Antihypertensive and Antioxidant Properties of Chicken Skin Protein Hydrolysates: *In vitro, in vivo,* and Metaboloics Studies

By

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ABSTRACT

The objective of this work was to produce bioactive peptides from the enzymatic hydrolysis of chicken skin proteins that could be used to treat hypertension, oxidative stress and associated health conditions using a metabolomics approach. Enzymatic hydrolysis of chicken thigh and breast muscle skin proteins was carried out using alcalase or a combination of pepsin/pancreatin (PP) at 1–4% enzyme concentrations. Chicken skin protein hydrolysates (CSPH) were each fractionated by membrane ultrafiltration into different molecular weight peptides (<1, 1–3, 3–5 and 5–10 kDa). Investigation of their *in vitro* antihypertensive and antioxidant activities showed that alcalase hydrolysates had significantly ($p < 0.05$) higher ACE-inhibitory activity compared to PP hydrolysates. ACE inhibition was inversely related to size of ultrafiltration membrane peptides. Renin-inhibitory activity varied from 15–36%, and was dependent on the type of protease; PP hydrolysates showed significantly ($p < 0.05$) higher inhibition than alcalase hydrolysates. CSPHs also significantly ($p < 0.05$) scavenged antioxidant radicals, increasing with enzyme concentration but decreased as peptide size increased. Kinetics studies revealed that peptide-dependent enzyme inhibition pattern was mostly of the mixed-type for both ACE and renin. Short-term (24 hr) oral administration of 100 mg peptides/kg body weight to spontaneously hypertensive rats (SHRs) led to maximum systolic blood pressure (SBP) reduction of –32.67 and –31.33 mmHg after 6 h for chicken thigh skin hydrolysate and chicken breast skin hydrolysate, respectively. During a 6-week feeding trial, CSPH at 1.0 and 0.5% feed substitutions had significant ($p<0.05$) antihypertensive effects in SHRs (-36 and -31 SBP reductions, respectively). SBP reduction was directly related to lower plasma ACE but not renin activity. Plasma total antioxidant capacity of the rats was also high. Metabolomics analysis revealed several metabolites with significant changes (≥ 2-fold changes, $p < 0.05$) in urine and

plasma of SHRs fed CSPH, such as Symmetric Dimethylarginine (SDMA), N2-acetyl-L-ornithine, buthionine sulfoximine, uric acid, Vitamin E succinate, L-isoleucine and phospholipids which may be considered important biomarkers/pathways for hypertension and oxidative stress. We conclude that CSPHs may be used as ingredients to formulate functional foods and nutraceuticals for the management of oxidative stress and hypertension-related diseases.

DEDICATION

This thesis is dedicated to my loving family (Rosemary, Stephen, Innocent and Janine).

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FOREWARD

The manuscript format was adopted in writing this thesis and it is composed of five manuscripts written according to the guidelines specific to each of the journals used. The thesis begins with a general introduction (Chapter 1) and a review of the relevant literature (Chapter 2). Manuscript one (Chapter 3) which describes the *in vitro* antihypertensive study was written according to the *Food Research International* journal format. Manuscript two (Chapter 4) discusses the *in vitro* antioxidant study and was written using the *Food Chemistry* journal format. Manuscript three (Chapter 5), on the kinetics and blood pressure lowering properties of chicken skin protein hydrolysates (CSPHs) and manuscript four (Chapter 6), on the *in vivo* antihypertensive and antioxidant enzymes study were written according to the *Journal of Functional Foods* format. Manuscript five (Chapter 7), which discusses the metabolomics study was written using the Journal of Chromatography format. Manuscripts 1-3 have been published while manuscripts 4 and 5 are presently at various stages of internal review prior to submission for journal publication. A transition statement has been added at the end of each manuscript in order to highlight what was done and relate it to the next study.

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LIST OF ABBREVIATIONS

AAA	Aromatic amino acids
ACE	Angiotensin converting enzyme
BCAA	Branched chain amino acids
BP	Blood pressure
BW	Body weight
CAT	Catalase enzyme
CBSH	Chicken breast skin hydrolysates
CSM	Chicken skin meal
CSPH	Chicken skin protein hydrolysates
CTSH	Chicken thigh skin hydrolysates
DH	Degree of hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
FAPGG	N-(3-[2-furyl]acryol-phenylalanylglycylglycine
FER	Feed efficiency ratio
FIU	Flourescence intensity unit
GSH	Glutathione
HAA	Hydrophobic amino acids
HMH	Hemp meal hydrolysate
HMW	High molecular weight
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration of enzyme/inhibitor
K_i	Enzyme-inhibitor dissociation constant

K_m	Michaelis constant (enzyme-substrate dissociation constant)
LC-QTOF-MS	Liquid chromatography quadrupole time of flight mass spectrometry
LMW	Low molecular weight
MHQ	MassHunter Qualitative
MPP	MassHunter Profiler Professional
MWCO	Molecular weight cut-off
NCAA	Negatively charged amino acids
NO	Nitric oxide
NTRs	Normotensive rats
ORAC	Oxygen radical absorption capacity
$^{\cdot}OH$	Hydroxyl radical
$O_2^{\cdot -}$	Superoxide radical
$ONOO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PCAA	Positively charged amino acids
PLS-DA	Principal least square discriminant analysis
PP	Pepsin-pancreatin
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
RP-HPLC	Reverse-phase high performance liquid chromatography
SBP	Systolic blood pressure
SHRs	Spontaneously hypertensive rats

SOD	Superoxide oxide dismutase
TAC	Total antioxidant capacity
TFA	Triflouroacetic acid
WKY	Wistar Kyoto rats

CHAPTER 1

INTRODUCTION

The incidence of hypertension or elevated blood pressure (BP) is increasing at an alarming rate worldwide, with an estimated prevalence of 30% in the adult population suffering from arterial hypertension and about 30 – 60% of diabetic patients (Ceriello, 2008; Cheng et al., 2008). Hypertension is one of the major known independent risk factors for cardiovascular diseases (CVD) such as atherosclerosis, coronary heart disease, and stroke, which has also been reported to be the leading cause of death in developing countries (Barbana & Boye, 2011). Approximately 600 million people with hypertension are likely to develop heart attacks and other heart-related complications such as strokes, kidney failure, diabetes, blindness, and dementia (Ahhmed & Muguruma, 2010). Hypertension is a chronic disease and is also often associated with metabolic abnormalities such as impaired glucose tolerance, insulin resistance, abdominal obesity, reduced high density lipoprotein-cholesterol (HDL), hyperglycemia and hyperlipidemia, especially in people suffering from this condition and as such, it may be considered a metabolic syndrome (Ceriello, 2008; Erdmann et al., 2008; Hu et al., 2011). Hypertension is considered to be a "silent killer" because of the multiple harmful effects on the body without showing any visible signs (Hu et al., 2011). Unfortunately, approximately 90% of people with hypertension are ignorant of these potential problems and consequences, thereby increasing the risk of death associated with the condition (Ahhmed & Muguruma, 2010).

