PRODUCTION OF HYDROLYSATES WITH ANTIOXIDANT AND ANGIOTENSIN 1-CONVERTING ENZYME INHIBITORYACTIVITIES FROM COWPEA (*Vigna unguiculata*)

BY

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MAY, 2021

DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. S. M. Abubakar and has not been presented anywhere for the award of degree or certificate. All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this Dissertation and the subsequent write-up by Adekale Idris Adediran, (SPS/16/MBC/00056) were carried out under my supervision.

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APPROVAL

This Dissertation has been examined and approved for the award of Master Degree in Biotechnology

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May Almighty Allah reward you all abundantly (Amen)

Adekale, Idris Adediran

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DEDICATION

This work is dedicated to Almighty Allah, the giver of knowledge, source of wisdom and through His guidance made it possible for me to commence this report and end it successfully. And also to my parents, Mr. and Mrs. Rufai Adekale and my wife, Mrs Adekale Zeenat for the love, moral and financial support they offered me in diverse points of my life.

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ABSTRACT

Legumes are good sources of proteins and their consumption has been associated with the management/prevention of chronic diseases due to their bioactive components. This study investigated cowpea (*Vigna unguiculata*) protein hydrolysate with angiotensin I converting enzyme (ACE) inhibitory and antioxidant activities. Cowpea (*Vigna unguiculata*) protein hydrolysate (CPH) was produced by sequential digestion with pepsin and pancreatin. The CPH was tested for antioxidant properties through DPPH radical scavenging activity, ABTS radical scavenging activity and metal ion chelation assays. The half maximal inhibitory concentration (IC_{50}) obtained for the different assays are; DPPH, 0.334 mg/ml; ABTS, 4.796 mg/ml; and metal ion chelation; 5.872 mg/ml.

CPH was then fractionated via G-50 gel filtration chromatography and reverse phase high performance liquid chromatography (RP-HPLC). The fractions were assayed for ACE inhibitory activity. The G-50 gel filtration chromatography fractions exhibited better ACE inhibitory activity as compared to CPH. The RP-HPLC fraction with the highest ACE inhibitory activity ($P < 0.05$) yielded an IC_{50} of 6.23 μg/ml. The amino acid profile of the RP-HPLC fraction containing peptide with most potent ACE inhibitory activity indicate high amount of hydrophobic amino acids. These results suggest that CPH may have potential for use in the development of functional food that may be useful in prevention and management of hypertension.

CHAPTER ONE

INTRODUCTION

1.1 Background

Hypertension is the leading risk factor of cardiovascular disease which has become a worldwide problem (Forouzanfar *et al*., 2017). It affects one out of four men and one out of five women (WHO, 2015). In fact, an estimated 1.13 billion people worldwide have hypertension, most (two-thirds) living in low- and middle-income countries. (WHO, 2015).

An Angiotensin converting enzyme (ACE) inhibitor is a drug used primarily for the treatment of hypertension and congestive heart failure, as they cause relaxation of blood vessels and decrease blood volume, which leads to lower blood pressure and decreased oxygen demand from the heart. ACE inhibitors inhibit angiotensin-converting enzyme. Angiotensin I - converting enzyme (ACE) (EC 3.4.15.1) which is a key enzyme that regulates blood pressure via the renin-angiotensin system (RAS) (Erdmann *et al*., *2008*; Chen *et al.*, 2009). ACE catalyzes the degradation of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II by cleavage of a dipeptide at the carboxyl-terminal site (Skeggs *et al*., 1956). It is also able to degrade bradykinin, a vasodilator, into inactive peptides (Yang and Erdos, 1967; Yang *et al*., 1970). This dual role enables overall elevation of blood pressure.

Reactive oxygen species (ROS) have been implicated in food deterioration as well as pathogenesis of various human diseases and aging-related disorders (Vidovic *et al*., 2010; Liu *et al*., 2018). Epidemiological studies have revealed the close association between

intake of food rich in antioxidants such as vegetables, fruits, and cereals with prevention of the aforementioned pathologies (Willet, 2001). Intake of exogenous antioxidants is crucial to maintaining an adequate level of antioxidants in order to balance the ROS especially when human *in vivo* antioxidant defense and repair systems are considered to be insufficient to totally prevent the damage. Oxidative stress has been linked to hypertension, imbalance in superoxide and nitric oxide production lead to reduced vasodilation (Munzel and Kearney, 2001; Elahi *et al*., 2009).

Treatment by administration of antihypertensive drugs, inhibitors of the angiotensin I-converting enzyme (ACE) aim to reduce blood pressure and lower the risk of hypertension complications. However, the use of synthetic ACE inhibitors such as captopril and enalapril results in serious side effects including dry cough, skin rashes, and allergic reactions (Choi *et al*., 2001). This situation has prompted the search for potential antioxidant and ACE inhibitors from natural sources.

Combination of both biological activities in one multifunctional preparation, especially in food, are useful for controlling the risk of cardiovascular diseases (Vercruysse *et al*., 2009). In fact, Munzel and Keaney (2001) proposed that ACE inhibitors possess novel antioxidant strategy by improving vasoconstriction, increasing bioactivity of nitric oxide, and inhibiting vascular superoxide production at its source. Therefore, consumption of antioxidant-rich foods which possess ACE inhibitory activity can be considered as an alternative therapy for treatment of hypertension especially for pre hypertensive patients whose blood pressure is marginally or mildly high but not high enough to warrant the prescription of blood-pressure-lowering medications (Chen *et al*., 2009).

Enzymatic hydrolysis of proteins can improve their chemical, functional and nutritional properties. Casein, whey and soy are the most commonly used protein sources in food protein hydrolysates production (Cho *et al*., 2004). Protein hydrolysates are used to obtain protein fractions and bioactive peptides, which are short peptide chains with 2 to 15 amino acid residues (Vioque *et al*., 2000).

Food-derived bioactive peptides with single activities have been well documented, yet only few peptides with multiple functions have been reported. Meanwhile, peptides with multifunctional bioactivities will be more preferred over single activity peptides as the former will simultaneously elicit multiple health benefits (García-Mora *et al*., 2017).

Legumes are known to contain bioactive peptides, beneficial nutrients and high-quality proteins. Legumes are especially promising due to their high protein content and diverse physiological activities in the human organism, including an antihypertensive effect (Ruiz-Ruiz *et al*., 2013). Legume-derived proteins are important food ingredients that can improve the nutritional and technological aspects of food formulations, as well as provide potential benefits to human health (Ruiz-Ruiz *et al*., 2013). Different peptides derived from several legumes (e.g. velvet bean Mucunapruriens (Chel-Guerrero *et al*., 2017) cowpea (*Vigna unguiculata*) and Jamapa bean (*Phaseolus vulgaris*), exhibit antihypertensive activities (Betancur-Ancona *et al*., 2015; Drago *et al*., 2016).

This research was undertaken to fractionate cowpea (*Vigna unguiculate*) hydrolysate and identify the amino acid composition of the peptide fraction responsible for the potent ACE inhibitory and antioxidant activities.

1.2 Statement of the Research Problem

There has been an increase in the incidence of hypertension and cardiovascular mortality in sub-Saharan Africa over the past years and is expected to double by the year 2030 (Atakite *et al*., 2015). In 2015, about 20 million cases of hypertension have been recorded among people aged at least 20 years in Nigeria with a prevalence of about 28%. (Adeloye *et al*., 2015). Inhibitors of angiotensin I-converting enzyme (ACE) are used as antihypertensive drugs in the treatment of hypertension by reducing blood pressure and lowering the risk of hypertension complications. Synthetic ACE inhibitors such as captopril and enalapril have been used in the management of hypertension. Both ACE inhibitors are equally active to increase activities of superoxide dismutase and glutathione peroxidase, hence they are also antioxidants (Bartosz *et al*., 1997). However, Captopril treatment bring unwanted side effects such as renovascular disease, cough, neutropenia, skin rashes, oedema and taste disturbance caused by the sulfhydryl moiety (Choi *et al*., 2001). Due to these unwanted side effects there is a need to explore natural products that can serve as both an ACE inhibitor and an antioxidant.

1.3 Justification

ACE inhibitors possess novel antioxidant strategy by increasing bioactivity of nitric oxide and inhibiting vascular superoxide production at its source (Munzel and Kearney, 2001). Therefore, consumption of antioxidant-rich foods which possess ACE inhibitory activity can be considered as an alternative in the management of hypertension (Munzel and Kearney, 2001). In recent years, peptides from various dietary sources such as legumes e.g. velvet bean (*Mucunapruriens*) (Chel-Guerrero *et al*., 2017) and Jamapa bean, (*Phaseolus vulgaris*) (Betancur-Ancona *et al*., 2015; Drago *et al*., 2016) have been shown to have clearly positive effects on health by functioning as antihypertensive and

antioxidants. However, there is paucity of information on the multifunctional properties (ACE inhibitory and radical scavenging properties) of the bioactive peptides present in Nigerian cowpea (*Vigna unguiculata*) hydrolysates.

1.4 Aim

The aim of this study was to produce hydrolysates with antioxidant and angiotensin 1-converting enzyme inhibitory activities from *Vigna unguiculata* (cowpea).

1.5 Objectives

1. To produce hydrolysates using pepsin and pancreatin from *Vigna unguiculata* (cowpea)
2. To fractionate the crude hydrolysates via RP-HPLC and to conduct amino acid profiling on the fraction.
3. To determine the antioxidant and ACE inhibitory activities of the crude hydrolysates and RP-HPLC fractions.

CHAPTER 2

LITERATURE REVIEW

2.1 Hypertension

Cardiovascular diseases (CVD) and metabolic disorders such as obesity, diabetes and atherosclerosis can provoke hypertension or high blood pressure (WHO, 2011). Hypertension is a very important chronic disease affecting one third of adults worldwide and causing about half of the total mortalities, mainly due to stroke and heart problems. In fact, it accounts for 9.4 million deaths worldwide every year (WHO, 2013). The global burden of hypertension is increasing at a disturbing rate with an estimated 25% increment in the next ten years (Mittal and Singh, 2010).

Hypertension is a disease of complex nature (polygenic and heterogenous), where the combined effect of mutations and polymorphisms at several genes determine blood pressure (Hingorani and Brown, 1996). These genes probably encode for proteins determining renal sodium reabsorption (i.e., components of the renin-angiotensin-aldosterone system, epithelial sodium channels, etc.), which may be affected by environmental factors such as stress and salt intake (Thibonnier and Schork, 1995; Lifton, 1996). It is not surprising then, that multiple mechanisms contribute to hypertension.

Hypertension can be subdivided into two;

i. Essential hypertension

ii. Secondary hypertension

2.1.1 Essential hypertension

Essential hypertension also referred to as primary or idiopathic hypertension affects about 95% of hypertensive patients, hence it is the most common type of hypertension (Carretero and Oparil, 2000). It is considered to be due to both environmental and genetic factors (Bűssemaker *et al*., 2010). The environmental factors that contribute to hypertension include low calcium intake, sedentary lifestyle, aging, low potassium intake, high salt intake etc. (Carretero and Oparil, 2000). Individuals with normal weight are less prone to hypertension compared to obese individuals. Obesity increases the risk of hypertension to by 5- folds and about two-thirds of hypertension cases are due to overweight. Individuals with Body Mass Index greater than 25 account for about 85% of hypertension cases (Haslam and James, 2005).

2.1.2 Secondary hypertension

Medical conditions such as renal parenchymal disease, renal artery stenosis, and hyperaldosteronism can cause secondary hypertension (Grossman and Messerli, 2012). Medications such as Non-Steroidal Anti-inflammatory Drugs (NSAIDs), corticosteroids, birth control pills etc. can also cause temporary high blood pressure. Increase in high blood pressure as well as interference with antihypertensive drugs e.g. beta blockers, diuretic agents as well as Angiotensin Converting Enzyme Inhibitors (ACEI) except for Ca^{2+} antagonist and central-acting drugs can be due to the usage of NSAIDs like Indomethacin, Naproxen, Piroxicam, etc. (Grossman and Messerli, 2012).

2.2 Renin Angiotensin Aldosterone System (RAAS)

The maintenance of blood pressure, fluid and water balance in mammals is regulated by a complex feedback circuit known as the Renin Angiotensin Aldosterone System (RAAS). It is involved in both short-term and long-term regulation of blood pressure. Hence, it is considered a hormonal regulator of blood pressure together with sodium, potassium and water balance. The development and maintenance of high blood pressure have been associated with the increasing activity of RAAS, hence there is an increasing interest in the pharmacological treatment of hypertension that involves the RAAS (Sparks *et al*., 2014).

2.2.1 Components of RAAS

The biosynthesis of renin by the juxtaglomerular cells that line the afferent arteriole (and occasionally the efferent) of the renal glomerulus, is the onset of the Renin Angiotensin Aldosterone hormonal cascade. Renin is synthesized as a prehormone and mature (active) renin is formed by the proteolytic removal of a 43 amino acids prosegment peptide from the N-terminus of prorenin, the proenzyme or renin precursor (Jia *et al*., 2013).

Renin regulates the initial, rate-limiting step of the RAAS by cleaving the N-terminal portion of a large molecular weight globulin, angiotensinogen, to form biologically inert decapeptide Ang1 or Ang1-10 (Jia *et al*., 2013). The liver is the primary source of systemic circulating angiotensinogen but angiotensinogen mRNA expression has also

been detected in many other tissues such as kidney, brain, placenta and adipose tissue (Morgan *et al*., 1996).

The inactive decapeptide Ang1 is hydrolyzed by the angiotensin- converting enzyme (ACE) which remove the C-terminal dipeptide to form the octapeptide AngII (Ang1-8) a biologically active, potent vasoconstrictor. ACE metabolizes a number of other peptides including the vasodilator peptides, bradykinin and kallidin to inactive metabolites (Carey and Siragy, 2003). Thus, the enzymatic actions of ACE potentially result in increased vasoconstriction and decreased vasodilation.

Ang II is the primary active product of RAAS but there is evidence of other metabolites of Ang I and Ang II with biological activities particularly in the tissues. Ang III and Ang IV are formed by the sequential removal of amino acids from the N-terminus of Ang II by action of aminopeptidases. They are most likely produced in tissues with high levels of aminopetidases A&N such as brain and kidney tissue (Reudelhuber, 2005). Ang III (Ang 2-8) a heptapeptide formed by removal of the first N-terminal amino acids is present in the central nervous system where it is thought to play an important role in tonic blood pressure maintenance and in hypertension (Reudelhuber, 2005). Ang IV (Ang 3-8) is a hexapeptide formed by further enzymatic degradation of Ang III (Reudelhuber, 2005). In the brain, Ang IV increases blood pressure by co-operating with Ang II on Ang II type 1 (AT_1) receptor signaling because its hemodynamic effects require the presence of both Ang II and functional AT_1 receptors (Reudelhuber, 2005).

