways to lower blood pressure such as dietary adjustments, exercise, calcium channel agonists, angiotensin II receptor blockers, diuretics, and ACE inhibitors (Fitzgerald and Murray, 2006). Researchers are looking at foods that naturally contain hypotensive peptides because synthetic drugs can induce side effects like cough, skin rashes, and angioedema (Roy *et al*., 2010). Some hypotensive peptides are located inside the parent protein structure and are released during the proteolysis process (Fitzgerald and Murray, 2006; Qian *et al*., 2007; Aluko, 2008; Wu and Muir, 2008).

Bioactive peptides and high-quality proteins are all found in legumes (Awika and Duodu, 2017; Chel-Guerrero *et al*., 2017). Legumes are being studied because of their high protein content and vast range of biological functions in the body (Chel-Guerrero *et al*., 2017). Nigeria produces about 2.5 million tons of cowpea, making it the world's top producer (Kamara *et al*., 2018). Cowpea is a high-protein, high-carbohydrate, and high-vitamin food legume (Awika and Duodu, 2017). Cowpea is a good source of protein extracts and hydrolysates as a raw material (Awika and Duodu, 2017). There are reports of ACE inhibitory activity in peptides from several dietary sources, such as velvet bean (*Mucuna pruriens*) and Jamapa bean (*Phaseolus vulgaris*) (Bentacur *et al*., 2015; Drago *et al*., 2016).

Plant proteins have been hydrolyzed using enzymes, and the resulting hydrolysates showed a high potential for blocking critical enzymes for CVD and T2D, as well as cancer cell proliferation and antioxidant qualities (Vital *et al*., 2014). With alcalase, flavorzyme, and pepsin-pancreatin, Segura Campos *et al*. (2010) hydrolyzed cowpea protein extract and obtained degrees of hydrolysis ranging from 38 to 59%, IC_{50} for angiotensin-converting enzyme inhibitory activity ranging from 1.4 mg/mL to 2.6 mg/mL, and hydrolysates also demonstrated significant antioxidant activity. Rudolph *et al*. (2017) found that hydrolyzing plant proteins with chymotrypsin and thermolysin resulted in lower ACE inhibitory activity values. Furthermore, the hydrolysis of Bambara bean protein with alcalase, thermolysin, and trypsin produced hydrolysates with high ACE inhibitory activity and significant antioxidant properties (Mune *et al*., 2018). The aim of this research was to investigate the amino acid composition of cowpea hydrolysate that can inhibit ACE-I. At the end of this work, the type of amino acids present in ACE inhibitory peptides produced by enzymatic hydrolysis of cowpea protein isolate will be provided.

MATERIALS AND METHODS

Cowpea (*Vigna unguiculata*) seed were obtained at Dawanau Market in Kano State, Nigeria, and authenticated by a Botanist at the Plant Science Department, Bayero University, Kano Herbarium in Kano State, Nigeria. Sigma supplied all of the chemicals and reagents utilized (St. Louis,MO, USA).

Preparation of Protein Concentrate

The cowpea seed were ground after drying. The flour that resulted was sieved to a fine pow der. The cowpea protein concentrate (CPC) was made utilizing the procedure outlined by Bentacur- Ancona *et al*. (2004). One kg cowpea flour was suspended in 1:6 (w/v) distilled water. To raise the pH to 11, 1 M of NaOH was added, and the mixture was mechanically

agitated for 1 hour before sieving. The protein-starch mixture was allowed to settle at room temperature for 30 minutes to get the starch and protein fractions. To attain the isoelectric point (pH 4.5), HCl (1 M) was used to adjust the pH of the separated solubilized proteins. After that, the sample was centrifuged at 1317 × g for 12 minutes. The supernatant was discarded, and the precipitate was freeze-dried until it could be used again.