Hypertension is a multifactorial disorder, though lifestyle related activities are believed to contribute more to the disease incidence and as such, changes in lifestyle patterns may help in prevention (Ahhmed & Muguruma, 2010; Erdmann et al., 2008). Several approaches are presently being used to treat and remedy hypertension. Antihypertensive medications (captopril,

enalapril, lisinopril, and aliskiren) known to target the renin-angiotensin system (RAS) are being used to effectively lower BP in patients without actually treating the underlying cause of hypertension (Aa et al., 2010; Ahhmed & Muguruma, 2010). They are however, synthetic in nature, expensive and exhibit some side effects such as dry cough, skin rashes, and pains, thereby making the search for natural compounds with potential nutraceutical and functional benefits as alternatives to synthetic drugs a great necessity (Aa et al., 2010; Erdmann et al., 2008; Fitzgerald et al., 2004; Lee et al., 2010). According to Huang et al. (2005), the intake of fruits and vegetables is inversely correlated with the incidence of chronic diseases such as inflammation, CVD, cancer and aging-related disorders. Consequently, a lot of attention is being increasingly placed on diet and lifestyle modifications as one of the strategies to address the issue of CVD-related problems like hypertension (Ahhmed & Muguruma, 2010; Erdmann et al., 2008). Of particular interest is the consumption of proteins which has been associated with reduced risk of hypertension and CVD. This has been attributed to the presence of biologically active amino acids and peptides with a wide range of nutritional, functional and bioactive properties (Erdmann et al., 2008; Samanarayaka et al., 2010; Vercruysse et al., 2005).

Bioactive peptides are short amino acid sequences (usually 2 – 20) or protein fragments that will influence metabolic or physiological processes and impart positive beneficial health effects (Di Bernardini et al., 2011; Ovelgonne et al., 2007). They are usually produced by proteolytic hydrolysis using either commercially purified enzymes or microbial fermentation processes and absorbed in the intestine when consumed where they enter into blood circulation directly and exert their physiological action *in vivo* (Di Bernardini, et al., 2011; Erdmann et al, 2008; Vermeirssen et al., 2004). Many food materials from both plants and animals such as milk, whey, meat, fish, pea, and soybean have been reported to be potent sources of bioactive peptides.

These foods could be used as potential ingredients in the development of functional foods and nutraceuticals for the prevention, management and treatment of chronic diseases, especially hypertension (Ahhmed & Muguruma, 2010; Di Bernardini et al., 2011; Erdmann et al., 2008). There is however, little information on the use of poultry processing by-products, especially chicken skin for the development of health-promoting bioactive peptides (Ovelgonne et al., 2007). According to Jamdar & Harikumar (2008), poultry processing is increasing rapidly resulting in a large volume of by-products that are high in proteins. However, most of these by-products are underutilized due to high amounts of fats, ash, fibre, and microbes in addition to poor aesthetic qualities. These poultry by-products are mostly discarded, which causes environmental pollution and loss of useful nutrients, especially proteins (Di Bernardini et al., 2011; Feddern et al., 2010; Piette et al., 2001). Enzymatic hydrolysis of these by-products of poultry processing industry, especially chicken skin to produce bioactive peptides will therefore, be an effective way to take advantage of the rich protein content to enhance nutrition and functional properties in addition to waste reduction and cost savings (Centenaro et al., 2011; Jamdar & Harikumar, 2008; Jamdar et al., 2012; Lasekan et al., 2013).

Another innovative and novel area that will be critical to this study will be the application of metabolomics profiling to understand fundamental aspects relating to the pathogenesis of hypertension, oxidative stress and associated chronic conditions. The role played by bioactive peptides obtained from the hydrolysis of chicken skin protein in regulating these metabolic processes is also important. Metabolomics is focused on the analysis of "small-molecule compound profiles that will provide information about the whole organism functional integrity over time after exposition of a perturbation" (Jiang et al., 2012). The use of metabolites profiling in hypertension has been generating increasing interest since it was first established that there is

an association between serum metabolite profiles and BP in clinically hypertensive patients (Aa et al., 2010; Brindle et al., 2003; Lu et al., 2008). Several attempts have been made at understanding the association between genetic and metabolic features as well as BP attenuation. This is with a view to discovering biomarkers useful for predicting and diagnosing hypertension using the spontaneously hypertensive rats (SHR) in comparison with the normotensive Wistar Kyoto (WKY) rats (Aa et al., 2010; Akira et al., 2013; Akira et al., 2012; Lu et al., 2008). However, to the best of our knowledge, no metabolomics profiling study has been conducted on the potential biomarkers of hypertension and oxidative stress using bioactive peptides obtained from chicken skin protein hydrolysates. Therefore, this work will assist our understanding of the molecular nature and mechanisms involved in hypertension and oxidative stress with the possibility to promote chicken skin protein hydrolysates (CSPHs) as suitable ingredients for the development of antihypertensive nutraceuticals and functional foods.

1.1 Research gaps/Justifications for the proposed study

From the foregoing, we have identified the following research gaps and justifications for this study;

I. To the best of our knowledge, no work has been reported on the development of chicken skin based foods with functional and health promoting values for use in the control, prevention, management and treatment of oxidative stress, hypertension and associated metabolic disorders.

II. There is a lack of information on the BP-lowering property of CSPHs.

III. There is a lack of information on the oxidative stress-reducing property of CSPHs.

IV. There is lack of information on the ability of food protein hydrolysates in general and CSPHs in particular to modulate metabolites associated with BP regulation.

V. There is equally a lack of information on the potential biomarkers of hypertension and oxidative stress as well as possible mechanisms for *in vivo* antioxidant and antihypertensive effects of CSPHs.