Ang 1-7, a heptapeptide fragment of Ang II can be formed from Ang II by action of carboxypeptidases (cleaves at C-terminal). Unlike ACE, these enzymes cannot convert

Ang I to Ang II and their activity is not affected by ACE inhibitors (ACEIs) (Carey and Siragy, 2003). ACE 2 can also cleave a single amino acid from the C-terminus of Ang I to form Ang 1-9, a peptide with no known function at the time.

Ang II is the primary effector of a variety of RAAS-induced physiological and pathophysiological actions. The AT1 receptor also mediates effects of Ang II on cell growth and proliferation, inflammatory responses, and oxidative stress (Ferrario, 2006). This receptor, which is typical of the G protein-coupled receptor superfamily containing 7 membrane-spanning sequences, is widely distributed on many cell types in Ang II target organs (Cristina *et al*., 2007).

The type 2 (AT_2) receptor is abundant during fetal life in the brain, kidney, and other sites, and its levels decrease markedly in the postnatal period (Carey and Siragy, 2003). There is some evidence that, despite low levels of expression in the adult, the AT_2 receptor might mediate vasodilation and antiproliferative and apoptotic effects in vascular smooth muscle and inhibit growth and remodeling in the heart (Carey and Siragy, 2003; Stanton, 2003). In the kidney, it has been proposed that activation of AT_2 receptors may influence proximal tubule sodium reabsorption and stimulate the conversion of renal prostaglandin E2 to prostaglandin F2α (Ferrario, 2006; Carey and Siragy, 2003). However, the importance of any of these AT_2-mediated actions remains uncertain.

The type 4 (AT_4) receptors are thought to mediate the release of plasminogen activator inhibitor 1 by Ang II and by the N-terminal truncated peptides (Ang III and Ang IV), but the function of the type 3 (AT_3) receptors is unknown (Stanton, 2003). Lastly, the putative effects attributed to the C-terminal truncated peptide Ang 1-7, including vasodilatation, natriuresis, antiproliferation, and cardiac protection, are presumed to be

mediated by a unique receptor that does not bind Ang II, most likely a product of the Mas proto-oncogene known as the Mas receptor (Stanton, 2003).

Ang II, via the AT_1 receptor, also stimulates the production of aldosterone by the zona glomerulosa, the outermost zone of the adrenal cortex (Cristina *et al*., 2007). Aldosterone is a major regulator of sodium and potassium balance and thus plays a major role in regulating extracellular volume. It enhances the reabsorption of sodium and water in the distal tubules and collecting ducts (as well as in the colon and salivary and sweat glands) and thereby promotes potassium (and hydrogen ion) excretion (Funder, 2002). Ang II, together with extracellular potassium levels, are the major regulators of aldosterone, but Ang II synthesis may also be stimulated by adrenocorticotrophic hormone (ACTH; corticotropin), norepinephrine, endothelin, and serotonin and inhibited by atrial natriuretic peptide and nitric oxide (NO). It is also important to note that Ang II is a major trophic factor for the zona glomerulosa, which can atrophy (reversibly) in its absence (Teresa *et al*., 2018).

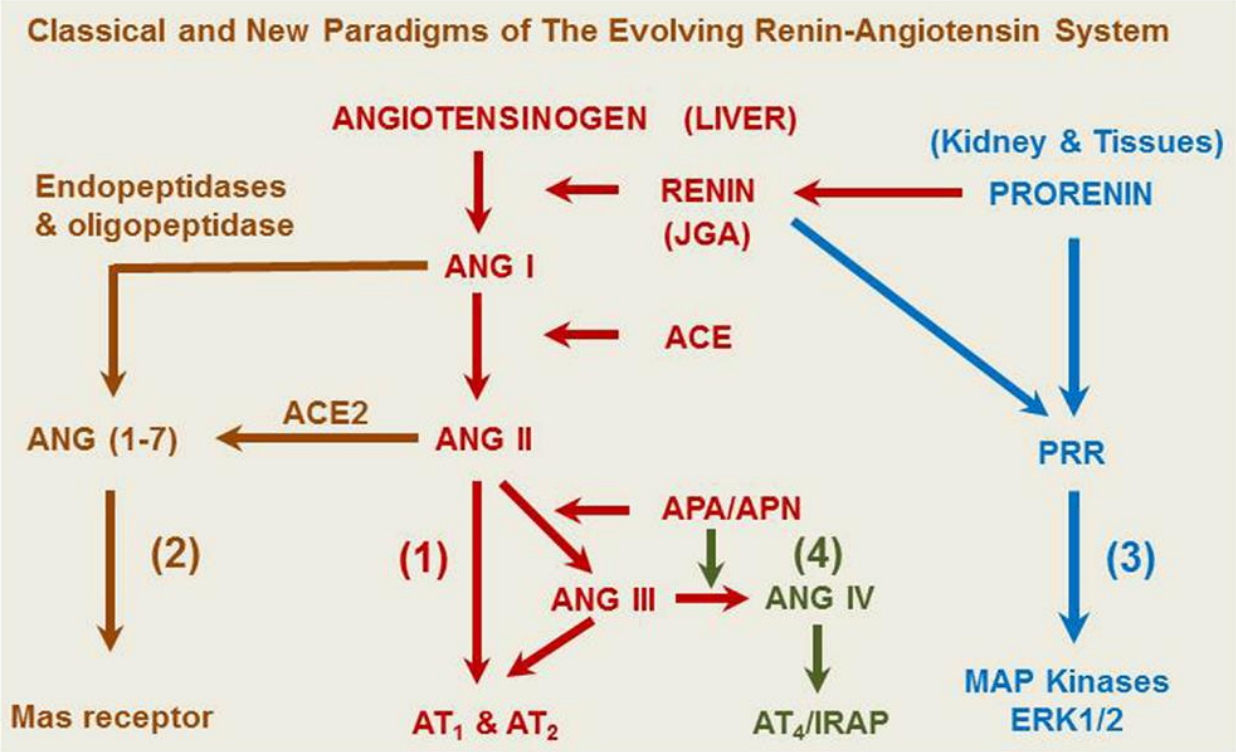


Figure 2.1: A representative overview of the renin-angiotensin system (Jia *et al*., 2013)

2.3 Angiotensin-Converting Enzyme (ACE)

The angiotensin-converting enzyme (ACE) is a zinc-containing dipeptidase, which was discovered in 1956 (Skeggs *et al*., 1956). Angiotensin I Converting Enzyme (ACE) [EC 3.4.15.1], belongs to the class of zinc proteases that are activated by the presence of zinc and chloride (Lee *et al*., 2010). ACE gene in human is located on chromosome 17 and consists of 25 exons (Igic and Skrbic, 2014). This enzyme exhibits important biological functions in the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor (Ng *et al*., 1967). It also shows functions in the degradation of bradykinin, a potent vasodilator, and other vasoactive peptides (Imig, 2004). These two actions endow this enzyme with critical roles in blood-pressure regulation. As a result, ACE has become a promising target in the treatment of hypertension, heart failure, and diabetic nephropathy.

ACE is epresssed in many different cell types, such as neuronal cells, monocytes, renal proximal tubular cells and epididymal cells, but its principal location is in endothelial cells. Since the vascular (especially capillary) endothelium covers a large surface area, the major site of angiotensin II production in the body is the blood vessels (Ruilope *et al*., 2007). ACE is attached to the endothelial surface membrane by an anchor peptide, which can be cleaved to release the soluble enzyme, although only a small percentage is released into the circulation (Ruilope *et al*., 2007). Therefore, the amount of ACE in the circulation is relatively low compared with that in the vascular endothelium.

2.3.1 Mechanism of action

In the presence of Zn^{2+} ACE hydrolyzes the peptide bond of Phe8-His9 of decapeptide angiotensin I (Ang I) to release octapeptide angiotensin II (Ang II) and C-terminal dipeptide His-Leu. Ang II is one of the most potent vasoconstrictor. Upon binding with its type 1 receptor, AngII contracts vascular smooth muscle, stimulates the secretion of aldosterone and Na+, K+-reabsorption in kidney, and induces water-sodium retention and blood volume increase. These subsequently result in blood pressure increase and the positive inotropic effect and chronotropic effect in heart. ACE inactivates the vasodilator by the sequential cleavage of two C-terminal dipeptides. It was named as kininase 2, and besides the substrates of angiotensin I and bradykinin, ACE can also hydrolyze a wide range of vasoactive peptide, such as enkephalin, substance P, and neurotensin (Skidgel and Erdös, 2004). ACE can inhibit amyloid-peptide overexpression in Alzheimer's disease *in vitro* (Hu *et al*., 2001).

2.4 Angiotensin-Converting Enzyme Inhibitors (ACEIs)

Angiotensin converting enzyme (ACE) inhibitors constitute an established therapy in the treatment of hypertension (Nyman *et al*., 1998). Early studies performed in the 1960s showed that peptides from the venom of the Brazilian arrowhead viper (*Bothrops jararaca*) inhibited kinase II, an enzyme that facilitates degradation of bradykinin, and which was later shown to be identical to ACE (Ferrario, 2006). Synthetic analogues of the peptide fraction of snake venom, such as the nonapeptide teprotide, were shown to lower blood pressure in patients with hypertension and produce beneficial hemodynamic effects in patients with heart failure (Ferrario, 2006). These findings encouraged the search for orally active inhibitors of ACE; the first of these, captopril, was designed based on known inhibitors of another zinc-containing metalloprotease, carboxypeptidase A, and included a sulfhydryl-containing amino acid to serve as ligand for the zinc moiety (Ferrario, 2006).

ACEIs competitively block the action of ACE and thus the conversion of Ang I to Ang II, thereby reducing circulating and local levels of Ang II (Grossman and Messerli, 2012). ACEIs also decrease aldosterone and vasopressin secretion and sympathetic nerve activity, but there is controversy regarding their efficacy in blocking other "tissue" actions of the RAAS (Lopez-Sendon *et al*., 2004). Short-term ACEI therapy is associated with a decrease in Ang II and aldosterone and an increase in renin release and Ang I. There is some evidence, however, that over the long term ACE inhibition may be associated with a return of Ang II and aldosterone toward baseline levels ("ACE escape")—perhaps, it is proposed, through activation of the so-called alternate pathways (Pitt, 1995; Lopez-Sendon *et al*., 2004). In general, short-term pharmacodynamic

responses to decreases in Ang II through inhibition of ACE include dose dependent reductions in cardiac preload and afterload, with lowering of systolic and diastolic blood pressure, but, in normotensive and hypertensive patients without cardiac dysfunction, little or no change in cardiac output. Because ACE is identical to kininase II, ACEIs may also lead to elevation of bradykinin levels in some tissues (but unlikely in the circulation); this effect is potentially associated with increased bradykinin-dependent release of NO and vasoactive prostaglandins, including prostacyclin and prostaglandin E2 (Lopez-Sendon *et al*., 2004). These actions may potentially contribute to the vasodilatory, antithrombotic, antiatherogenic, and antiproliferative effects of ACEI, although the importance of this pathway is debated (Lopez-Sendon *et al*., 2004).

In 40% to 60% of patients with mild-to-moderate hypertension, ACEI monotherapy produces a satisfactory reduction in blood pressure (Ibrahim, 2006). In this population, ACEIs contribute to reversal of cardiac hypertrophy, and do so with significantly greater efficacy than beta blockers (Klingbeil *et al*., 2003). ACEI therapy is generally well tolerated by most patients but is nonetheless associated with some significant side effects. Most frequent among these is a dry cough, which has been attributed to accumulation of substance P (which is normally degraded by kininase II). More serious side effects common to all ACEIs include angioedema (which is potentiated by decreased catabolism of kinins) and foetal abnormalities and mortality (Wong *et al*., 2004). Other "physiologic" consequences of ACE inhibition may include hypotension, deterioration of renal function, and hyperkalemia. Lastly, toxic effects, associated mainly with captopril, include abnormal (metallic or salty) taste, rash, neutropenia, hepatic toxicity, and proteinuria (membranous nephropathy) (Wong *et al*., 2004).

2.5 Reactive Oxygen Species (ROS) and Hypertension

In the past decade, it has become widely recognized that ROS contribute to hypertension. An initial study showed that bolus administration of a heparin binding form of Superoxide dismutase (SOD) acutely lowered blood pressure in hypertensive rats (Nakazono *et al*., 1991). Studies have shown that membrane-targeted forms of SOD lower blood pressure in angiotensin- II-induced hypertension (Fukui *et al*., 1997; Landmesser *et al*., 2002; 2003; Laursen *et al*., 1997).

It appears that the NADPH oxidases are critical sources of ROS in hypertension (Mueller *et al*., 2005). These enzymes, also known as the Nox enzymes, are a predominant vascular source of ROS not only because they directly produce ROS, but also because they stimulate other ROS-generating enzymes (Mueller *et al*., 2005). The Nox proteins represent the catalytic subunits of the NADPH oxidases, and vary in terms of their mode of activation and need for co-factors (Lassegue *et al*., 2003). In vascular cells, Nox1 is minimally expressed under basal conditions, but stimuli such as platelet de-rived growth factor (PDGF), angiotensin II, and serum can increase its expression substantially (Lassegue *et al*., 2003)

A variety of pathological stimuli, such as angiotensin II, stretch, endothelin-1, thrombin, and catecholamines, acutely activate the NADPH oxidases in both vascular smooth muscle and endothelial cells, likely by promoting phosphorylation of p47*phox* and perhaps other subunits (Groemping and Rittinger, 2005). Interestingly, pathophysiological stimuli such as angiotensin II, hypercholesterolemia, growth factors,

and serum can also increase expression of several of the NADPH oxidase subunits, including p22*phox* and Nox1 and Nox4, further promoting ROS production (Lassegue *et al*., 2003).

At first glance, a compelling explanation for how ROS might cause hypertension is that the increased O_2 oxidizes NO to peroxynitrite and subsequently nitrite and nitrate. This results in a loss of "bioactive" NO-mediated vasodilatation, an increase in vasoconstriction, and subsequently an increase in systemic vascular resistance (Figure 2.2A). Assuming that cardiac output is unchanged (the usual situation in hypertension), this increase in systemic vascular resistance would elevate blood pressure. This hypothesis has been supported by studies in which we made mice with targeted overexpression of the NADPH oxidase in vascular smooth muscle cells and showed that these animals have augmented hypertension in response to angiotensin II infusion (Weber *et al*., 2005).