Enzymatic Hydrolysis of Protein Concentrate

The method of Guzman-Mendez *et al*. (2014) was used to perform enzymatic hydrolysis. CPC was digested sequentially for 60 minutes with pepsin (40 mg/mL) and then for 60 minutes with pancreatin (40 mg/mL). The hydrolysis conditions include an enzyme-substrate ratio of 1:20 (w:w), temperature control at 37^0C, pH 2 for pepsin (adjusted with 0.1M HCl), and pH 7.5 for pancreatin (adjusted with 0.1M NaOH). The hydrolysis reaction was stopped by boiling for 20 minutes in a water bath at 80 °C, then centrifuging for 12 minutes at 1317 g to remove the insoluble component.. The protein hydrolysate was then freeze-dried.

Determination of Degree of Hydrolysis

The procedure reported by Nielsen *et al.* (2001) for measuring free amino groups with o-phthaldialdehyde (OPA) was employed to determine the degree of hydrolysis (DH)

Determination of ACE-I Inhibitory Activity

The CPH potential to inhibit ACE-I was determined based on the procedure of Wu *et al*. (2011). The CPH, Hippuryl-His-Leu (HHL) and ACE used were prepared in 0.1 M borate buffer containing 0.3 M sodium chloride at pH 8.3. The sample solution of 10 µL was pre-incubated for 5 minutes at 37^0C with a 45 µL 5mM HHL. The cells were then treated with 10 µL ACE for 30 minutes at 37^0C. The reaction was halted by adding 85 µL of 1M HCl to the samples. 1000 µL of ethyl acetate was used to extract the hippuric acid. After that, 800 µl of ethyl acetate were collected and evaporated at 100^0C in a dry oven. Absorbance was measured at 228 nm after 800 µL of distilled water was used to dissolve the residue.

To calculate inhibitory activity, the following equation was used:

Percentage ACE inhibition = AB_C-AB_S/AB_C-AB_B × 100

AB_C, AB_S, and AB_B represent absorbance without sample, absorbance with sample, and blank absorbance, respectively.

The IC_{50} value was determined as the concentration required to inhibit 50% of ACE activity.

Gel Filtration Chromatography

The CPH was separated on Sephadex G-50 column, 1.6 cm × 60 cm. At a flow rate of 1.0 mL/min, the column was eluted with distilled water. Fractions were measured at 220 nm after being collected in 2 mL tubes. The fractions potential to inhibit ACE-I was determined based on the procedure of Wu *et al*. (2011). The fractions that exhibited high inhibitory activities were pooled and further purified.

REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

After gel filtration chromatography, the most active fractions were pooled together and purified using RP-HPLC on a Shimadzu C18 column , 7 µm, 4.6 × 250 mm . The column was eluted with an 80/20 mix of acetonitrile and water at a flow rate of 1.0 mL/min. The absorbance at 220 nm was used to capture all of the peaks. The fractions potential to inhibit ACE-I was determined based on the procedure of Wu *et al*. (2011).

Determination of Amino Acid Profile

The method reported by Benitez (1989) was employed in determining the amino acid composition of CPH. The sample was defatted, digested and, evaporated in a rotary evaporator. Afterwards, it was analyzed using an Applied Biosystems PTH Amino Acid Analyzer. Because tryptophan decomposes chemically during acid hydrolysis, it is a difficult amino acid to recognize in proteins and peptides (Maria *et al*., 2004). It is important to remember that tryptophan is destroyed during hydrolysis by 6 N HCl. As a result, 4.2 M NaOH was used to hydrolyze tryptophan (Maria *et al*., 2004).