1.2 Hypothesis of the proposed study

The approximately 20% protein in chicken skin (usually considered a waste material) could be used to add value through enzymatic hydrolysis to produce CSPHs with potential physiological health benefits for the control and management of chronic disease conditions, especially hypertension and oxidative stress. The specific hypotheses for this research study are;

I. Chicken skin proteins can be hydrolyzed enzymatically to yield peptides with potential bioactivities.

II. CSPH will also possess *in vitro* antihypertensive activities measured as modulations of the renin angiotensin system.

III. CSPH will exhibit *in vitro* antioxidative properties determined as the ability to scavenge free radicals, chelate or reduce metal ion.

IV. CSPH will reduce blood pressure and oxidative stress after oral administration to SHR.

V. BP and oxidative stress reductions in SHR by CSPH will correlate with plasma levels of specific metabolites considered as potential biomarkers of hypertension and oxidative stress as well as potential mechanistic pathways.

1.3 Objective of the proposed study

Therefore, the general objective of this work is to produce antioxidant and antihypertensive peptides through enzymatic hydrolysis of chicken skin proteins. The mechanism of action of these peptides can be determined using a metabolomics approach. The specific objectives of the proposed study are;

I. Optimize the enzymatic hydrolysis of chicken skin proteins to produce antioxidant and antihypertensive peptides.

II. Determine the potential antihypertensive properties of the CSPHs by measuring their *in vitro* inhibitory activities against renin and angiotensin converting enzyme (ACE).

III. Determine the potential *in vitro* antioxidative properties of CSPH and their ultrafiltration fractions.

IV. Determine the BP– and oxidative stress-lowering effects of CSPH after long-term oral administration to SHR.

V. Determine correlations between BP and plasma metabolite profiles in order to identify potential biomarkers for hypertension and oxidative stress as well as potential mechanisms for BP regulation.

1.4 Significance of the proposed research

The significance of this research proposal will be to:

I. Promote the value-added utilization of chicken skin proteins. This will have multi-sectorial benefits especially, to the consumers, farmers, processors, government, research institutes and the academia.

II. Offer new opportunities for the functional foods and nutraceuticals industries to develop food products or supplements that will actively improve treatment and management of hypertension and other oxidative stress-induced metabolic disorders.

III. Obtain results from animal studies that will form the basis for developing human clinical trials to evaluate the BP-reducing efficacy of CSPH-based functional foods and nutraceuticals.

IV. Perform metabolomics studies that will serve as the basis for developing biomarkers of oxidative stress, hypertension and CVD. The work will provide important information on potential mechanisms of action of the CSPH as well as the regulatory pathways involved in BP or oxidative stress attenuation by CSPH.

CHAPTER 2

LITERATURE REVIEW

2.1 Poultry industry

According to Bhat et al. (2013), the poultry industry has undergone a great transformation into a "vibrant, organized and scientific sector" involved in the production of quality animal protein products at cheaper rates. This transformation has resulted in a great increase in global production and consumption of poultry meat and products, especially in countries where the industry has significant economic importance (Badr, 2005; Bonifer & Froning, 1996; Jamdar & Harikumar, 2008; Lasekan et al., 2013). The United States, China and Brazil are reported to be world leaders in poultry production with approximately 19,852, 18,102 and 12,200 thousand metric tons, respectively in 2011. China and Brazil lead in chicken meat production with estimated outputs of 13,000 and 11,750 thousand metric tons, respectively in 2011 (Feddern et al., 2010; Lasekan et al., 2013). The consumption of poultry is further expected to increase to 40% or more of total meat consumption in the United States and Canada due probably to the consumption of value added processed products (Bonifer & Froning, 1996; Piette et al., 2001). This increase may also be attributed to the price, availability and the fact that poultry products, especially chicken are not forbidden by any religion, necessitating their incorporation into other food products in order to improve nutrition (Barbut, 2012; Feddern et al., 2010).

Within the poultry group, chicken has been reported to be the most frequently consumed worldwide due to its high meat yield, lack of shrinkage during cooking, ease of cooking and low cost (Badr, 2005). This has also led to an increasing variety of value-added processed chicken products, especially in ready-to-eat forms such as cooked chicken, bologna sausage, chicken nuggets and frozen meals in recent years (Bonifer & Froning, 1996; Cliche et al., 2003).

Associated with these increases also is the generation of waste and under-utilized by-products such as organs, viscera, feet, head, bones, blood, feathers and mechanically separated poultry meat. This creates a huge problem for the food and poultry industry since these by-products greatly exceed the industry capacity for food product formulation (Di Bernardini et al., 2011; Piette et al., 2001). It is therefore, very imperative to find ways of handling these by-products, especially reutilizing them in making other products considering their rich lipid and protein contents (Feddern et al., 2010).

2.2 Utilization of chicken skin

Chicken skin, which constitutes approximately 4% of the live weight and 12% of carcass weight of the animal, has been reported to be one of the most under-utilized residues of chicken meat industries (Bhat et al., 2013; Feddern et al., 2010). This creates additional cost required for disposal and consequently, a huge environmental problem as well as loss of potential nutritional benefits that might be derived. According to Lasekan et al. (2013) animal wastes including chicken skin were previously converted to livestock feed and organic fertilizers which led to development of rendering, composting, and other treatment processes. However, some of these processes have their inherent drawbacks necessitating the need for an alternative and appropriate processing technology. Most of the excess chicken skin was previously used with other poultry wastes to produce inedible rendered fat and meals of varying degrees of quality. However, due to the large volumes and the improved sanitary conditions of the poultry processing plants, the chicken skin is presently used as a starting material for edible fat manufacture (Piette et al., 2001).

In addition to rendered edible fat, attempts have also been made to develop novel products from chicken skin in other to diversify its utilization, reduce waste and cost and