Despite the attractiveness of this scheme, there is evidence that ROS production in organs other than the vessel can contribute to hypertension. For example, all NADPH oxidase subunits have been identified in the kidney and there is up-regulation of p47*phox* and p67*phox* in the kidneys of spontaneously hypertensive rats (Chabrashvili *et al*., 2002). In rats, chronic angiotensin II infusion increases expression of Nox1 and p22*phox* and decreases expression of extracellular SOD (ec- SOD) in the kidneys (Wilcox, 2005). In renal tubular cells, increased levels of O_2 and diminished NO promote sodium reabsorption and alter tubuloglomerular feedback, leading to sodium- and volume-dependent hypertension (Wilcox, 2003; 2005). Increased superoxide activity has been associated with nuclear factor _B activation and apoptosis in tubulointerstitial cells

(Quiroz *et al*., 2003). Together, these effects of ROS cannot only stimulate sodium and volume retention but cause nephron loss, leading to hypertension (Figure 2.2B).

There is also evidence that ROS can contribute to central causes of hypertension. Researches have shown that intracerebroventricular (ICV) administration of angiotensin II stimulates O_2 production in the circumventricular organs (Zimmerman *et al*., 2002; 2004). These authors also showed that ICV injection of an adenovirus expressing SOD (which reaches the circumventricular organs because of the poorly developed blood-brain barrier) prevents both the hypertension and dipsogenic effect of angiotensin II (Zimmerman *et al*., 2002). In a subsequent study, they also demonstrated that overexpression of SOD in the circumventricular organs markedly reduced the hypertension caused by systemic angiotensin II infusion (Zimmerman *et al*., 2004). More recently, the NADPH oxidase in the hypothalamus has also been implicated in angiotensin-II-induced hypertension (Erdos *et al*., 2006). Taken together, these studies indicate that centrally produced ROS can stimulate sympathetic outflow, leading to hypertension (Figure 2.2C).

Finally, there is evidence that carotid baroreflex resetting, an important consequence of hypertension, is mediated by ROS (Li *et al*., 1996). This can prevent the normal bradycardia and sympathetic withdrawal that accompanies elevations of pressure, and thus play a permissive role in hypertension (Figure 2.2D).

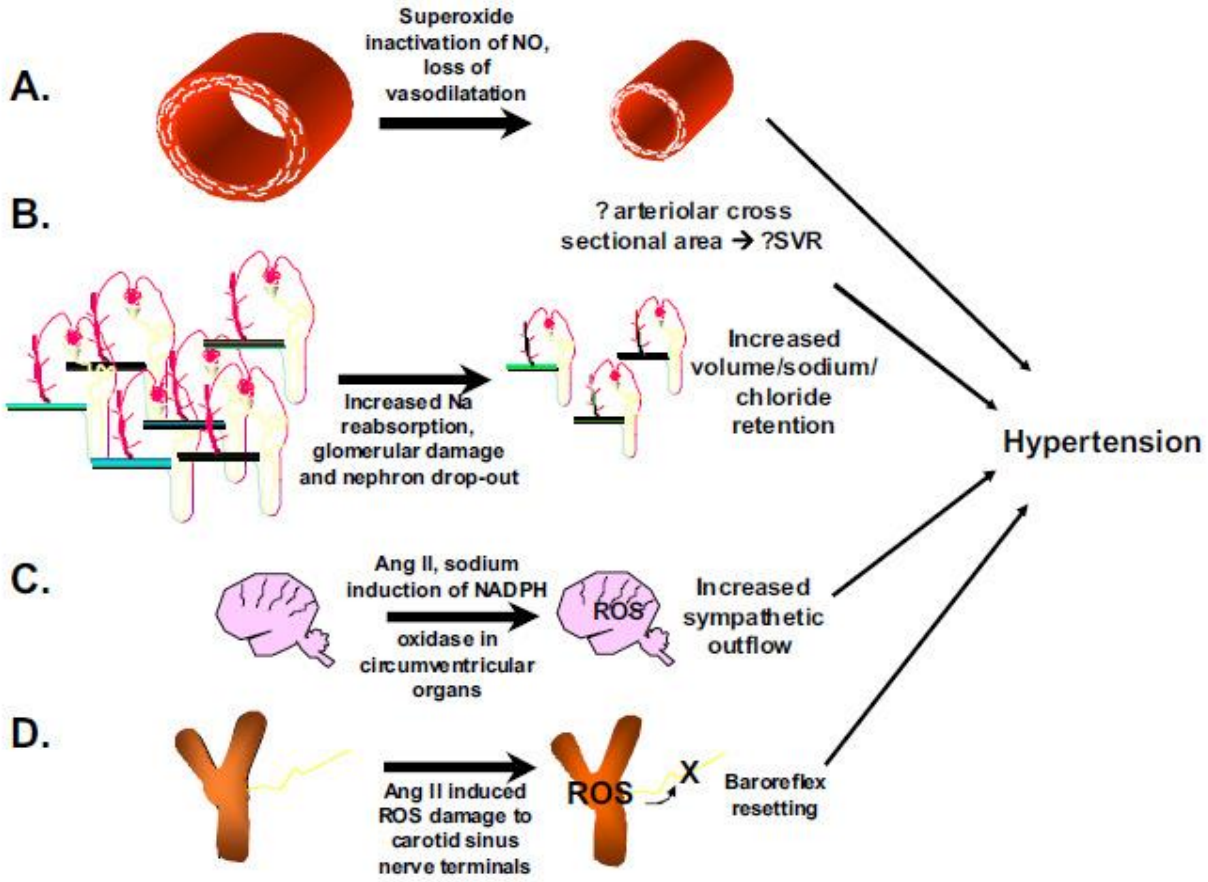


Figure 2.2: Effect of reactive oxygen species (ROS) on various organs leading to hypertension.
(A) ROS can promote vasoconstriction by inactivation of nitric oxide (NO), leading to an increase in systemic vascular resistance. (B) ROS can cause nephron damage and drop-out, promoting hypertension. (C) In the central nervous system, ROS in the circumventricular organ stimulates sympathetic outflow and hypertension. (D) ROS produced in the vessel wall can damage afferent nerve terminals, leading to alteration of the baroreflex (Harrison *et al*., 2007).

2.6 Antioxidants

Oxidation reactions that produce free radicals start the chain reaction in damaging cells while a molecule called antioxidant slows or prevents the oxidation, donates one of its own electrons to end the electron stealing reaction hence neutralizing the free radicals (Cheeseman and Slater, 1993). Potential antioxidants especially natural antioxidants have received great attention and have been studied in fruits, teas, vegetables, cereals and medicinal plants, as they are effective free radical scavengers and assumed to be less toxic compared to synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT) that were also suspected as carcinogenic agents and can cause liver damage (Ito *et al*., 1986). Antioxidants have a potential protective role on cardiovascular system that focused on reversing the endothelial dysfunction caused by oxidative stress (Escobales *et al*., 2005).

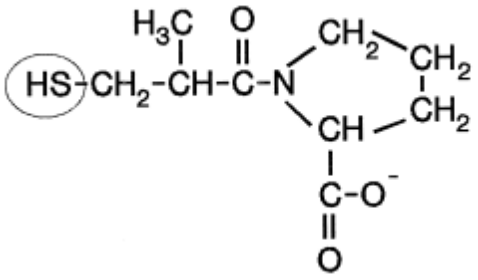
2.6.1 ACE inhibitors are also antioxidants

Captopril ([2S]-1-[3-mercapto-2-methyl-propionyl]-L-proline) (Figure 2.3(A)) was the first ACE inhibitors found and has important role in reducing hypertension. It protects erythrocytes from haemolysis caused by 2,2'-azobis (2- amidinopropane) (AAPH) and hypochlorite, inhibited ascorbate autoxidation caused by Cuprum (Cu^{2+}) that indicates the role of Captopril as antioxidant (Bartosz *et al*., 1997). Together with Enalapril (Figure 2.3(B)), both ACE inhibitors are equally active to avert oxidation of Low Density

Lipoprotein (LDL) and increase activities of superoxide dismutase and glutathione peroxidase in experimental animal's liver, while activities of erythrocyte Na^+, K^+-ATPase and Ca^{2+} -ATPase in hypertensive patients is increased as well (Bartosz *et al*., 1997). Compared to Enalapril, Captopril has advantages as it has the ability to prevent arrhythmias caused by ischemia or reperfusion, to protect cultured endothelial cells against free radical injury as well as anti-inflammatory activity (Bartosz *et al*., 1997).

However, Captopril treatment is associated with unwanted side effects such as renovascular disease, cough, neutropenia, skin rashes, angioneurotic oedema and taste disturbance due to the sulfhydryl moiety (Choi *et al*., 2001). Due to these unwanted side effects there is a need to explore natural products that can serve both as ACE inhibitor and an antioxidant.

A.



B.

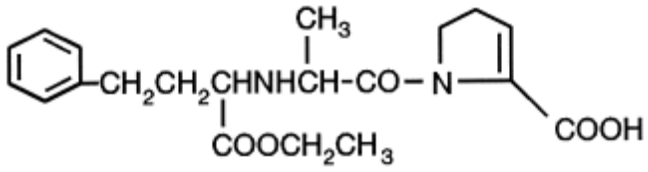


Figure 2.3: Chemical structures of ACE inhibitors, A. Captopril and B. Enalapril (Bartosz *et al*., 1997).

2.7 Bioactive Peptides

Bioactive peptides are specific protein fragments with beneficial impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). For several years, identification of bioactive peptides in plant and animal sources have been increasing due to new scientific methods. These bioactive peptides have high nutraceutical potential with great benefits for human health; i.e, on blood pressure and lipid metabolism; as well as anticancer, immunomodulatory, analgesic, antimicrobial, antioxidants, anti-thrombosis, anti-atherosclerotic, opioid and anti-inflammatory; some of them have more than one activity. They are also useful to improve the absorption of minerals (Hartmann and Meisel 2007; Cicero *et al*. 2011; Udenigwe and Aluko 2012; Aluko 2015; Cicero and Colletti 2015; Maestri, *et al*., 2016; Bouglé and Bouhallab 2017). The inherent amino acid composition and sequence of these peptides determines their activity. The size of active sequences may vary from two to twenty amino acid residues and multifunctional properties are exhibited by many of these peptides (Meisel and FitzGerald, 2003).

Bioactive peptides are product of the hydrolysis by gastrointestinal digestive enzymes (pepsin, trypsin, and chymotrypsin), or by *in vitro* producers with enzymes, temperature or pH. Characteristics of the bioactive peptides are; the presence of hydrophobic amino acids in their sequences, a positive charge, the resistance to digestion by proteases and peptidases, and a proline C terminal. Small peptides with a dipeptide of proline-proline at their C terminal are the most resistant to degradation by proteases and peptidases of stomach, pancreas or intestine. Outside the intestinal epithelium, large peptides (with 6 or more) are usually more active (Galvez *et al*. 2001; Korhonen and Pihlanto 2003; Panchaud *et al*., 2012; Perez-Espitia *et al*. 2012; Maestri *et al*., 2016). Models (*in silico, in vitro, ex vivo* and *in vivo*) have been reported, with over 3566 different potential bioactive peptides and distinct biological activities; this information has been compiled into a peptide database (BIOPEP database) (Minkiewicz *et al*. 2008). Bioactive peptides are identified by mass spectrometry, and used for comparative proteomics.

2.8 Production of Bioactive Peptides

The most common methods to produce bioactive peptides have been by enzyme hydrolysis of food proteins or fermentation (Lee and Hur, 2017). In few situations however, water extracts of mushrooms and some plant parts have been found to contain bioactive peptides (Geng *et al*., 2016).

2.8.1 Enzymatic hydrolysis

The protein material is subjected to enzyme hydrolysis at a given temperature and pH (Norris and FitzGerald, 2013; He *et al*., 2016; Huang *et al*., 2016; E Silva *et al*., 2017).

The use of enzymatic hydrolysis to produce bioactive peptides is preferred than microbial fermentation due to the short reaction time, ease of scalability and predictability. More than a single proteolytic enzyme (whether purified or crude) can be used to hydrolyze the protein to produce the hydrolysate containing short peptide sequences. However, addition of the enzymes (whether simultaneously or sequentially) would depend on the optimal pH and temperature of the enzymes (Khiari *et al*., 2014; Zhang *et al*., 2016; Cai *et al*., 2017; Sangsawad *et al*., 2017). Though no specific proteolytic enzymes are known to produce specific bioactive peptides in foods, subtilisin hydrolysis tends to yield low molecular weight peptides some of which are bioactive (Huang *et al*., 2017). For instance, it was found that *Achatina fulica* (snail) foot muscle protein hydrolyzed using subtilisin had a higher number of small molecular weight peptides than samples hydrolyzed by papain and trypsin (Huang *et al*., 2017). Subtilisin hydrolysed rice bran proteins generated the highest number of low molecular weight peptides and showed higher biological activity than samples hydrolyzed with a cysteine endopeptidase, papain and pepsin (Zhang *et al*., 2012). However, the enzyme to substrate ratio is an important factor to consider so as to obtain a good degree of hydrolysis. Peptide sequences and their biological activities may differ depending on the type of enzyme used (Mojica and de Mejia, 2016). Low molecular weight peptides (<10 kDa) have been found to be more effective antioxidants and antihypertensive peptides (Fernandez-Musoles *et al*., 2013; Ruiz-Ruiz *et al*., 2013; Garcia-tejedor *et al*., 2014; Wattanasiritham *et al*., 2016) than high molecular weight peptides and hence proteases that yield low molecular weight peptides would be helpful for commercial production of antioxidant and antihypertensive peptides.

The type of enzyme used, the temperature and the time allowed for hydrolysis affect the extent of hydrolysis and may also affect the type of peptides generated in the hydrolysates. For instance, it was observed that stronger anti-tyrosinase and anti-inflammatory activities after hydrolyzing rice derived proteins with bacillolysin than samples hydrolyzed with subtilisin while cysteine endo-peptidase, papain and leucyl aminopeptidase hydrolyzed samples showed the lowest bioactivity (Ferri *et al*., 2017). Bacillolysin hydrolysed samples had the strongest anti-angiotensin converting enzyme (ACE) activity followed by cysteine endopeptidase, and then subtilisin (Ferri *et al*., 2017). However, other studies found that chymotrypsin or cysteine endopeptidase hydrolyzed rice proteins had strong antioxidant abilities (Zhang *et al*., 2010; Dei pui *et al*., 2014). After enzymatic hydrolysis, the mixture was centrifuged to separate the supernatant which contains low molecular weight peptides from the precipitates (Ahn *et al*., 2009; Nimalaratne *et al*., 2015; Zhang *et al*., 2016). The peptides may be recovered by freeze-drying, desalting (Zhang *et al*., 2012), cross-flow membrane filtration (Ferri *et al*., 2017), and membrane ultrafiltration or column chromatography.