RESULTS AND DISCUSSION

Enzymatic Hydrolysis

The hydrolysis of protein concentrate is an important step in the synthesis of peptides (Akbarian *et al*., 2022). Hydrolysis might be enzymatic or microbiological, depending on your interests. The CPC was hydrolyzed for 120 minutes using pepsin and pancreatin successively. CPH with a DH of 20.10±0.2% were produced by sequential hydrolysis with pepsin and pancreatin. These results are higher than the reported values for 60 minute (11.0%) and 180 minute (17.0%) pancreatin-treated soy protein hydrolysates (Qi *et al*., 1997), but lower than the values obtained for cowpea protein hydrolysates (35.74%) (Segura-Campos, 2010). The variations in results could be attributable to sample preparation, isolation method, source of protein, or cowpea varieties (Segura Campos *et al*., 2010). All these factors have an impact on the DHs value. The CPH is a collection of peptides that are similar to those produced by an organism's digestion of cowpea proteins (Segura Campos *et al*., 2010). The proteolytic enzyme pepsin is the most important in the stomach, whereas the pancreas produces pancreatin (which includes trypsin, chymotrypsin, and elastase). The peptides produced are bioavailable because they are resistant to pepsin and pancreatin digestion (Jin *et al*., 2016). As a result, they are able to perform their biological functions.

ACE-I Inhibitory Activity

In this study, CPH, had a low ACE-I inhibitory activity (22.58%). It is possible that this is due to the low concentration of small peptides and the large peptides' blocking effect on small peptides' antihypertensive effects (Olagunju *et al*., 2018). Gel filtration chromatography was used to fractionate CPH. Figure 1 depicts the hydrolysate elution profile. The fractions with ACE-I inhibitory activities greater than or equal to 50% were pooled together and further fractionated using the RP-HPLC

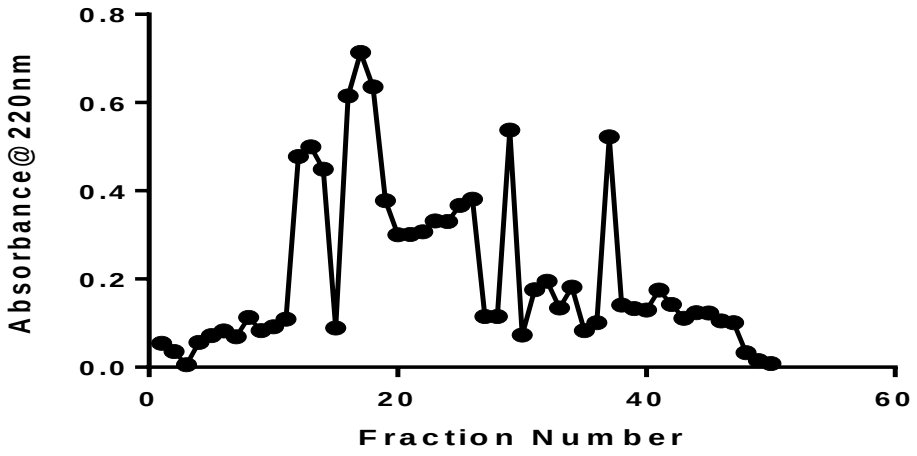

Figure 1: Gel Chromatography Elution Profile of Cowpea Hydrolysate.

The activity of ACE-I was inhibited by the majority of the fractions (Figure 2). Fraction 11 exhibited the highest inhibitory activity (65.68%). ACE-I inhibitory fractions are often detected in the middle or end of an RP-HPLC chromatogram, correlating to peptides with a lower molecular mass and a larger proportion of hydrophobic amino acids (Yust *et al*., 2003; Mallikurjan-Gouda *et al*., 2006). These findings are supported by our findings. Fraction 11 had an IC_{50} of 6.23 µg/mL. This value is lower than the 19.3 µg/mL found in small red bean (Phaseolus vulgaris) peptide using RP-HPLC (Xin *et al*., 2013). This fraction was collected for amino acid profiling.