contribute to nutrition (Babji et al., 1998; Bhat et al., 2013; Biswas et al., 2007; Bonifer & Froning, 1996; Cliche et al., 2003). Chicken skin was incorporated up to 50% level into lean chicken meat from spent hen to make coated and uncoated meat balls with very good sensory palatability (Bhat et al., 2013). Though the enrobing process was reported to significantly improve sensory qualities of the meat balls, both stored well in low density polyethylene (LDPE) pouches for 14 days in the refrigerator without detrimental loss of quality. Collagen with valuable and unique functional properties has been extracted and characterized from chicken skin in response to the increasing demand for alternative sources other than bovine collagen and the low levels of antigens in poultry collagen (Cliche et al., 2003). Chicken skin contains a high amount of collagen (3%) which is a huge potential raw material for the manufacture of a variety of value-added products, especially in the cosmetics and medical industries (Bonifer & Froning, 1996; Cliche et al., 2003). The extraction yields of the collagen after heat treatment at 40^{o}C depend on the solubilisation process, with approximately 39 and 25% for pepsin and ethylene diamine respectively. Chicken skin has also been incorporated as a source of fat and also to contribute to improved texture in the manufacture of sausage in an attempt to reduce the cost of sausage and diversify utilization of chicken skin (Biswas et al., 2007). It was observed that as the level of incorporation of chicken skin and chicken fat increased, the physicochemical and sensory qualities of the sausages decreased. However addition of chicken skin up to a level of 20% during sausage preparation had no adverse or objectionable physicochemical and sensory effects. Similarly, chicken skin was incorporated into mechanically deboned frankfurters up to 20% following separation of the fat from the protein or collagen by cooking at low (40 -45^{o}C) heat (Babji et al., 1998). It was reported that increasing chicken skin levels resulted in increased moisture, ash, protein, fat, cholesterol and color but reduced lipid oxidation and cooking loss.

Overall, the taste and texture sensory parameters of the frankfurters (10-20%) were acceptable even after 3 months of frozen storage.

Several convenient chicken skin based foods and products are currently being developed to meet the ever increasing demand, reduced cost and protect the environment against waste and pollution. However, research on the development of chicken skin based foods with functional and health promoting values still remains highly unexplored. Chicken skin is rich in fat, protein (about 20%, dry weight basis), enzymes and other nutritionally important biomolecules that have the potentials to be recovered and processed into useful products (Badr 2005; Bonifer & Froning, 1996; Jamdar & Harikumar, 2008; Lasekan et al., 2013). The high protein contents of chicken skin especially can be used as a very good source of bioactive peptides with functional and health promoting values. For example, bioactive peptides can be used to alleviate symptoms of oxidative stress, hypertension and dyslipidemia, which are coronary heart disease risk factors in addition to contributing to good nutrition (Samanarayaka et al., 2010).

2.3 Bioactive peptides-containing food protein hydrolysates

Food proteins are known to contribute to nutrition by their amino acid content as well as the utilization of some specific amino acids after digestion and absorption (Ryan et al., 2011). The protein content could also be hydrolyzed by enzymes to yield bioactive peptides with functional applications as nutraceuticals and pharmaceuticals for the prevention and management of chronic diseases such as hypertension or high blood pressure, cancer, obesity, hypercholesterolemia and other chronic conditions (Di Bernardini et al., 2011). Functional foods have been defined as either conventional or endogenous foods consumed as part of a regular diet that however, contain bioactive compounds and nutraceuticals with demonstrated physiological benefits of reducing the risk of chronic diseases beyond basic nutritional functions (Ahhmed &

Muguruma, 2010). However, Erdmann et al. (2008) defined biologically active peptides as "food-derived peptides that exert, beyond their nutritional value, a physiological, hormone-like effect in humans". They consist of natural sequences of amino acids (2 – 20 amino acid residues) encrypted in the parent or natural protein molecule and their action is often a function of their amino acid composition as well as the sequences of the amino acids. They are usually inactive within the protein sequence but released during gastrointestinal digestion or *in vitro* hydrolysis by enzymes (proteases) and the liberated peptides thereafter play important roles to regulate and modulate intestinal metabolism (Aluko, 2008a). They thus have the potential of being used as metabolic supplements in the production of nutraceuticals and functional food ingredients for the promotion of health and prevention of diseases (Di Bernardini et al., 2011; Martinez-Maqueda et al., 2012). This will also help to meet the current growing demand for food-derived (natural) bioactive compounds with potential health benefits as alternatives to drugs since they are not known to exhibit any of the adverse side effects (cough, skin rash and pain) associated with synthetic compounds (Di Bernardini et al., 2011; Je et al., 2004; Shahidi & Zhong, 2008).

Bioactive peptides have been isolated from various food sources; milk (FitzGerald et al., 2004), whey (Hernandez-Ledesma et al., 2005), eggs (Erdmann et al., 2008), meat (Di Bernardini et al., 2011; Ryan et al., 2011), fish (Centenaro et al., 2011; Ryan et al., 2011), quinoa seeds (Aluko & Monu, 2003), lentil proteins (Barbana & Boye, 2011), pea proteins (Pownall, Udenigwe, & Aluko, 2010) and several other foods. Protein hydrolysates derived from these edible foods have been used over a long period of time to provide nutritional, functional and bioactive properties. However, the continuous use of these foods as sources of protein hydrolysates is unsustainable due to the growing world population and incidence of food insecurity, thereby creating the need for alternative low cost by-products from food processing

plants, especially fish and chicken bones and skins (Je et al., 2004; Lasekan et al, 2013; Nakade et al., 2008). The use of non-toxic food processing by-products also has the added advantage of being environmentally friendly, as it helps to prevent, reduce and control pollution.

The possible regulatory effects of bioactive peptides relate to nutrient uptake, antihypertensive, antioxidant, anticancer, antithrombotic, opiates or antiproliferative as well as antimicrobial activities (Erdmann et al., 2008; Samaranayaka & Li-Chan, 2011). Many of the known bioactive peptides exhibit multifunctional properties, exerting more than one function simultaneously and are easily absorbed (Erdmann et al., 2008; Lee et al., 2010). However, focus has been more on the antihypertensive and antioxidative activities of bioactive peptides and protein hydrolysates because of their potential roles in the prevention of oxidative stress, hypertension and associated CVD (Samanarayaka et al., 2010). Moreover, oxidative stress due to free radical generation during cellular metabolism and respiration is invariably associated with hypertension (Vaziri, 2008).

2.4 Hypertension and high blood pressure

Hypertension has been defined as a condition in which the "blood pressure in the arteries is elevated, usually is persistently at or above 140 mmHg of the systolic blood pressure (SBP) or 90 mmHg diastolic blood pressure (DBP) or both" (Ahhmed & Muguruma, 2010). It is a chronic condition that has been reported to also be a leading risk factor for CVD, stroke, end stage of renal disease and premature death globally (Erdmann et al., 2008; Girgih et al., 2013). Hypertension affects about 24% of the adult population in the developed world especially the US and is also rapidly becoming a major problem in developing countries (Erdmann et al., 2008; Lee et al., 2010). Though the cause of primary (essential) hypertension in about 85 – 95% of cases is unknown, lifestyle may actually support its development. Therefore, diet and lifestyle

modifications are being increasingly targeted as effective ways to minimize the risk of hypertension and CVD related conditions (Ahhmed & Muguruma, 2010; Vermeirssen et al., 2004). Increased consumption of protein has been notably reported to lower the risk of hypertension and heart diseases probably due to the presence of biologically active peptides (Vercruysse et al., 2005). As a multifactorial disorder, hypertension may be caused by a complex combination of genetic and environmental factors (diet, salt intake, age, race, gender, smoking, lack of exercise, stress and excessive alcohol intake). Diets rich in fruits and vegetables, fibre grains, lean meat, low-fat dairy but low in salts have also been reported to significantly reduce BP in both hypertensive and normotensive individuals (Ahhmed & Muguruma, 2010).