2.8.2 Microbial fermentation

This involves culturing some bacteria or yeast on protein substrates to hydrolyze the proteins with their enzymes as they grow. The growing bacteria or yeast secret their proteolytic enzymes into the protein material to release peptides from the parent proteins (Lee and Hur, 2017). Usually, the bacterium of choice is grown to its exponential phase in a broth at a temperature suitable for the bacterial growth. The cells are then harvested, washed and suspended in sterile distilled water (usually containing glucose) and used as a

starter to inoculate a sterilized protein substrate (Aguilar-Toalá *et al*., 2017; Rizzello *et al*., 2017) The extent of hydrolysis would depend on the strain used, the type of protein and the fermentation time. We observed that whey fermented by *Lactobacillus brevis* had a stronger ACE inhibitory ability than those fermented with Lb. acidophilus, *Lb. bifermentan, Lb. casei, Lb. helveticus, Lb. lactis, Lb. paracasei, Lb. plantarum* and *Lb. reuteri* (Ahn *et al*., 2009). This shows that the functionality of protein hydrolysates may differ between cultures since microorganisms have different proteolytic systems (Daliri *et al*., 2016).

Apart from bacteria starter, yeast (Chaves-Lopez *et al*., 2012; García-Tejedor *et al*., 2014; García-Tejedor *et al*., 2015; Rai *et al*., 2016) and filamentous fungus (Giri *et al*., 2012; Hou *et al*., 2014; Lima *et al*., 2015) have also been used in producing bioactive peptides. Proteins can be co-cultured using a combination of different bacteria or even yeast and bacteria to accelerate the proteolytic process (Chaves-Lopez *et al*., 2014). After fermentation, the mixture is centrifuged and the supernatant recovered. The supernatant may then be subjected to further hydrolysis using proteolytic enzymes to obtain shorter peptide sequences (Babini *et al*., 2017). Alternatively, the low molecular weight peptides in the supernatant can be recovered by solvent extraction or other methods, purified and their amino acid sequences determined by mass spectrometry.

2.9 Functional Properties of Bioactive Peptides

The composition of amino acids presents in each peptide and the different reactivity confer the wide range of benefits, bioactive peptides have on the human body (García-Mora *et al*., 2017). The most studied are antihypertensive, anti-cholesterolemic,

antioxidant, anti-inflammatory, anti-cancer, anti-microbial and immunomodulatory properties. Some peptides have multimodal activities and hence, exert more than one beneficial effect (Meisel 2004; Hartmann and Meisel 2007; Santiago-Lopez *et al*. 2016). This review will be limited to the antihypertensive and antioxidant properties of bioactive peptides

2.9.1 Antihypertensive properties

The use of food derived drugs in the treatment or prevention of this condition will be of great impact in reducing the side effects of antihypertensive drugs (Sirtori *et al*., 2015; Borghi and Cicero 2017).

The renin-angiotensin aldosterone system (RAS) is the main mechanism to regulate blood pressure. This system plays an important role in the control and regulation of blood pressure and salt balance. Antihypertensive drugs, like Captopril, Lisinopril and Enalapril, inhibit the renin-angiotensin system but produce some side effects (sleep apnea, dry cough, angioedema, etc). Some bioactive peptides show competitive and/or noncompetitive ACE inhibition, which is responsible of the conversion of angiotensin I in angiotensin II. Angiotensin-I catalyzes the conversion to the vasoconstrictor angiotensin II, which increases the peripheral vascular resistance, inducing hypertensive action. ACE also determines the cleavage and inactivation of bradykinin, a vasodilator peptide. The increased of protein intake has been associated with a reduced risk of increased blood pressure and coronary heart diseases.

Food-derived peptides have been identified as potential antihypertensive agents. Peptides with ACE inhibitory activity are small and have hydrophobic amino acids (W, Y, F, and P) in C-terminal which determine the affinity to the active site of the enzyme. In addition,

the effectiveness of these peptides depends on their resistance to degradation, to the absorption into the blood stream and to amino acid sequence. Many peptides have been identified with this activity in protein of plants, animals and microorganisms, and the beneficial effect on blood pressure and vascular system has been confirmed in animal and human models (Foltz *et al*. 2007; Aluko 2015; Mora and Hayes 2015; Cicero *et al*. 2016; Bhat *et al*., 2017; Bougle and Bouhallab 2017).

In general, there are confirmatory results from clinical trials in both normotensive and hypertensive patients that peptides, derived from any type of food, have significant antihypertensive effect in humans (Cicero *et al*. 2016).

2.9.2 Antioxidant properties

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are free radicals produced by endogenous oxidation reduction (REDOX) reactions, i.e., through normal respiration in aerobic organisms (Castro and Freeman 2001). ROS/RNS are useful to facilitate biological processes such as gene expression, cell proliferation, angiogenesis, programmed cell death, senescence and defense against infections. Under normal conditions, the body antioxidant defense systems can remove reactive species; however, in certain circumstances the endogenous defense system fails, resulting in oxidative stress (Johansen *et al*. 2005). When accumulated ROS/RNS exceeds the threshold of the body, cellular damage is induced, which results in macromolecular damage such as atherosclerosis, arthritis, hypertension, cancer, diabetes, neurodegenerative disorders, heart disease, stroke, and aging (Castro and Freeman 2001; Hancock *et al*., 2001; Evans *et al*. 2003; Johansen *et al*. 2005; Valko *et al*. 2007; Dowling and Simmons 2009; Scherz-Shouval and Elazar 2011; Liu *et al*. 2016).

Once the redox balance is interrupted, it is necessary to reinforce the intake of antioxidants from food sources. Vitamin C, herbal extracts and artificial antioxidants have been marketed for a long time as natural antioxidants. However, amino acids and peptides could also provide antioxidant effect to protect cells against oxidative damage (Sarmadi and Ismail 2010). Peptides with antioxidant capacity are safe, healthy, stable and without dangerous immunological reactions (Shahidi 2000; Sarmadi and Ismail 2010).

Antioxidants peptides comprise 4 to 16 amino acids and approximately 400 – 2000 Da molecular weight. Low molecular weight peptides (<3 kDa) present better antioxidant potential than high ones. Small molecules can easily cross the gastrointestinal barrier and enter peripheral tissue. They can be digested by gastrointestinal proteases, and their antioxidant capacity increases because they can be absorbed more easily (Rubas and Grass 1991; Roberts *et al*. 1999; Wang *et al*. 2008; Liu *et al*. 2010). Peptides with strong antioxidant activities have been found in crude albumin proteins. The egg white peptide TAQQRTPIL, with ACE-inhibitory activity, also exhibited a high radical scavenging activity (Davalos *et al*. 2004; Bhat *et al*., 2017).

2.10 Food-Derived Peptides

Food-derived peptides are protein fragments derived from food with beneficial impact on body functions or conditions and may ultimately influence health (Iwaniak *et al*., 2014) Food-derived peptides can be categorized into three groups according to their sources: animal-derived, plant-derived and microorganism-derived ACE inhibitory peptides. Little has been reported on the microorganism- derived ACE inhibitory peptides (Lee and Hur,

2017). Due to the objective of this research, we limit the discussion to plant-derived ACE inhibitory peptides and multifunctional peptides (peptides with both ACE inhibitory and antioxidant properties).

2.10.1 Plant-derived ACE inhibitory peptides

Some plant proteins are a source of various bioactive ACEI peptides with anti-hypertensive activity (He *et al*., 2005; Lo and Li-Chan, 2005). ACE inhibitory properties of different enzymatic hydrolysates of glycinin, the major storage protein of soybean, have been demonstrated (Mallikarjun *et al*., 2006). Sesame peptide powder (SPP) could competitively inhibit ACE activity. A reconstituted sesame peptide mixture according to their content ratio in SPP showed a strong antihypertensive effect on spontaneously hypertensive rats (SHR). Repeated oral administration of SPP also lowered both systolic blood pressure (SBP) and the aortic ACE activity in SHR (Nakano *et al*., 2006). Mung bean protein isolates also showed ACE inhibitory activity (Li *et al*., 2006). After Cha fed 100mg soy-protein hydrolysate/kg body weight to SHR for 1 month, blood pressure decreased by 38 mmHg (Cha *et al*., 2005). The peptide, which was isolated from wheat gliadin hydrolysate, inhibited the hypertensive activity of angiotensin I with intravenous injection, and decreased the blood pressure significantly with intraperitoneal administration (Motoi and Kodama, 2003). The fruiting body of *Tricholoma giganteum* has many pharmaceutical uses, some similar to that of ACE inhibitory peptides. The maximum ACE inhibitory activity was obtained from the fruiting body of *T. giganteum.* The purified ACE inhibitor from *T. giganteum* competitively inhibited ACE and it maintained inhibitory activity (Hyoung *et al*., 2004).

As a part of the daily diet, these peptides appear to be more natural and safe for patients. Those foods source ACE inhibitory peptides are present in an inactive form within the primary sequences of proteins (Furushiro *et al*., 1993). They may be released and degraded in the animal or human body through either *in vitro* or *in vivo* proteolysis of the parent protein molecule, e.g., during gastrointestinal digestion or food processing. Therefore, antihypertensive peptides must be resistant to *in vivo* proteolysis once they are within the body in order to reach the desired target tissue or organ and exert antihypertensive effect (Furushiro *et al*., 1993).

2.11 Multifunctional Peptides

Single peptides with multifunctional bioactivities will be more preferred over single activity peptides as the former would simultaneously elicit multiple health benefits. For this reason, lentil proteins were hydrolyzed using Savinase® (Novozymes, Bagsvaerd, Denmark) to search for peptides with multiple functions (García-Mora *et al*., 2017). They observed that peptides LLSGTQNQPSFLSGF, NSLTLPILRYL and TLEPNSVFLPVLLH present in the hydrolysate have strong antioxidant and antihypertensive effects. YSK from rice bran protein (Wang *et al*., 2017), WVYY and PSLPA from hemp seed protein hydrolysates (Girgih *et al*., 2014) also showed both antioxidant and antihypertensive effects. Four other peptides, YINQMPQKSRE, YINQMPQKSREA, VTGRFAGHPAAQ and YIEAVNKVSPRAGQF isolated from egg yolk show antidiabetic, ACE inhibitory and antioxidant activities (Zambrowicz *et al*., 2015). These peptides could be important in managing metabolic diseases such as diabetes and hypercholesterolemia.

2.12 Measurement of Bioactive Peptides with ACE Inhibitory Activities

In order to facilitate the identification and isolation of ACE inhibitors peptides, establishment of a simple, sensitive and reliable in vitro inhibition assay is desirable. Numerous methods for the measurement of ACE activity have been reported, including spectrophotometric, fluorometric, radiochemical, high-performance liquid chromatography and capillary electrophoresis methods.

Different substrates are suitable to measure ACE activity among them synthetic peptides hippuryl-L-histidyl-L-leucine (HHL) and furanacryloyl-L-phenylalanylglycyl-glycine (FAPGG) are the most commonly used but other peptides can be used like fluorescent molecules (for example, o-aminobenzoylglycyl-pnitrophenylalanylproline) for specific detection and quantification (Carmel and Yaron, 1978; Sentandreu and Toldra, 2006).

In most of the previous cited works, the assay used for ACE activity was based on the method developed by Cushman and Cheung (1971). The amount of hippuric acid (HA) formed from HHL by action of ACE is extracted with ethyl acetate and the concentration is determined by a spectrophotometric assay. Different modifications of this method have been reported in which the ethyl acetate extraction was replaced by a specific binding of His-Leu with 2,4,6- trinitrobenzene sulphonate (TNBS) (Matsui *et al*., 1992) or a specific reaction of HA with benzene sulfonyl chloride (Li, *et al*., 2005). Although this assay has been very useful for decades, it has some limitations such as the required extraction of the product from the reaction mixture with an organic solvent which is an additional source of error.

Another method was described by Holmquist, *et al*., (1979) using FAPGG as substrate. This method, involves the measurement of the decrease in absorbance due to substrate

hydrolysis by action of ACE, was adopted for testing ACE-inhibitory activity of peptides (Vermeirssen *et al*., 2002). Different modifications were also reported with fixed time conditions (Murray *et al*., 2004), using 96-wells microtiter plate (Otte *et al*., 2007) or HPLC determination (Anzenbacherova *et al*., 2001).

Both methods showed similar performance but HHL substrate in solution seemed to be less stable than FAPGG substrate and the assay with FAPGG consumed less chemicals per sample. So, it is important to control the ACE-activity in the assays or to use the same enzyme preparation to compare the ACE-inhibitory activity of different samples.

2.13 **Cowpea** (*Vigna unguiculata*)

2.13.1 Description of cowpea

Cowpea (*Vigna unguiculata*) is a dicotyledonous annual herb belonging to the family fabaceae, with a strong principal root and many spreading lateral roots in surface soil (Adegbite and Amusa, 2008). The growth forms vary and may be erect, trailing, climbing or bushy, usually indeterminate under favourable conditions. Leaves are alternate and trifoliate usually dark green and first pair of them is simple and opposite. Stems are striate, smooth or slightly hairy, sometimes tinged with purple (Aveling, 1999). Flowers are self-pollinating and may be white, dirty yellow, pink, pale blue or purple in colour. They are arranged in raceme or intermediate inflorescences in alternate pairs. Flowers open in the early day and close at approximately midday, after blooming they wilt and collapse (McGregor, 1976). Fruits are pods that vary in size, shape, colour and texture

whichmay be erect, crescent-shaped or coiled, usually yellow when ripe, but may also be brown, black or purple in colour. There are usually 8 - 20 seeds per podand seeds vary considerably in size, shape and colour. The seeds are relatively large, 2-12 mm long and weigh 5 - 30 g/100 seeds as seed shape could be reniform or globular. The testa (the coat covering the grain) may be smooth or wrinkled; white, green, red, brown, black, speckled, blotched, or mottled in colour (Aveling, 1999).

Figure 2.4: Image of the cowpea (*Vigna unguiculate*) used for the research

2.13.2 Origin and distribution of cowpea

Cowpea certainly evolved in Africa, as wild types only exist in Africa and Madagascar (Steele, 1976). Interestingly, while West Africa appears to be the major centre of diversity of cultivated forms of cowpea (Ng and Padulosi, 1988) and was probably

domesticated by farmers in this region (Ba *et al*., 2004), the centre of diversity of wild *Vigna* species is south-eastern Africa (Padulosi and Ng, 1997). Some evidence that domestication occurred in north eastern Africa, based on studies of amplified fragment length polymorphism (AFLP) analysis, has also been presented (Coulibaly *et al*., 2009). The wild cowpea is the likely progenitor of cultivated cowpea (Pasquet, 1999). The crop was first introduced to India during Neolithic period, and therefore India is the secondary centre of genetic diversity (Pant *et al*., 1982). "Yardlong beans," a unique cultivar of cowpea that produces very long pods widely consumed in Asia as a fresh green or "snap" bean, evolved in Asia and is rare in the African landrace germplasm. Cowpea has been cultivated in southern Europe at least since the 8th century BC and since prehistoric times (Tosti and Negri, 2002). Cowpea was introduced to the West Indies in the 16th century by the Spanish and was taken to the USA about 1700, presumably it was introduced into South America at about the same time (Purseglove, 1977).