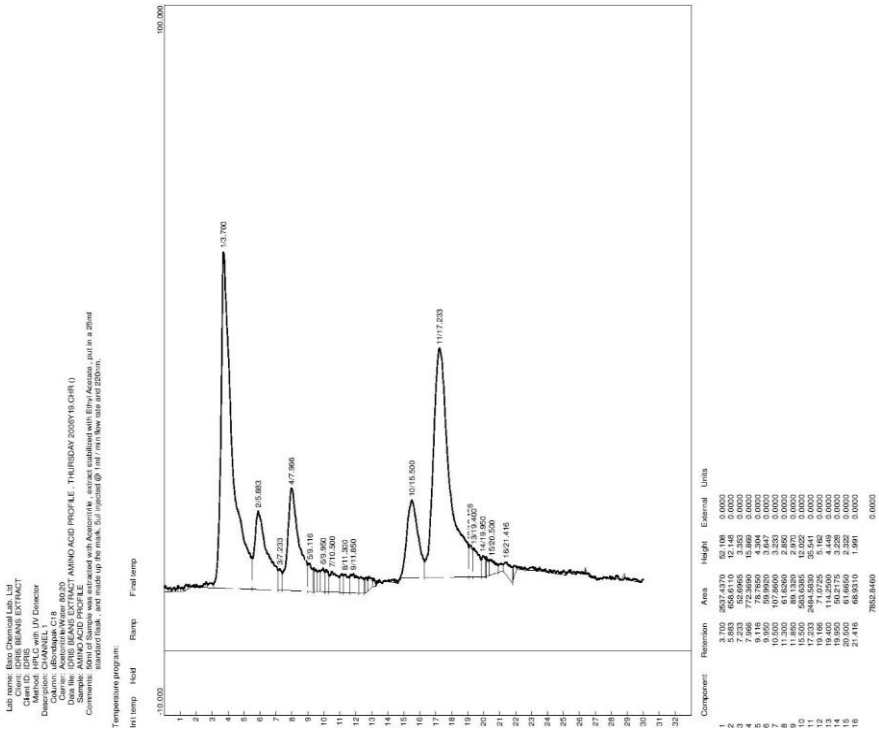

Figure 2: RP-HPLC Elution Profile of the Gel chromatography product

Amino Acid Profile

The most prevalent amino acids in the most effective fraction were determined to be glutamic acid, aspartic acid, lysine, leucine, arginine, and phenylalanine (Table 1). The fraction showed a well-balanced amino acid profile, making it suitable for human consumption (FAO/WHO, 2007). The amount of hydrophobic amino acids (HAA) detected in the fraction, 42.16 g was larger than that reported by Olagunju *et al* (2018) for Pigeon pea hydrolysate, 35.42 g, hydrolyzed sequentially with pepsin-pancreatin system. Peptides having considerable aromatic residues (tyrosine, phenylalanine, tryptophan) at the C-terminus and basic residues (lysine, histidine, and arginine) at the N-terminus have also been shown to exhibit robust and competitive ACE-I inhibitory action (Hwang and Ko, 2004). Aromatic amino acids also help to suppress ACE by binding with three subsites at the enzyme's active site. Other researchers suggest that amino acids including leucine, isoleucine, and valine may help to boost ACE-I inhibitory function (Ruiz *et al*., 2004). As a result, this research suggests that the presence of aromatic and hydrophobic amino acids contributes to cowpea hydrolysate's ACE-I inhibitory effect.

Table 1: Amino acids present in the most potent ACE inhibitory fraction

Amino Acid	Concentration (g/100 g)
Leucine	8.69
Lysine	6.18
Isoleucine	4.41
Phenylalanine	6.30
Trytophan	0.86
Valine	4.50
Methionine	2.60
Proline	6.15
Arginine	7.32
Tyrosine	0.35
Histidine	2.16
Cysteine	0.35
Alanine	4.75
Glutamic acid	14.77
Glycine	3.90
Threonine	4.22
Serine	3.83
Aspartic acid	12.10

CONCLUSION

The cowpea protein hydrolysate had a low ACE-I inhibitory activity but upon purification via gel filtration and reverse phase chromatography, the ACE-I inhibitory activity increased. Also, the RP-HPLC fraction containing the peptide with the highest ACE-I inhibitory activity contains a large proportion of hydrophobic amino acids. Hence, this study discovered that *Vigna unguiculata* hydrolysate has ACE-I inhibiting action *in vitro.* To be efficient ACE-I inhibitors, peptides must escape digestion and enter the circulatory system before getting to the target cells. As a result, more study into *in vivo* and clinical antihypertensive efficacy will be required to back up the findings.

Conflict of Interest

The authors have all declared that they have no conflicts of interest.

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