Although, BP control is a physiological balance maintained by the nervous system and the kidney, however, when these control mechanisms fail, due to a variety of factors, hypertension develops. Hypertension is also often caused by the loss of elasticity of the walls of the larger arteries which becomes rigid, creating less space for the flow of blood and leading to increased pressure of fluid (Ahhmed & Muguruma, 2010). As had been previously discussed, hypertension is a multifactorial disorder. However, an in-depth discussion of two of the notable pathways, the renin angiotensin system (RAS) and the oxidative stress-induced pathway will be made here in order to highlight the roles of bioactive peptides and protein hydrolysates in the inhibition of these reactions and consequently the control of hypertension and related health conditions.

2.4.1 Renin-Angiotensin system and hypertension.

The renin-angiotensin system (RAS) has been reported to be a major regulator of BP, CVD such as congestive heart failure, hypertension and renal function (Fujita et al., 2000; Schmieder et al., 2007). RAS plays vital roles in the maintenance of BP homeostasis, fluid and

electrolyte balance in the human body (Girgih et al., 2011; Lee et al., 2010). The RAS system exerts strong antiatherosclerotic effects due to their antihypertensive, anti-inflammatory, antiproliferative and antioxidative properties. Therefore, the function of the antihypertensive peptide is to lower BP by reducing RAS activity, which causes arterial vessel relaxation and allowing more flexibility (Ahhmed & Muguruma, 2010; Erdmann et al., 2008; Schmieder et al., 2007). RAS is mainly controlled by two enzymes, renin and angiotensin-I-converting enzyme (ACE). Renin (an aspartyl protease) catalyzes the initial step of the reaction, converting angiotensinogen to angiotensin I. The angiotensin I is subsequently converted to angiotensin II by ACE (EC 3.4.15.1). ACE belongs to a class of zinc proteases located in the vascular endothelial linings of the lungs (Je et al., 2004). ACE is present in several body tissues where it serves as an integral part of BP moderation and control of normal heart functions (Ryan et al., 2011). As a decapeptidylcarboxylase that cleaves the dipeptides from the C-terminus of oligopeptides, ACE catalyzes the hydrolysis of the inactive angiotensin I to form angiotensin II, the potent vasoconstrictor (Martinez-Maqueda et al., 2012).

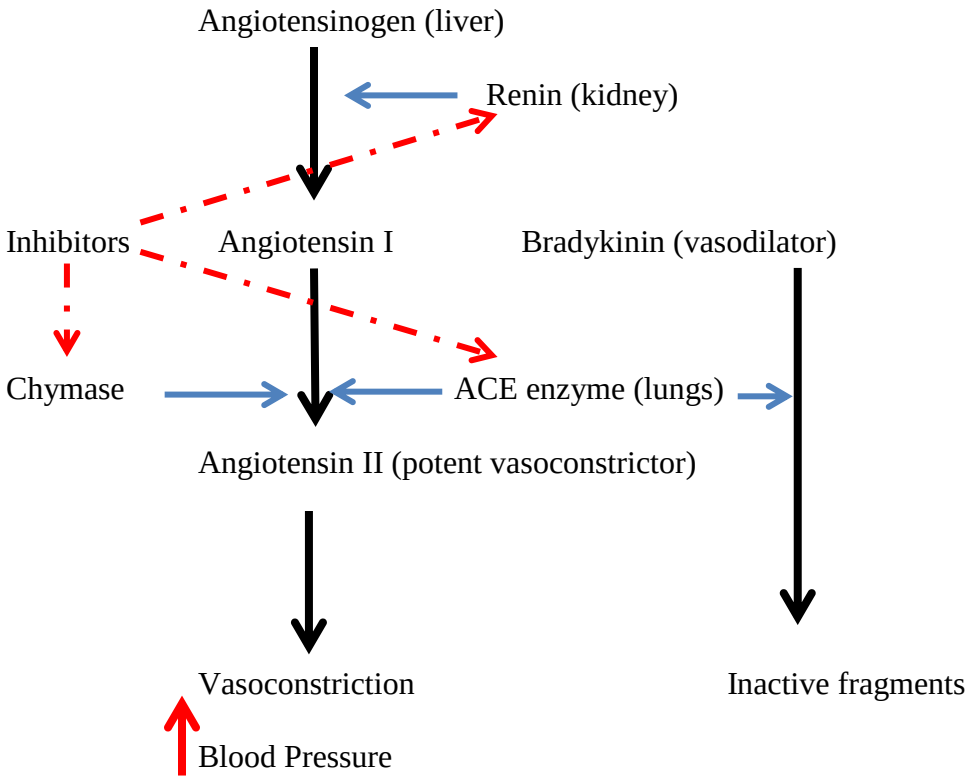
ACE is also responsible for the inactivation of the potent vasodilator, bradykinin, resulting in blood pressure elevation (Je et al., 2004; Vercruysse et al., 2005). Angiotensin II acts directly on vascular smooth muscles and expands vascular volume by retaining sodium and fluids contents, thereby constricting the vessels. Bradykinin on the other hand is responsible for the contraction of uterine and ileal smooth muscles, thereby enhancing vascular permeability, activation of peripheral and C fibres as well as increasing secretion of mucous. Bradykinin is also responsible for the vasodilation of blood vessels by contributing to the assembly of arachidonic acid metabolites, nitric oxide (NO) and endothelium-derived hyperpolarizing factor in the vascular endothelium (Ryan et al., 2011).