2.13.3 Cowpea production

More than 11 million hectares are harvested worldwide, 97% of which is in Africa (Oyewale and Bamaiyi, 2013). Nigeria produces the most quantity of cowpea grains annually at approximately 2.14 million metric tonnes (FAO STAT, 2017). The crop can be harvested in three stages; while the pods are young and green, mature and green and dry. The grain yield of cowpea in Nigeria is 578kg/ha (FAO STAT, 2017). The highest production of cowpea comes from the northern states of Nigeria (about 1.7 million tonnes from 4 million hectares) (Oyewale and Bamaiyi, 2013). The main producing areas in Nigeria are within the Guinea and Sudan savannas (Mongo, 1996). Even though

appreciable quantities are grown in the rain forest belts, particularly in the South West, which has two (2) growing seasons, namely; early (March – July) and late (August – November) (Oyewale, and Bamaiyi, 2013). The major producing states in Nigeria include; Kaduna, Katsina, Zamfara, Bauchi, Sokoto, Kebbi, Plateau, Borno, Yobe, Jigawa, Niger, Benue, Nasarawa and Kano where most cowpeas are traditionally grown as intercrops with cereals such as millet, maize and sorghum (Chemada, 1997; Oyewale, and Bamaiyi, 2013; Emechebe and Singh, 2013). In this system, the yields are low; inter-specific competition is high, population density is undetermined and harvesting is complicated by differing maturities of the intercrops (Singh, *et al.,* 1997) estimated a world total area of about 12.5 million hectares grown to cowpea annually. Cowpea is grown across the world on an estimated 14.5 million hectares of land planted each year and the total annual production is 6. Million metric tonnes (Erana and Zelalam, 2020).

2.13.4 Nutritional value of cowpea

The nutritional profile of cowpea grain is similar to that of other pulses, with a relatively low fat content and a total protein content that is two to four times greater than cereal and tuber crops. In a study of 100 cowpea breeding lines in the IITA collection, seed protein content ranged from 23 to 32 % of seed weight (Nielson *et al*., 1993). Similarly, protein content of 12 West African and United States cultivars ranged from 22 to 29 %, with most accessions having protein content values between 22 and 24 % (Hall *et al*., 2003). It contains about 62 % soluble carbohydrates, and small amounts of other nutrients. Thus, most of its nutritional value is provided by proteins and carbohydrates (Nielson *et al*., 1993). According to Purseglove (1977), dry cowpea seed contains 56.8 % carbohydrate,

23.4 % protein, 11.0 % water, 39.9 % fibre, 3.6 % ash and 1.3 % fat. Cowpea is especially rich in lysine, but it is deficient in sulphurous amino acids. Compared to other legumes, methionine and tryptophan levels are high. Except for total sulphurous amino acids, and to a lesser extent isoleucine, levels of essential amino acids are at least as high as those in soybean (Singh *et al*., 1997). The high protein content represents a major advantage in the use of cowpea in nutritional products, for infant and children's food, and to compensate for the large proportion of carbohydrates often ingested in African diets (Ajeigbe *et al*., 2008).

2.13.5 Importance and uses of cowpea

Cowpea is the most economically important indigenous African legume crop that is of vital importance to the livelihood of people in West and Central Africa. Rural families that make up the larger part of the population of these regions derive from its production, food, animal feed, alongside cash income (Dugje *et al*., 2009). Cowpea is a valuable source of protein for human and animal nutrition. It is mainly grown to produce dry grains, but about 25 % is consumed as fodder or marketed as green pods (Cisse and Hall, 2002). The grain and fresh peas have high protein content (about 25 % on a dry weight basis) and an amino acid profile that complements the proteins present in rice, millet, maize or wheat. Thus, a combination of 75 % cereal and 25 % cowpea diet, on a dry weight basis, provides sufficient proteins for adults (Cisse and Hall, 2002). Besides, cowpea hay has a higher protein content than grass hay and thus constitutes a valuable feed for livestock (Tarawali *et al*., 1997). There is a big market for the sale of cowpea grain and fodder in West Africa. In Nigeria, farmers who cut and store cowpea fodder for

sale at the peak of the dry season increase their annual income by 25 % (Dugje *et al*., 2009). Cowpea does not require a high rate of Nitrogen fertilization; its roots have nodules in which a soil bacterium, *Rhizobia* helps to fix nitrogen from the air (Aikins and Afuakwa, 2008). The crop can fix about 240 kg ha-1 of atmospheric nitrogen and make available about 60 - 70 kg ha-1 nitrogen for succeeding crop in rotation (Aikins and Afuakwa, 2008). Traditionally in West and Central Africa, cowpea is grown on small farms, often intercropped with cereals such as millet and sorghum. The cereal is planted first, followed by the cowpea. The fast growth and spreading habit of traditional cowpea varieties suppress weeds, and soil nitrogen is increased which improves cereal growth. The two crops are harvested at different times, distributing available labour force (Carlos, 2004).

Cowpea can be used at all stages of growth as vegetable crop, and the leaves contain significant nutritional value (Ahenkora *et al*., 1998) The tender green leaves are an important food source in Africa and are prepared as a pot herb, like spinach. Immature green pods are used in the same way as snap beans, often being mixed with cooked dry cowpeas or with other foods. Nearly mature "fresh-shelled" cowpea grains are boiled as a fresh vegetable or may be canned or frozen. Dry mature seeds are also suitable for boiling and canning (Tarawali *et al*., 2002). In Nigeria, cowpea is consumed in the form of bean pudding, bean cake, baked beans, fried beans, bean soup amongst others. The industrial uses of cowpea is facing some major constraints: primary processors do not exist; the quality of the grain available in open market is poor, with high percentage of physical defects and risk of pesticide residue contamination; strong price fluctuations along the

years are forcing procurement during a short period; the protein content of available cowpea is low compared to that of soybean (Nyankori, 2002).

The literatures reviewed revealed that the use of gastrointestinal enzymes (pepsin and pancreatin) sequentially for *in vitro* gastrointestinal digestion of cowpea is under explored. Also, the effect of time on the degree of hydrolysis and bioactivity have not been fully elucidated.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and Preparation of Bean Samples

Sample of *Vigna unguiculata* (Cowpea) was purchased from Dawanau Market, Kano State, Nigeria. The sample was authenticated by a botanist at Plant Science Department, Bayero University, Kano Herbarium, Kano State, Nigeria. Accession Number BUKHAN 99 was assigned to the sample.

Preparation of sample was done according to the method described by Pérez-Navarrete *et al*., (2006). Briefly, impurities were manually removed from the beans sample. The beans sample was dried at 60 °C using an oven for 4 hours. The dried beans sample was then ground to flour using a Thomas Hammer mill. The resulting flour was sieved to fine powder.

3.2 Equipment and Reagents

All chemicals and reagents used in this study were of analytical grade, and the details are in the appendix.

3.3 Preparation of Protein Concentrate

Protein Concentrate was prepared according to Bentacur-Ancona *et al.* (2004) with some modifications. Briefly, 1 kg of the bean flour was suspended in distilled water at a 1:6 (w/v) ratio. The pH was adjusted to 11 with 1 M NaOH which was stirred for 1hour with a shaker at 400 x g and then sieved (with a mesh cloth). The protein-starch suspension is allowed to sediment for 30 min at room temperature to recover the starch and protein fractions.

The pH of the separated solubilized proteins was adjusted to the isoelectric point (4.5) with 1 M HCl. The suspension was then centrifuged at 1317 x g for 12 min. The supernatants were discarded and the precipitate, freeze-dried until further use.

3.4 Proximate Composition

The Cowpea Flour and the Cowpea Protein Concentrate (CPC) were analyzed chemically for proximate contents according to the official methods of analysis described by the Association of Official Analytical Chemist (A.O.A.C., 2005). All analyses were carried out in duplicate.

3.4.1 Crude protein determination (AOAC Official Method 988.05)

Principle: The principle is built on that strong acid helps in digestion of food. Consequently, nitrogen is released which can be determined by a suitable titration method. The concentration of nitrogen is used to calculate the amount of protein present.

Procedure: The crude protein in the samples were determined by the routine semi-micro Kjeldahl, procedure/technique. This consists of three techniques of analysis namely Digestion, Distillation and Titration.

Digestion**:** Finely ground dried samples (0.5 g each) were weighed carefully into the Kjeldahl digestion tubes to ensure that all sample materials got to the bottom of the tubes. To this were added 1 Kjeldahl catalyst tablet and 10 ml of Concentrated H_2SO_4. These were set in the appropriate hole of the Digestion Block Heaters in a fume cupboard. The digestion was left on for 4 hours, after which a clear colourless solution was left in the tube. The digest was cooled and carefully transferred into 100 ml volumetric flask, thoroughly rinsing the digestion tube with distilled water and the flask was made up to the mark with distilled water.

Distillation**:** The distillation was done with Markham Distillation Apparatus which allows volatile substances such as ammonia to be steam distilled with complete collection of the distillate. The apparatus was steamed out for about ten minutes. The steam generator was then removed from the heat source to allow the developing vacuum remove condensed water. The steam generator was then placed on the heat source (i.e. heating mantle) and each component of the apparatus was fixed up appropriately

Determination**:** Portion of the digest above (5 ml) was pipetted into the body of the apparatus via the small funnel aperture. To this was added 5 ml of 40% (W/V) NaOH through the same opening with the 5 ml pipette. The mixture was steam-distilled for 2 minutes into a 50 ml conical flask containing 10 ml of 2% Boric Acid plus mixed indicator solution placed at the receiving tip of the condenser. The Boric Acid plus indicator solution changes colour from red to green showing that all the ammonia

liberated have been trapped. The green colour solution obtained was then titrated against 0.01 N HCl contained in a 50 millimeter burette. At the end point or equivalent point, the green colour turns to wine colour which indicates that all the Nitrogen trapped as Ammonium Borate [$(NH_4)_2BO_3$] have been removed as Ammonium chloride (NH_4Cl).

The percentage nitrogen was calculated using the formula:

% N = Titre value x Atomic mass of Nitrogen x Normality of HCl used x 4

or % N = Titre value x Normality/Molarity of HCl used x Atomic mass of

N x Volume of flask containing the digest x 100

Weight of sample digested in milligram (mg) x Vol. of digest for steam distillation. The crude protein content is determined by multiplying percentage Nitrogen by a constant factor of 6.25 i.e. % CP = % N x 6.25.

3.4.2 Fat or Ether Extract Determination (AOAC Official Method 2003.06)

Principle: the principle is based on the soxhlet extraction method. Lipid is soluble in organic solvent and insoluble in water. Because of this, organic solvents like hexane, petroleum ether have the ability to solubilize fat. Fat is extracted from food in combination with the solvent. Later, the fat is collected by evaporating the solvent.

Procedure: Dried samples of 1 g each were weighed into fat free extraction thimble and pug lightly with cotton wool. The thimble was placed in the extractor and fitted up with reflux condenser and a 250 ml soxhlet flask which has been previously dried in the oven, cooled in the desicator and weighed. The soxhlet flask is then filled to $^3/_4$ of its volume with petroleum ether (b.pt. 40 ^oC – 60 ^oC), and the soxhlet flask. Extractor plus condenser

set was placed on the heater. The heater was put on for six hours with constant running water from the tap for condensation of ether vapour. The set was constantly watched for ether leaks and the heat source was adjusted appropriately for the ether to boil gently. The Ether was left to siphon over several times (say over at least 10 – 12 times) until it was short of siphoning. It was after this was noticed then any ether content of the extractor was carefully drained into the ether stock bottle. The thimble containing sample was then removed and dried on a clock glass on the bench top. The extractor, flask and condenser were replaced and the distillation continued until the flask was practically dry. The flask which now contains the fat or oil was detached, its exterior cleaned and dried to a constant weight in the oven.

If the initial weight of dry soxhlet flask is W_0 and the final weight of oven dried flask + oil/fat is W_1, percentage fat/oil is obtained by the formula:

$$\frac{W_1 - W_0}{\text{Wt. of Sample taken}} \times 100$$

3.4.3 Dry matter and moisture determination (AOAC Official Method 967.08)

Principle: This is based on oven drying methods. The oven drying consists of heating by convection with forced or circulating hot air.

Procedure**:** The samples (2 g each) were weighed into a previously weighed crucible. The crucible (plus sample taken) was then transferred into the oven set at 100 ^{o}C to dry to a constant weight for 24 hours overnight. At the end of the 24 hours, the crucible plus sample were removed from the oven and transferred to dessicator, cooled for ten minutes and weighed.

The dry matter was obtained using the relation:

$$(\%\ DM)\ \%\ \text{Dry Matter} = \frac{W_3 - W_0}{W_1 - W_0} \times 100$$

The moisture was obtained using the relation:

$$\%\ \text{Moisture} = \frac{W_1 - W_3}{W_1 - W_0} \times 100$$

$$\text{Or } \%\ \text{Moisture} = 100 - \%\ DM.$$

Where: W_0 = weight of empty crucible

W_1= weight of crucible plus sample

W_3= weight of crucible plus oven-dried sample

3.4.4 Determination of ash (AOAC Official Method 942.05)

Principle: The principle of ashing is to burn off the organic matter and to determine the inorganic matter removed. Heating is carried out in 2 stages, first to remove the water present and to char the sample thoroughly. Finally, ashing is done in a muffle furnace at 550^0

Procedure**:** The samples (2 g each) were weighed into a porcelain crucible. This was transferred into the muffle furnace set at 550 ºC and left for about 4 hours. About this time it had turned to white ash. The crucible and its content were cooled to about 100 ºC in air, then room temperature in a dessicator and weighed. This was done in duplicate. The percentage ash was calculated from the formula below:

$$\text{Ash content} = \frac{\text{weight. of ash}}{\text{Original weight of sample}} \times 100$$

3.4.5 Fibre determination (AOAC 958.06)

Principle: This involves acid-alkali digestion. This is achieved by treating a sample with ether to remove fat, then boiling it alternatively in a weak acid and weak alkali.

Procedure**:** The samples (2 g each) were accurately weighed into the fibre flask and 100 ml of 0.255 N H_2SO_4 added. The mixture was heated under reflux for 1 hour with the heating mantle. The hot mixture was filtered through a fibre sieve cloth. The filtrate obtained was thrown off and the residue was returned to the fibre flask to which 100 ml of 0.313 N NaOH was added and heated under reflux for another 1 hour. The mixture was filtered through a fibre sieve cloth and 10 ml of acetone added to dissolve any organic constituent. The residue was washed with about 50 ml hot water on the sieve cloth before it was finally transferred into the crucible. The crucible and the residue were oven-dried at 105 ºC overnight to drive off moisture. The oven-dried crucible containing the residue was cooled in a dessicator and later weighed to obtain the weight W_1. The crucible with weight W_1 was transferred to the muffle furnace for ashing at 550 °C for 4 hours.