2.4.1.1 Mechanisms of action of ACE inhibitors

The mechanism of ACE inhibitors in BP regulation is the maintenance of a balance between the vasoconstrictive plus salt retentive abilities of angiotensin II and the vasodilatory property of bradykinin. This is done by decreasing the production of angiotensin II while at the same time reducing the hydrolytic degradation of bradykinin (Ryan et al., 2011). Inhibition of these processes has long been the basis for the use of synthetic ACE inhibitors such as captopril, enalapril, lisinopril, and fosinopril in the treatment of hypertension, congestive heart failure and myocardial infarction (Erdmann et al., 2008; Fujita et al., 2000). Captopril, for instance, also known as peptidyl-dipeptidase A or (2*S*)-1-(3-mercapto-2-methylpropanoyl)-L-proline is a potent ACE inhibitor for the control of elevated blood pressure through formation of angiotensin II (vasoconstrictor) and destruction of bradykinin (vasodilator). However, while the synthetic ACE inhibitors work by directly by blocking the action of ACE by binding to the enzyme active site, bioactive peptides with inhibitory actions against ACE on the other hand are effective by reacting with ACE, making it unavailable to cleave angiotensin I and subsequently preventing its hydrolysis to the potent vasoconstrictor, angiotensin II (Ryan et al., 2011). Therefore, naturally occurring bioactive peptides are increasingly becoming more attractive as potential antihypertensive treatment agents since they are food-derived, and exhibit no known negative side effects common with synthetic drugs (Je et al., 2004).

Several *in vivo* studies in SHR models have been used to explain the mechanisms of action responsible for BP-lowering ability of some renin and ACE-inhibitory peptides (Erdmann et al., 2008; Fujita et al., 2000; Girgih et al., 2011; Lee et al., 2010). Potency of the renin and

Figure 2.1 Mechanism of action of ACE inhibitors (Adapted from Erdmann et al., 2008)



ACE-inhibitory peptides is usually expressed in terms of the IC_{50} value, defined as the inhibitor concentration that cause a 50% inhibition of renin or ACE activity (Erdmann et al., 2008). However, for the peptide to act as an inhibitor, it has to be absorbed through the intestinal walls in intact form and also reach the target organ or system, for instance, the cardiovascular system without a loss of activity. However, there are usually some problems with regards to determining or extrapolating the efficacy of potential renin and ACE-inhibitory peptides *in vivo* in humans using the SHR model probably due to differences in bioavailability of nutrients between humans and rats (Fujita et al., 2000; Ryan et al., 2011). Equally important are dosage and nutrient form, time of administration, state of the subjects as well as the control diets (Ryan et al., 2011).

Another problem usually encountered is the likelihood of peptides with demonstrated *in vitro* ACE-inhibitory activity but devoid of *in vivo* activity. This may be as a result of possible changes in peptide form before reaching the target site for activity (Fujita et al., 2000; Martinez-Maqueda et al., 2012; Ryan et al., 2011).

Based on this, ACE-inhibitory peptides are classified according to their metabolic fate in the presence or absence of gastrointestinal enzymes into 3 groups (Fujita et al., 2000). The first group referred to as "substrates" are peptides that inhibit *in vitro* ACE activity but are hydrolysed by ACE or gastrointestinal tract enzymes when consumed orally, which results in weak inhibitory activity or are ineffective as BP-lowering agents. The second group called the "true inhibitors", inhibit *in vitro* ACE activity and also lower BP when consumed orally (the IC_{50} value is not affected by preincubation with ACE or gastrointestinal tract enzymes). The third group known as the "pro-drug", has increased activity against ACE as they are converted to "true inhibitors" by ACE or gastrointestinal tract enzymes to a peptide with higher inhibitory property than the original peptide (Aluko, 2008b; Fujita et al., 2000). The Caco-2 cell monolayer offers an ideal *in vitro* test of ACE-inhibitory peptide activity as it is accepted to predict the permeability of intestinal cells to pharmaceuticals (Ryan et al., 2011).

The mechanism of action of the majority of renin and ACE-inhibitory peptides is considered to be as competitive substrates (Ryan et al., 2011). The structure-activity relationships of these peptides are still not fully understood due to variations in amino acid sequences. However, it was originally believed to be due to the presence of proline at the C-terminal, as is the case with the peptides Val-Pro-Pro and Ile-Pro-Pro (Erdmann et al., 2008; Ryan et al., 2011). However Saiga et al. (2006) observed that the peptides that do not contain proline are also capable of acting as ACE inhibitors with adequate antihypertensive activity.

Short-chain peptides (di or tripeptides), such as those with C-terminal proline or hydroxyproline are more resistant to enzymes of the digestive tracts and are also more rapidly absorbed than the free amino acids (Erdmann et al., 2008). Peptides with hydrophobic or positively charged amino acids at the C-terminal position such as Trp, Phe, Pro, Lys or Arg exhibit better ACE-inhibitory potency (Je et al., 2004). This is possibly as a result of interaction occurring at the active sites of the ACE protein molecule (Lee et al., 2010). The better potency of these peptides may also be due to the presence of zinc binding and hydrogen bond donation (Erdmann et al., 2008). The presence of hydroxyproline has also been observed to be vital for the peptides to bind to ACE active sites, especially, in the case of peptides that contain more than three amino acid residues (Ryan et al., 2011). In addition, overall hydrophobicity of the peptide is vital and hydrophilic peptides act as weak inhibitors or have no inhibitory activity since they are not compatible with required interactions with the ACE active site hydrophobic residues (Erdmann et al., 2008).

2.4.1.2 Antihypertensive peptides and protein hydrolysates

Antihypertensive peptides, notably the ACE inhibitors are the best known and researched among the different groups of bioactive peptides (Ryan et al., 2011; Vercruysse et al., 2005). Bioactive peptides from protein hydrolysates with demonstrated inhibitions of renin and ACE activities have been isolated and studied extensively in different foods such as milk, soybean, hemp seed, flaxseed, eggs, whey, meat, fish and other animal by-products as well from chicken bone extracts, chicken breast muscle extracts, and chicken collagen (Aluko & Monu, 2003; Cheng et al., 2008; Erdmann et al., 2008; Girgih et al., 2011; Nakade, et al., 2008; Saiga et al., 2008; Samanarayaka et al., 2010; Udenigwe et al., 2009; Vercruysse et al., 2005). The antihypertensive properties of hemp seed protein hydrolysates (HPH) and peptide fractions have been evaluated following simulated gastrointestinal digestion using pepsin and pancreatin

(Girgih et al., 2011). The HPH was reported to have significantly higher *in vitro* renin and ACE inhibitory activities than the peptide fractions. This observed trend was attributed to a loss of synergistic effects when the peptides were fractionated which was in contrast with other studies in which the potency of bioactive peptides was reported to increase with decreasing peptide sizes (Cheng et al., 2008; Saiga et al., 2008; Udenigwe et al., 2009). Kinetics studies revealed a mixed pattern of inhibition for the hydrolysates and the ultrafiltration membrane fractions, suggesting peptide ability to bind to sites other than the enzyme active site. The hydrolysates were also observed in a short-term oral administration study to decrease SBP to a maximum of –30 mmHg when administered at 200 mg/kg body weight dosage to SHRs after 8 hrs when compared to the weaker effects of <1 and 1 – 3 kDa peptides (Girgih et al., 2011). The results suggest that HPH may be used as an effective food protein-based agent to ameliorate elevated BP levels.

Lentil protein hydrolysates have also been investigated for their ACE inhibitory activities using different enzymes for the protein digestion (Barbana & Boye, 2011). Electrophoretic and size exclusion chromatrography analysis of the hydrolysates revealed significantly high content of low molecular weight peptides in the range of 0.244 – 1.06 kDa, suggesting a high degree of hydrolysis (DH). The DH is a parameter generally used to monitor proteolysis and it is the most widely used indicator for comparing different protein hydrolysates as well as an indication of peptide yield (Adlernissen, 1979; Barbana & Boye, 2011; Cheng et al., 2008). The IC_{50} value of the hydrolysates ranged from 0.053 – 0.190 mg/ml with the gastrointestinal simulated hydrolysates been the most potent, suggesting their ability to resist further gastrointestinal breakdown after consumption and consequently exert *in vivo* potency. The observed differences in ACE inhibition may be due to differences in the type of peptides liberated by the hydrolytic actions of the enzymes and the conditions during the hydrolytic process (Barbana & Boye, 2011;

Vermeirssen et al., 2004). The mechanism of ACE inhibition by the hydrolysates was shown to be non-competitive, suggesting the peptides may not bind at the ACE active site but at other sites on the ACE molecule, which will likely generate an inactive (enzyme-substrate-inhibitor) complex. They concluded that the results indicate potential new applications in the utilization of lentils (Barbana & Boye, 2011).

The protein (61%) from Alaska Pollock frame (skeleton), a by-product of fish processing has also been hydrolyzed with pepsin and fractionated using ultrafiltration membranes to yield ACE-inhibitory peptides (Je et al., 2004). The <1 kDa fraction was observed to exhibit the highest ACE-inhibitory activity and was subsequently selected for size exclusion chromatography to yield 5 additional fractions with the fraction IV having the strongest potency against ACE (IC_{50} value of 0.11 mg/ml). The active fraction was further separated and purified using reverse phase high performance liquid chromatography (RP-HPLC) and the most active purified peptide obtained was identified to have amino acid sequence as Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala with IC_{50} value of 14.7 μM. The mode of ACE inhibition using the Lineweaver-Burk plots was determined to be non-competitve (Je et al., 2004). Similar results were obtained for protein hydrolysates from tuna frame (Lee et al., 2010), tuna dark muscle (Qian et al., 2007) and tilapia (Raghavan & Kristinsson, 2009) though the amino acid sequences differs depending on the enzyme used for hydrolysis, the DH and the peptide size.

The ability of protein hydrolysates from hemp seed meal to be used as a preventive and treatment agent against high BP has also been demonstrated in a diet-intervention study with SHRs (Girgih et al., 2014). The diets containing 0.5 and 1.0% (w/w) hemp seed meal protein hydrolysate (HMH) substitutions were fed to young and growing rats for 8 weeks in the preventive phase of the study and 4 weeks in the treatment phase of the study. It was reported

that HMH was able to significantly ameliorate the normal increases in SBP, which resulted in an average ~ 120 mmHg value for the treated growing SHRs when compared to ~ 158 mmHg value for the control diet group. The results) indicate potential of the HMH to be used as a preventive therapy against hypertension. Similar results were also obtained for the 4 weeks treatment phase with ~119 mmHg SBP for the treatment group when compared to ~150 mmHg for the control group). Plasma ACE and renin levels were shown to be significantly suppressed by the HMH intervention diets with values ranging from 0.047 – 0.059 U/ml and 0.040 – 0.054 μg/ml, respectively. The authors concluded that the inhibition of plasma ACE and renin activities could be one of the major mechanisms responsible for the antihypertensive effects of HMH (Girgih et al., 2014).

2.4.1.3 Antihypertensive protein hydrolysates from poultry and chicken by-products

Bioactive peptides containing protein hydrolysates with potent ACE-inhibitory activities have also been produced from several by-products of poultry (chicken) processing (Cheng et al., 2008; Cheng et al., 2009; Cheng et al., 2008; Nakade et al., 2008; Saiga et al., 2003). Oral administration of two chicken breast muscle extracts from untreated and microbial (*Aspergillus*) protease-hydrolysed samples was reported to significantly lower BP of SHRs to a maximum value of 50 mmHg, which is stronger than previously observed (Saiga et al., 2003). The effect was observed after 1 hr and continued for at least 4 hs post-administration. Purification of the hydrolysate active peptides using RP-HPLC yielded 4 peptides upon identification. The peptides with the strongest ACE-inhibitory activity had an amino acid sequence of GFHypGTHypGLHypGF and an IC_{50} value of 42.4 μM. Subsequent work was carried out to determine the peptide's action mechanisms against ACE by an intravenous administration of a synthetic form of the most active peptide. Administration of the synthesized peptide resulted in a

significant drop in the BP of SHRs. They reported that the peptide showed high affinity toward ACE with only 10% of the total peptide decomposed, and a stronger activity (IC_{50} value of 10 μM) than the original peptide (IC_{50} value of 46 μM). The mode of inhibition was reported to be non-competitive. However, removal of phenylalanine (Phe) from the C-terminus significantly increased the IC_{50} (25000 μM), thereby weakening the ACE-inhibitory activity. The results suggest that the Phe presence at the C-terminus is very vital for this peptide's activity (Saiga et al., 2006).