The crucible containing white or grey ash (free of carbonaceous material) was cooled in the dessicator and weighed to obtain W_2. The difference between $W_1 - W_2$ gives the weight of fibre. The percentage fibre was obtained from the formula:

$$\% \text{ Fibre} = \frac{W_1 - W_2}{\text{weight of sample}} \times 100$$

3.4.6 Nitrogen-free extract (NFE) or carbohydrate by difference determination

The NFE was determined by difference. This was done by subtracting sum of (Moisture % + % Crude Protein + % Ether Extract + % Crude Fibre + % Ash) from 100 i.e. (100 – (% M + % CP + % EE + % CF + % Ash).

3.5 Enzymatic Hydrolysis of Protein Concentrate

Enzymatic hydrolysis was performed according to the method of Guzman-Mendez *et al*. (2014). Briefly, cowpea protein concentrate (CPC) was digested sequentially with pepsin (40 mg/ml) for 60 minutes followed by digestion with pancreatin (40 mg/ml) for 60 minutes. The conditions for hydrolysis were; Enzyme-substrate ratio (1:20, w:w), temperature control at 37 ^{o}C, pH 2 for pepsin (adjusted with 0.1 M HCl) and pH 7.5 for pancreatin (adjusted with 0.1 M NaOH). The hydrolysis reaction was stopped by heating at 80 °C for 20 min in a water bath followed by centrifuging at 1317 x g for 12 min, to remove the insoluble portion. Protein hydrolysates was then freeze-dried.

Crude protein determination of the hydrolysate was done according to the AOAC official method 988.05 (A.O.A.C., 2005). The analysis was carried out in duplicate.

3.6 Determination of Protein Concentration

Principle: It is used to measure the concentration of total protein in a sample. The principle of this assay is that binding of protein molecules to coomassie dye under acidic conditions results in a colour change from brown to blue.

Procedure: The protein content of the crude samples, hydrolysate and the fractions was determined by the binding assay method of Bradford (1976) using bovine serum Albumin (BSA) as standard protein.

Protein extract of 100µl was diluted into two different concentrations of the extract. 5ml of dye reagent was added and mixed well. At the same time, a set of standards containing 5, 10, 20, 30, 40, 50, and 100µl of Bovine Serum Albumin (BSA 2.0mg/ml stock in extraction buffer) were prepared in separate tubes. The extraction buffer was added to each tube to bring the volume to 100 µl. To these tubes, 5ml of dye reagent was added and mixed well by vortexing. Then, read the absorbance at 595nm (OD595) against the reagent blank (100 µl of extraction buffer with 1 ml of dye reagent). The protein concentration in the extract was calculated by comparison with the standard curve for BSA.

Different proteins show considerable variation in their dye-binding capacities and so give different responses in the assay. In particular, bovine serum albumin gives a high OD595 value and so it is not totally representative of proteins. It is used here for convenience with total leaf extract. If you wish to measure the concentration of a specific protein, it is advised to use a purified form of the same protein as standard.

3.7 Determination of Degree of Hydrolysis (DH)

Degree of hydrolysis (DH) was calculated by determining free amino groups with o-phthaldialdehyde (OPA) following method of Nielsen *et al*., (2001). Details of the procedure is in the appendix

Principle: OPA reacts with primary amino groups and a SH-compound (dithiothreitol, DTT) to form a compound that will absorb light at 340 nm.

Calculation: Determination of h:

Serine-NH_2 = OD sample – OD blank/OD standard – OD blank ×0.9516 meqv/L ×0.1×100/ X×P

Where serine-NH_2 = meqv serine NH_2/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in liter (L). h is then: h = (serine-NH_2 - b) / a meqv/g protein.

Calculation of DH: DH = h / h_{tot} ×100%

h_{tot} is the total number of peptide bonds per protein equivalent, h_{tot} is dependent on the amino-acid composition of the raw material and,

h is the number of hydrolyzed bonds.

3.8 Determination of Antioxidant Activity

3.8.1 DPPH (2, 2- diphenyl-1-picrylhydrazyl hydrate) radical scavenging assay

Principle: 1, 1 Diphenyl 2- Picryl Hydrazyl is a stable (in powder form) free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The radical scavenging ability of the cowpea hydrolysate was

determined using the stable radical DPPH (2, 2- diphenyl-1-picrylhydrazyl hydrate) as described by Brand- Williams *et al*., (1995). The reaction of the DPPH with an antioxidant compound which can donate hydrogen, leads to its reduction (Blois, 1958).

Procedure: Different volumes (2 - 20µl) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 min. After 20 min, the absorbance of the mixture was read at 517 nm. 3ml of DPPH was taken as control.

3.8.2 $ABTS^{+}$ radical scavenging assay

Principle: The free radical scavenging activity of the cowpea hydrolysate was determined by the ABTS radical cation decolourisation assay (Re *et al*., 1999). The principle is based on the reduction of ABTS radical.

Procedure: The ABTS radical cation ($ABTS^{+}$) was produced by reacting ABTS stock solution (7 mM) with 7 mM potassium persulphate in a ratio 2:1 and the mixture was allowed to stand in the dark at room temperature for 16h before use. 2 ml of ABTS working solution was mixed with 50 µl of different concentration of trolox standard and the absorbance was measured after 20 min at 734 nm.

3.8.3 Metal ion chelating activity

Principle: Ferrozine can quantitatively chelate with Fe2+ and form a red coloured complex. This reaction is limited in the presence of other chelating agents and results in a decrease of the red colour of the ferrozine-Fe2+ complex. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions. The antioxidants present in plant extract forms a coordinate complex with the metal ions (chelating activity) and inhibit the transfer of electrons. Thus oxidation reaction is

arrested and no free radicals are produced. The metal ion chelating activity of the cowpea hydrolysate was determined according to the method of Singh and Rajini (2004).

Procedure: 100µl of plant extract (10mg in 1ml DMSO) was added to 50µl of 2mM ferrous chloride and 200µl of 5mM ferrozine solution. The solution was mixed thoroughly and incubated in dark at room temperature for 10 min. The absorbance was read at 562 nm. 100µl EDTA (10mg/1ml DMSO) was used as standard.

3.9 Determination of ACE Inhibitory Activity

The ACE inhibitory activity was measured according to the method of Wu *et al*., (2011). Sample solution of 10 µl of with 45 µl Hippuryl-His-Leu (HHL) solution (5 mM HHL in 0.1 M borate buffer containing 0.3 M NaCl, pH 8.3) was pre-incubated at 37 ^{o}C for 5 min, then incubated with 10 µl ACE at 37 ^{o}C for 30 min. The reaction was stopped by adding 85 µl of 1M HCl to the samples. The hippuric acid formed was extracted with 1000 µl ethyl acetate. Ethyl acetate layer of 800 µl was then collected and evaporated in a dry oven at 100 ^{o}C. The residue was dissolved in 800 µl distilled water and its absorbance was measured at 228 nm. The inhibitory activity was calculated using the following equation:

ACE inhibitory activity (%) = C-S/C-B

Where C, S and B are the absorbance without sample, the absorbance with sample, and the absorbance of blank, respectively.

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

3.10 Fractionation of ACE Inhibitory Peptides

3.10.1 Gel filtration chromatography

The Cowpea Protein Hydrolysate (CPH) was separated on Sephadex G-50 column (1.6cm × 60cm). The column was eluted with distilled water at flow rate of 1.0 ml/min. Fractions were collected at 2ml per tube and measured at 220nm. The fractions with the highest absorbance were combined and the ACE inhibitory activity (according to the method of Wu *et al*., (2011)) was determined. The fractions exhibiting the highest ACE inhibitory activity were pooled together and further purified by RP-HPLC on a C_{18} column.

3.10.2 Reversed-phase high performance liquid chromatography (RP-HPLC)

The most active fractions pooled together after gel filtration chromatography were further purified by RP-HPLC on a C18 column (7 µm, 4.6 mm × 250 mm, Shimadzu). The column was eluted with acetonitrile/water (80/20) at a flow rate of 1.0ml/min. All the peaks were collected by monitoring absorbance at 220nm. The ACE inhibitory activity according to the method of Wu *et al*., (2011) was determined for the fractions. The fraction with the highest ACE inhibitory activity was used in further analysis (Amino acid profile).

3.11 Determination of Amino Acid Profile

The Amino Acid profile in the known sample was determined using the method described by Benitez (1989). The known sample was dried to constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator (RE-52-3, Biocotek, China) and loaded into the Applied Biosystems PTH Amino Acid Analyzer.

The sample was defatted using chloroform/methanol mixture (ratio 2:1). About 2.0 g of the sample were put in extraction thimble and extracted for 15 hours in soxhlet extraction apparatus (99036-00, Cole-Parmer, UK).

A small amount (150 mg) of ground sample was weighed, wrapped in whatman filter paper (No.1) and put in the Kjeldahl digestion flask. Concentrated sulphuric acid (10 ml) was added. Catalyst mixture (0.5 g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) (in the ratio of 10:5:1) was added into the flask to facilitate digestion. Six pieces of anti-bumping granules were added. The flask was then put in Kjeldahl digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100 ml in standard volumetric flask. Aliquot (10 m1) of the diluted solution with 10 ml of 45 % sodium hydroxide was put into the Markham distillation apparatus and distilled into 10 ml of 2 % boric acid containing 4 drops of bromocresol green/methyl red indicator until about 70 ml of distillate was collected. The distillate was then titrated with standardize 0.01 N hydrochloric acid to grey coloured end point.

$$\text{Percentage Nitrogen} = \frac{(a-b) \times 0.01 \times 14 \times V \times 100}{W \times C}$$

Where:

a. = Titre value of the digested sample

b. = Titre value of blank sample

v. = Volume after dilution (100 ml)

W. = Weight of dried sample (mg)

C. = Aliquot of the sample used (5 ml)

14. = Nitrogen constant in mg.

Hydrolysis of the sample: A known weight (mentioned in the calculation sheet) of the defated sample was weighed into glass ampoule. 7 ml of 6 N HCl was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis e.g methionine and cystine). The glass ampoule was then sealed with Bunsen burner flame and put in an oven preset at 105 ^{o}C± 5 ^{o}C for 22 hours. The ampoule was allowed to cool before broken open at the tip and the content was filtered to remove the humins. It should be noted that tryptophan is destroyed by 6 N HCL during hydrolysis.

The filtrate was then evaporated to dryness using rotary evaporator. The residue was dissolved with 5 ml to acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer. The amount loaded was 60 microlitres. This was dispensed into the cartridge of the analyzer. The analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysates.

Method of calculating amino acid values

An integrator attached to the Analyzer calculates the peak area proportional to the concentration of each of the amino acids.

NOTE: g/100 g Protein = g/16 g N

3.11.1 Determination of tryptophan

Since, tryptophan is destroyed by 6 N HCl during hydrolysis, tryptophan in the known sample was hydrolyzed with 4.2 M Sodium hydroxide (Maria *et al.*, 2004). Then a known amount of the dried to constant weight, defatted, hydrolyzed, evaporated sample (in a rotary evaporator) and loaded into the Applied Biosystems PTH Amino Acid Analyzer.

3.12 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). The data were subjected to analysis of variance using GraphPad Prism version 6.0(GraphPad Software, San Diego, CA, USA) with level of significance at $P< 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Proximate Composition (%) of Cowpea Flour and Protein Concentrate

Table 4.1 shows the proximate composition of the Cowpea flour and Cowpea protein concentrate (CPC). The Cowpea flour was found to have high moisture, crude fat, crude fiber, ash and carbohydrate content than the Cowpea protein concentrate. The crude protein was significantly ($p<0.05$) higher in CPC than in the Cowpea flour.

Table 4.1: Proximate composition (%) of Cowpea flour and its protein concentrate

Components/Samples	**Cowpea Flour**	**Cowpea protein concentrate (CPC)**
Moisture	10.68 ± 0.02^{a}	5.38 ± 0.02^{b}
Crude Protein	22.73 ± 0.06^{b}	68.83 ± 0.06^{a}
Crude fat	2.00 ± 0.05^{a}	0.47 ± 0.04^{b}
Crude fiber	3.93 ± 0.02^{a}	1.20 ± 0.02^{b}
Ash	4.24 ± 0.03^{a}	1.65 ± 0.03^{b}
Carbohydrates	56.44 ± 0.04^{b}	22.49 ± 0.05^{a}

Different letters in rows indicate significant difference at $p<0.05$

4.2 Antioxidant Activities of Cowpea Protein Hydrolysate (CPH)

4.2.1 DPPH radical scavenging activity

Figure 4.1 presents the radical scavenging activity of CPH and ascorbic acid (standard). The IC_{50} (0.334 mg/ml) of CPH was significantly higher ($p < 0.05$) than that of the standard, ascorbic acid (0.055 mg/ml)

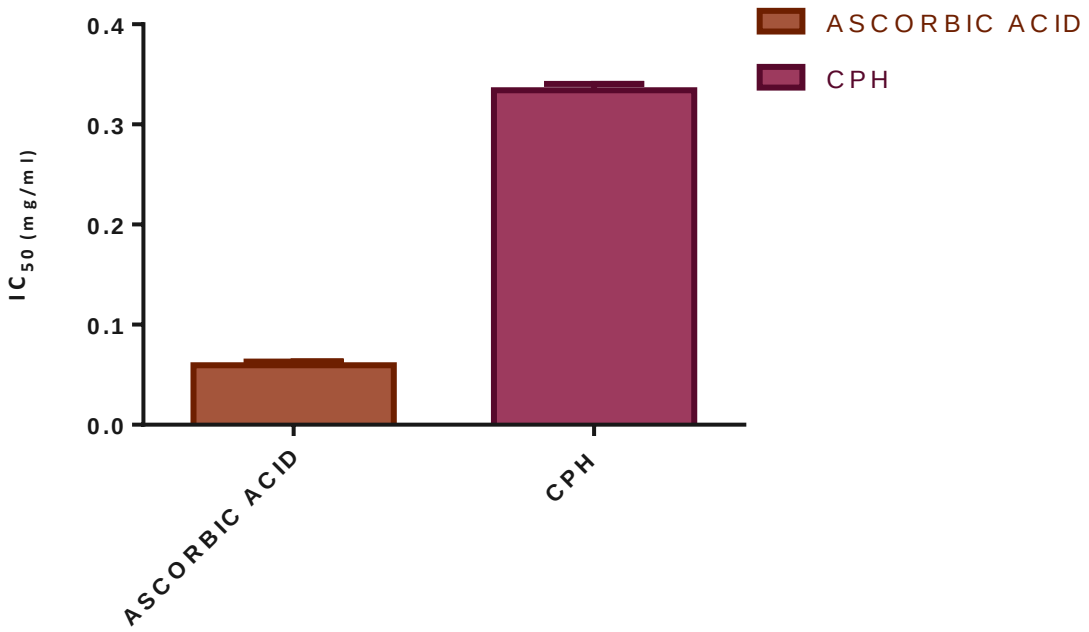


Figure 4.1: DPPH Radical Scavenging Activity of Cowpea Protein hydrolysate (CPH). Values are represented in mean ± S.D (n = 3)

4.2.2 ABTS radical scavenging activity

Figure 4.2 shows the radical scavenging activity of crude CPH and Trolox (means ± standard deviation of IC_{50} (mg/ml)). The IC_{50} (4.796 mg/ml) of CPH was significantly higher ($p < 0.05$) than that of the standard, trolox (0.170 mg/ml)

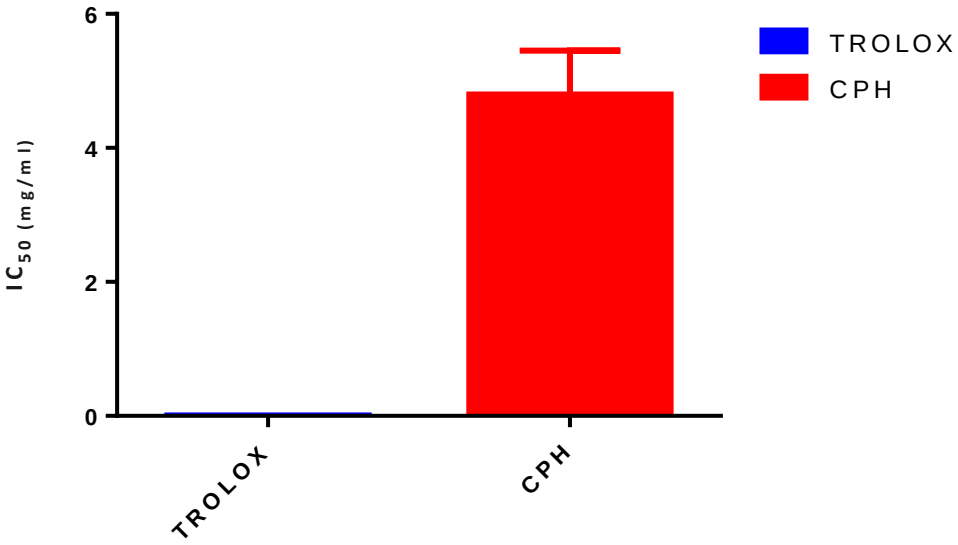


Figure 4.2: ABTS Radical Scavenging Activity of Cowpea Protein hydrolysate (CPH). Values are represented in mean ± S.D (n = 3)

4.2.3 Metal chelating activity

Figure 4.3 shows metal chelating activity o rude CPH and EDTA (the means ± standard deviation of IC_{50} (mg/ml)). The IC_{50} (5.872801 mg/ml) of CPH was significantly higher ($p < 0.05$) than that of the standard, EDTA (0.352 mg/ml)

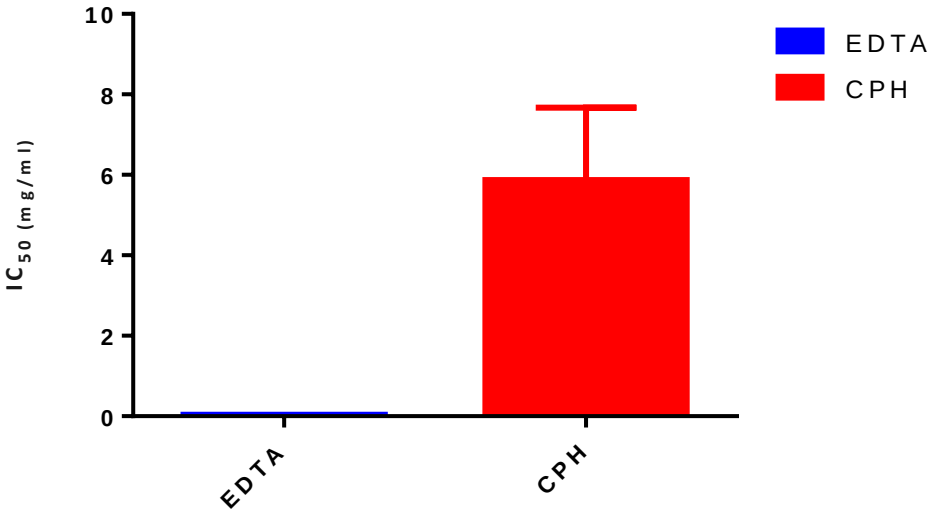


Figure 4.3: Metal Chelating Activity of Cowpea Protein hydrolysate (CPH).
Values are represented in mean ± S.D (n = 3)

4.3 Fractionation of ACE Inhibitory Peptide

4.3.1 Gel filtration chromatography

Figure 4.1 the percentage (%) ACE inhibition obtained from CPH and the first purification step by gel filtration chromatography. Five fractions were isolated at 220nm. The ACE inhibitory activity of fraction A was significantly higher ($P<0.05$) than that of fraction B, D, and E but not significantly different form fraction C. Fraction A, B, C exhibited ACE inhibition above 50%. These fractions were pooled together for the next purification step.

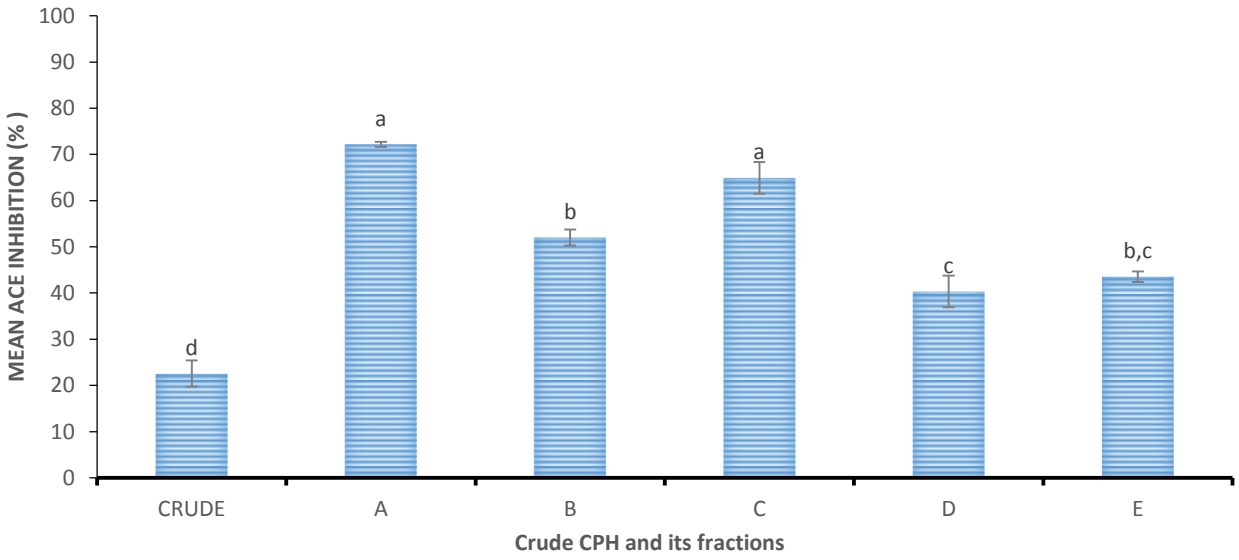


Figure 4.4: ACE inhibitory activities of CPH and the fractions collected from G-50 Gel Filtration Chromatography.

[a, b,c,d] Different letters are significantly different at $p < 0.05$

4.3.2 Reversed-phase high performance liquid chromatography (RP-HPLC)

Figure 4.2 shows percentage (%) ACE inhibition obtained from fractions A, B, C pooled together purified via RP-HPLC. The ACE inhibitory activity of fraction F5 is significantly higher ($P < 0.05$) than fractions F1, F2, F3, and F4. There is no significant difference between fractions F1, F2, F3, and F4. Fraction F5 had the highest ACE inhibition (%)

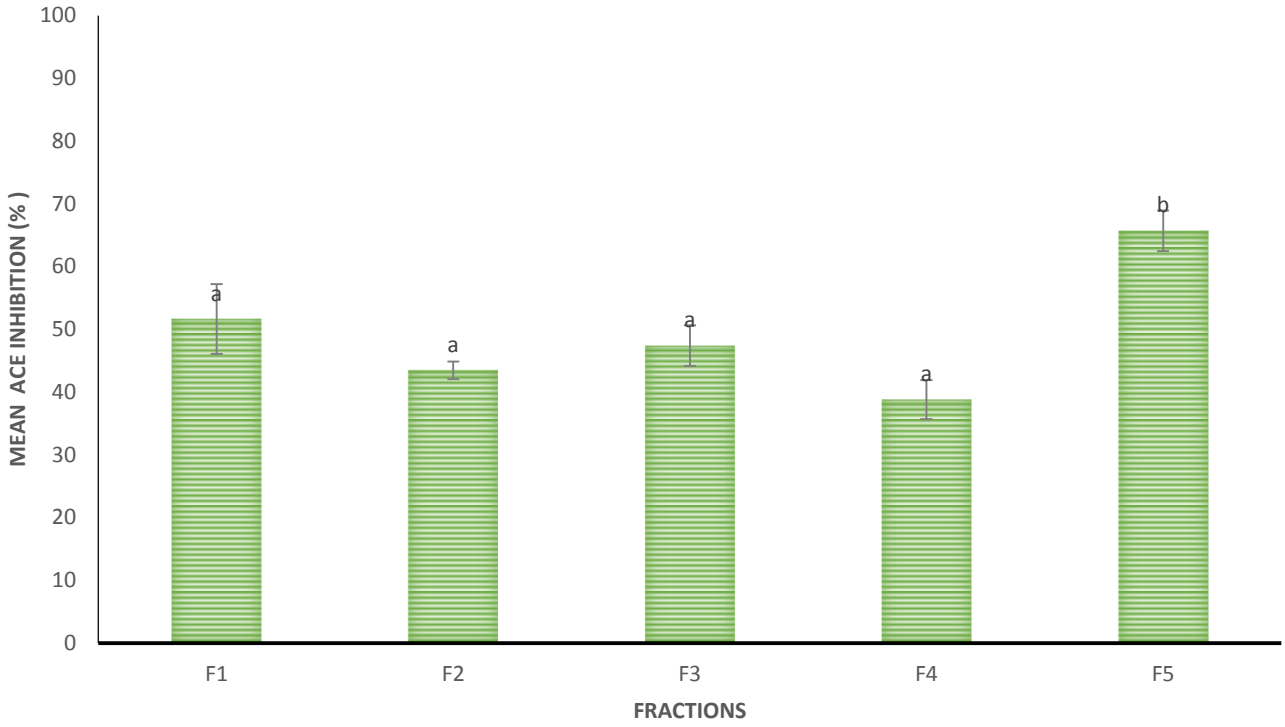


Figure 4.5: ACE inhibitory activities of the fractions obtained from RP-HPLC

[a, b,] Different letters are significantly different at $p < 0.05$

4.5 Amino Acid Composition

Table 4.3 shows the amino acid composition of the fraction that exhibited the highest ACE inhibitory activity after RP-HPLC. It contains eighteen amino acids with glutamic acid having the highest concentration (14.77 g/100 g) and cysteine and tyrosine (each 0.35g/100g) being the least.

Table 4.3: Amino acids present in the most potent ACE inhibitory fraction (RP-HPLC).

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

4.6 Discussion

Hypertension is recognized as major risk factor for the development of many chronic diseases (Kearney *et al*., 2005; Forouzanfar *et al*., 2017) and since RAS plays a significant role in the pathogenesis of hypertension, ACE inhibitors have long been known for their beneficial effects in reducing high blood pressure (Brown and Hall, 2005). ACE inhibitors reduce blood pressure by preventing the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor (Grossman and Messerli, 2012). They also degrade bradykinin, either by binding to the active site of the enzyme or as a non-substrate, hence, inhibiting the activity of this enzyme ultimately results to low blood pressure (Viernes *et al*., 2012).

The use of synthetic ACE inhibitors has led to some unwanted side effects such as renovascular disease, cough, neutropenia, skin rashes, oedema and taste disturbance. Hence a cheap safer alternative is sought. Food derived bioactive peptides such as from legumes have been reported to have multifunctional properties such as antihypertensive, anti-carcinogenic and anti-oxidative properties. In the present study, the potential ACE inhibitory and DPPH radical scavenging activities of cowpea hydrolysates were studied.

The protein contents of the cowpea flour (22.73±0.06%) obtained in the present study was higher than that reported by Alayande *et al* (2012) (17.91 ± 0.09%). Moreover, the protein content in the concentrate (68.83%) was also higher than that reported for *P. vulgaricus* (63.8%) by Torruco – Uco *et al*., (2009). The variations in protein contents of different protein concentrates could be due to the extent of soluble proteins present in the raw materials as well as extraction method used etc. (Chel- Guerrero *et al*., 2012).

Hydrolysis of protein concentrate is a major step in peptide production. Depending on interest, hydrolysis could be enzymatic or microbial (Lee and Hur, 2017). In this study, the cowpea protein concentrate (CPC) was hydrolysed sequentially using pepsin and pancreatin for 120min. Sequential hydrolysis with pepsin-pancreatin produced cowpea protein hydrolysates (CPH) with degree of hydrolysis (DH) of 20.10±0.2%. This result is higher than the reported values for soy protein hydrolysates produced with pancreatin for 60 minutes (11.0%) and 180 min (17.0%) by Qi *et al*., (1997) but lower than the values reported for cowpea (35.74%) by Segura-Campos *et al*., (2013). The differences in results might be due to the fact that the preparation of samples, the method used for isolation, source of protein or varieties of cowpea could have an influence on the DHs value. The CPH represents a pool of peptides resembling those generated during digestion of cowpea proteins in an organism. Pepsin is the main proteolytic enzyme generated in the stomach during food digestion, while pancreatin includes proteases such as trypsin, chymotrypsin and elastase, which are released by the pancreas in the small intestine. The resulting peptides, are therefore, resistant to pepsin and pancreatin, suggesting that they might be absorbed by digestive epithelial cells in the small intestine and probably might be bioavailable and exercise their biological activity.

Antioxidant activity is an essential property since oxidants are known to be involved in many human diseases and aging processes (Escobales *et al*., 2005). The cowpea hydrolysate (CPH) showed higher scavenging activity (IC_{50} = 0.334 mg/ml) for DPPH radical than wheat germ protein hydrolysates (IC_{50} = 1.50 mg/ml) (Zhu *et al*., 2006). The ABTS radical scavenging activity was high but not as high as that of trolox (used as standard). Ferrous ions (Fe^{2+}) chelation may render important antioxidant effects by

retarding metal catalyzed oxidation (Gülçin *et al.*, 2010). Although the ferrous ions chelating capability of cowpea hydrolysate in the present study was lower than that of EDTA, cowpea hydrolysate has an effective capacity for chelating ferrous ions. Some researchers claim that proteins possess antioxidant properties and protein insufficiency aggravates lipid peroxidation and reduces antioxidative enzyme activities in rats (Hui *et al*., 2008).

CPH in the present study displayed a very low ACE inhibition (22.58%). This could be due to the low concentration of small peptides and blocking effect of large peptides on the antihypertensive activity of small peptides (Olagunju *et al*., 2018). The most potent fraction, F5 had an IC_{50} of 6.23 μg/ml. This value is lower than 19.3 μg/ml reported for peptide of small red bean (*Phaseolus vulgaris*) purified by RP-HPLC (Xin Rui *et al*., 2013).

Amino acid composition shows the amount of amino acids present in a sample. The amino acid composition of F5 fraction showed that glutamic acid, aspartic acid, lysine, leucine, arginine and phenylalanine were predominant. The F5 fraction has a balanced amino acid composition that makes them appropriate as a protein source in human nutrition (FAO/WHO, 2007). The amount of hydrophobic amino acids (HAA) in the fraction (42.16g) was higher than Pigeon pea hydrolysate, 35.42g (hydrolyzed sequentially with pepsin-pancreatin system.) reported by Olagunju *et al*., (2018). HAA have been reported to have a relationship with antioxidant activity (Valdez-Ortiz *et al*., 2012). Hydrophobic amino acids have strong radical scavenging activity in oxidative reactions. Hydrophobic amino acids means low solubility which induces low ABTS and high hydroxyl radical scavenging activity (Tang-Bin *et* al., 2016). Also, the presence of

peptides with significant aromatic residues (tyrosine, phenylalanine, tryptophan) at C-terminal and basic residues (Lysine, histidine and arginine) at the N- terminal have been reported to have strong and competitive ACE inhibitory activity (Hwang and Ko, 2004). AAA also contributes to inhibition of ACE by interacting between three subsites at the active site of ACE. Other authors have also suggested that amino acids such as leucine, isoleucine and valine can contribute to increased ACE inhibitory activity (Ruiz *et al*., 2004). Hence, this study suggest that the presence of aromatic and hydrophobic amino acids is a valuable contributor to the ACE inhibitory activity of cowpea hydrolysate. This could be as result of the structure of these amino acids. The indole and pyrrolidine ring in tryptophan and proline respectively, serve as hydrogen donors via hydroxyl groups. Thus, acting as hydroxyl radical scavengers.

The result of this study can be applied in the production of nutraceuticals that can be employed in the treatment or management of diseases. The hydrolysate contain a considerable high amount of essential amino acids. This could be incorporated in diet or individuals that lack such amino acids. SARS-COV-2 down regulates ACE2 expression that decreases the protective effects of ACE2 on different organs. ACE inhibitors could prevent COVID 19 viral entry by stabilizing ACE2-AT1R interaction and preventing viral protein ACE2 interaction and internalization (Awgichew and Teshome, 2020). Natural ACE inhibitors could also be exploited for this purpose; this can only be achieved via further research on bioactive peptides derived from plant/food crops.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

Bioactive peptides derived from legumes such as beans are important food ingredients that can improve the nutritional and technological aspects of food formulations, as well as provide potential benefits to human health. In order to fractionate cowpeas (*Vigna unguiculata*) hydrolysate that has antioxidant potentials and identify the amino acid composition of the peptide fraction responsible for the potent ACE inhibitory, the cowpea protein concentrate was extracted via wet fractionation method. The cowpea protein concentrate was enzymatically hydrolyzed using pepsin-pancreatin sequentially, the resulting hydrolysate was then fractionated via G-50 gel filtration chromatography and reverse phase high performance liquid chromatography (RP-HPLC). Antioxidant assays; DPPH radical scavenging, ABTS radical scavenging and metal chelation assay were performed on the crude hydrolysate. ACE inhibitory assay was performed on the crude hydrolysate and the fractions from G-50 gel filtration chromatography and reverse phase high performance liquid chromatography. Afterwards, the amino acid profile of the most potent fraction (RP-HPLC fraction) was determined. The results obtained suggest that cowpea hydrolysates possess antioxidant and ACE inhibitory properties

5.2 Conclusion

Based on the result from this study, cowpea hydrolysate contains peptides that may possess antioxidant and ACE inhibitory properties. Therefore, enzymatic hydrolysis with pepsin and pancreatin sequentially, may be used to produce cowpea hydrolysates and peptides with potential to be used as ingredients for the formulation of functional foods that will provide potential benefits to human health especially in cases of cardiovascular diseases.

5.3 Recommendations

1. Further investigation should be carried out to elucidate the structure and sequence of the fraction with the most potent ACE inhibitory fraction
2. The mode of inhibition should be studied i.e. how the peptide inhibits the ACE
3. Finally, *in vivo* studies of the active peptides and bioavailability should be studied to further exploit the potentials for use as antihypertensive agents.

5.4 Contributions to knowledge

1. The production of hydrolysates with antioxidant and ACE inhibitory potentials. This hydrolysates could be employed in the production of nutraceuticals for management/prevention of diseases.
2. The hydrolysates are rich in essential amino acids. This could be incorporated in diets.
3. The hydrolysates are also rich in hydrophilic amino acids which could be used in cosmetics industries.

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APPENDICES

APPENDIX I: LIST OF EQUIPMENT, GLASS WARES AND CHEMICALS

CHEMICAL/REAGENTS

1. Angiotensin converting enzyme (rabbit lungs)
2. Hippuryl-histidyl-leucine (HHL)

3. Pepsin
4. Pancreatin
5. Serine
6. Di-Natetraborate decahydrate
7. Na-dodecyl-sulfate (SDS)
8. O-phthaldialdehyde 97% (OPA)
9. Dithiothreitol 99% (DTT)
10. 2, 2- diphenyl-1-picrylhydrazyl hydrate
11. Concentrated sulphuric acid
12. Hydrochloric acid
13. Sodium hydroxide
14. Boric Acid Solution
15. Methyl Red
16. Kjeldahl Catalyst tablet.

All other reagents used were of analytical grade.

EQUIPMENT

1. Refrigerated centrifuge MSB005.CR2.K (made in U.K)
2. HPLC Machine Adept, on a C18 column (7µm, 4.6mm×250mm, shimadzu)
3. JENWAY 6300 spectrophotometer (Bibby scientific limited, UK)
4. Genlab oven OV/100
5. Genlab incubator MINI/50/VIS,

6. pH meter
7. UV spectrophotometer
8. PTH Amino Acid Analyzer (Applied Biosystems)
9. Electrical weighing balance, Freeze dryer H 06150030 (Labogene ApS, Denmark).
10. Thomas Hammer Mill

APPENDIX II: METHODOLOGY

Determination of Degree of Hydrolysis (DH)

The OPA solution was prepared as follows: 7.62 g di-Natetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) was dissolved in 150 ml deionized water. The reagents were completely dissolved, then 160 mg o-phthaldialdehyde 97% (OPA) was dissolved in

4 mL ethanol. The OPA solution was then transferred quantitatively to the above-mentioned solution by rinsing with deionized water. 176 mg dithiothreitol 99% (DTT) was added to the solution. The solution was made up to 200 mL with deionized water. The serine standard was prepared as follows: 50 mg serine was diluted in 500 ml deionized water. The sample solution was prepared as follows: X g sample was dissolved in 100 mL deionized water. X was 0.1 to 1.0 g sample containing 8% to 80% protein. The Degree of Hydrolysis (DH) of the sample also influences the amount required.

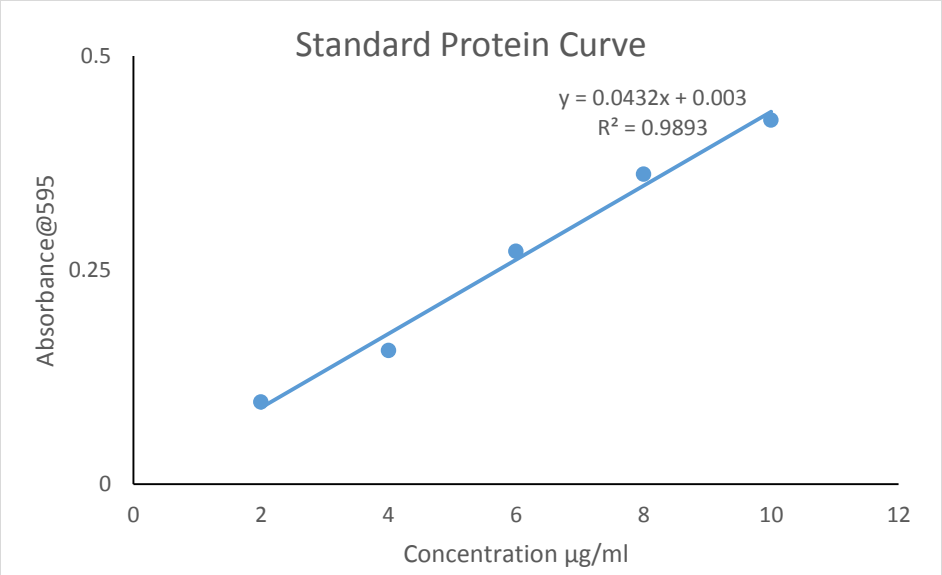
All spectrophotometer readings were performed at 340 nm using deionized water as the control. OPA solution (3 ml) were added to all test tubes. As absorbance changes somewhat with time, it is important the samples stand for exactly the same time (2 min) before measuring. The assay was carried out at room temperature.

Standard measuring: 400 µl serine standard was added to a test tube (time 0) containing 3 ml OPA reagents and mixed for 5 s. The mixture stood for exactly 2 min before being read at 340 nm in the spectrophotometer. The typical value of the standards is OD about 0.8.

Blank measuring: Blanks were prepared from 400 µl deionized water and treated as described above. The typical value of a blank is OD about 0.07.

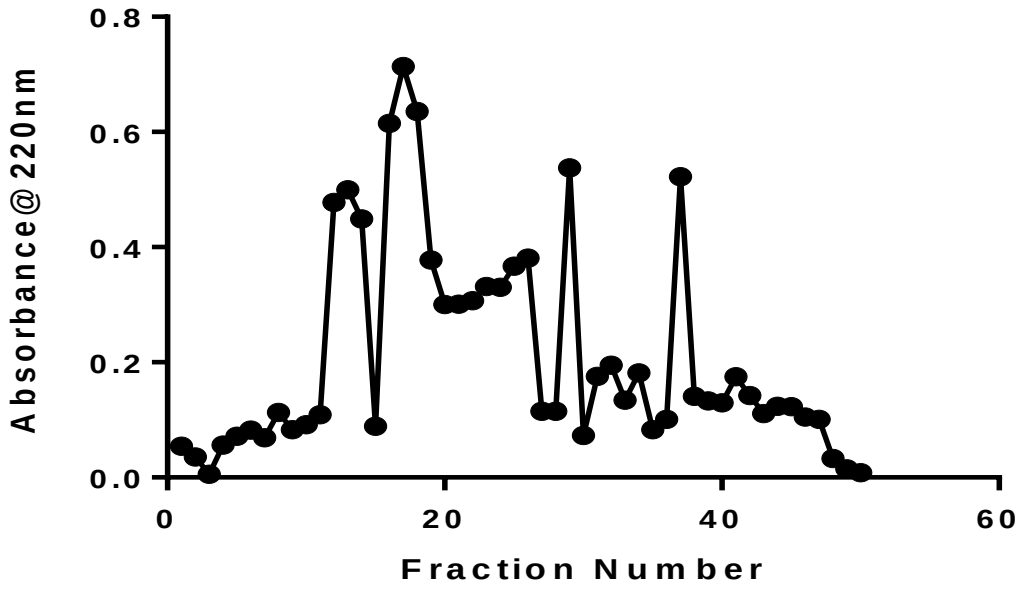
Sample measuring: Samples were prepared from 400 µl sample.

APPENDIX III: STANDARD CURVE

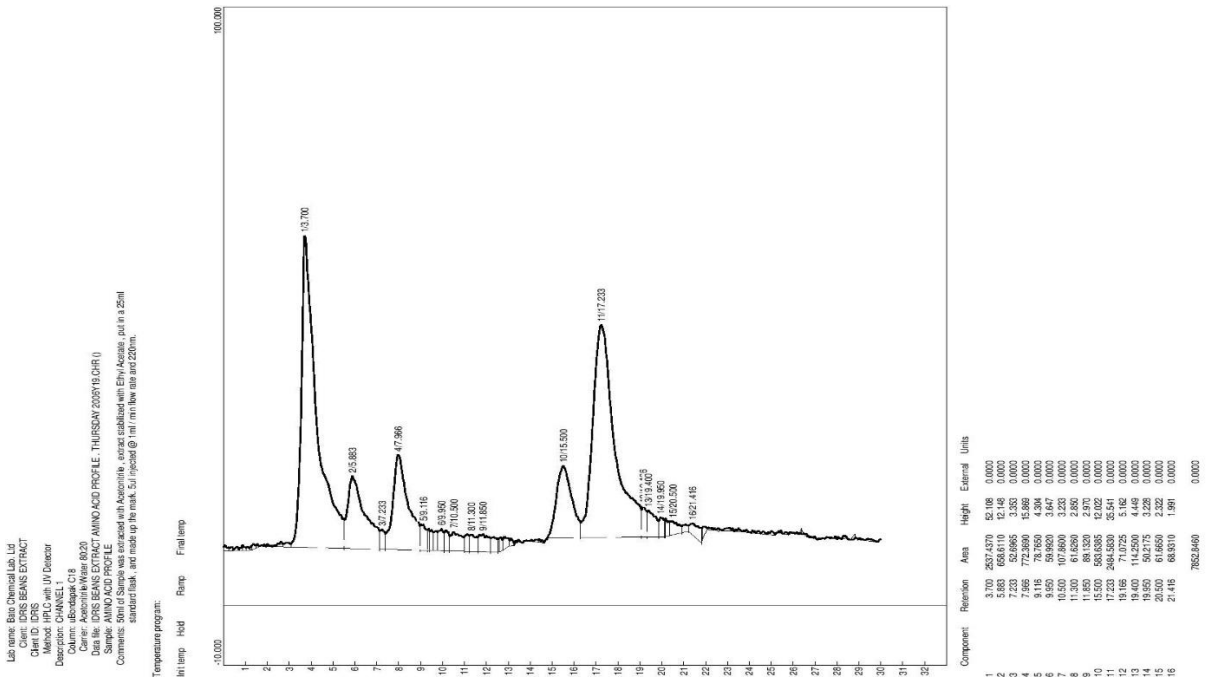


Standard Protein Curve

APPENDIX IV:



Gel Chromatography Elution Profile of Cowpea Hydrolysate.



RP-HPLC Chromatogram of the Gel chromatography product

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