Chicken bones from a meat processing factory were hydrolysed with alcalase, pepsin, and trypsin for 12 hrs to produce ACE-inhibitory peptides (Cheng et al., 2008a; Cheng et al., 2009; Cheng et al., 2008b; Cheng et al., 2008c). Alcalase hydrolysates exhibited the highest peptide content and degree of hydrolysis, were more active in inhibiting in vitro ACE activity with IC_{50} values of 1.960 and 0.945 μM. Effectiveness of the peptides was attributed to the lower molecular weights in the alcalase hydrolysates, suggesting that chicken leg bone has a high potential to be used as an ingredient for the development of antihypertensive functional foods (Cheng et al., 2008a). Oral administration of the hydrolysates to SHRs (50 mg/kg body weight/day) led to a maximal SBP reduction of about 26 mmHg after 4 hrs, which was maintained up to 8 hrs. Treatment for 8 weeks also led to a significant SBP reduction, which is comparable to captopril's effect (Cheng et al., 2008b). The hydrolysate was shown to gradually reduce the development of hypertension and cardiovascular hypertrophy in SHRs. After HPLC purification and separation, 18 fractions were detected with most of them showing inhibitory activities. The amino acid sequence of fractions having >50% ACE-inhibitory activity revealed 10 peptides consisting of 5 – 10 amino acid residues. Three of the peptides were similar to peptides obtained from collagen type I and chicken muscle proteins (Cheng et al., 2009).

A novel ACE-inhibitory peptide was isolated and purified from chicken bone extract using enzyme (peptic) hydrolysis (Nakade et al., 2008). The purified peptide exhibited antihypertensive activity when administered orally to SHRs indicating that it has the ability to significantly reduce SHR SBP over a short time (3 hrs) and the blood pressure reduction was sustained for the same duration of time. Amino acid sequence analysis revealed that the peptide is a tetra-peptide with the amino acid sequence Tyr-Tyr-Arg-Ala and a molecular weight of 571.67 Da. The synthesized peptide exhibited an IC_{50} value of 33.9 µg/ml, which is comparatively stronger than other ACE-inhibitory peptides reported for chicken bone extracts, sake lees, dried bonito, sardine muscle, fermented milk, porcine troponin C and cottonseed proteins.

2.4.2 Oxidative stress and hypertension

Another important area in which biologically active peptides have been used extensively is antioxidative activity due to their potential roles in the prevention and treatment of chronic oxidative stress-induced diseases (Di Bernardini et al., 2011; Girgih et al., 2011; Girgih et al., 2014; Ryan et al., 2011). Free radical generation during normal cellular metabolism and respiration has become a major health concern. Although oxidation is known to be essential to all living organisms to fuel biological processes, uncontrolled production of free radicals (superoxide, hydroxyl, singlet oxygen, peroxyl) associated with it is of great concern to human health (Erdmann et al., 2008). Oxidative stress, defined as the "increased production of reactive oxygen species (ROS) in combination with outstripping of endogenous antioxidant defense mechanisms", has been implicated in the initiation or progression of many vascular diseases (Erdmann et al., 2008; Kregel and Zhang, 2007). This is because of the ROS ability to damage critically important biological molecules such as proteins, lipids and DNA. ROS also modify low

density lipoprotein (LDL), thereby leading to increased atherogenicity of oxidized LDL and development of atherosclerotic plaque (Erdmann et al., 2008). This can consequently cause many terminal degenerative diseases such as CVD, diabetes, cancer, Alzheimer's disease and so many other conditions (Di Bernardini et al., 2011; Erdmann et al., 2008; Ryan et al., 2011).

Oxidative stress resulting from the imbalance between free radical generation and the body's antioxidant defense mechanisms has been closely associated to the pathogenesis of hypertension (Ceriello, 2008; Kregel and Zhang, 2007; Nabha et al., 2005; Vaziri, 2008; Young and Woodside, 2001). More worrisome is the evidence that oxidative stress (and its associated inflammation) and hypertension are in an intrinsically self-perpetuating vicious cycle with damaging consequences on cellular organs and tissues (Vaziri, 2008). ROS molecules are usually produced *in vivo* during normal cellular metabolism primarily from the mitochondrial electron transport chain as well as from the activities of several oxidase enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cyclooxygenase, lipoxygenase, glucose oxidase, uncoupled nitric oxide synthase, NOS and xanthine oxidase (Young and Woodside, 2001). Superoxide anion which is the primary ROS produced in the body by a single electron reduction of molecular oxygen is short-lived, very reactive and toxic and can react with the vascular relaxation factor, nitric oxide (NO) secreted by the endothelium to form peroxintrite (OONO-), a potent vasoconstrictor (Vaziri, 2008; Nabha et al., 2005). The superoxide radical is usually converted to hydrogen peroxide by superoxide dismutase (SOD) and then water by either catalase or glutathione peroxidase. The hydrogen peroxide (H_2O_2) can however also undergo conversion in the presence of metal ions such as Fe^{2+} and Cu^{2+} to a highly reactive cytoxic hydroxyl (.OH) radical, causing further damages to biomolecules (Kregel and Zhang, 2007; Vaziri, 2008; Young and Woodside, 2008).

According to Ceriello (2008), superoxide anion is a major determinant of the biosynthesis and bioavailability of NO since it causes inactivation of NO and as such, may modify endothelial functions. As a consequence, free radicals may reduce the NOS activity, and thereby decrease NO either by direct reduction of NO synthesis from NOS or disruption of endothelial receptor signal transduction and increased blood pressure (Nabha et al., 2005). They can also act directly on NO and make it less available. NOS, especially endothelial nitric oxide (eNOS) has equally been found to cause oxidative stress by "NOS uncoupling" whereby the production of NO is decreased in favour of an increase in the production of superoxide radical (Ceriello, 2008). This process potentiates oxidative stress with attendant damaging effects on endothelial and vascular functions arising from the oxidation of proteins, enzymes, lipids and other biomolecules which consequently results in hypertension (Ceriello, 2008; Vaziri, 2008).

Evidences also support an association between oxidative stress, hypertension and inflammation, as demonstrated by increased biomarkers of lipid peroxidation and oxidative stress and reduced levels of antioxidants as well as antioxidant enzymes (Ceriello, 2008; Simao et al., 2011; Vaziri, 2008; Young and Woodside, 2001). Hypertension is however reversed or improved when oxidative stress is treated by administration of antioxidants or increased levels of antioxidant enzymes. ROS production through the action of NADPH oxidase also causes Angiotensin II induced hypertension through binding to the angiotension I receptor and consequently, the production of inflammation (Ceriello, 2008).

In addition to body cell damages, oxidative changes of food lipids commonly referred to as rancidity can cause deterioration and loss of food quality as well as reduction in the shelf life or keeping quality of food materials. Consumption of such foods containing oxidation products of lipids has also been associated with various chronic diseases (Di Bernardini et al., 2011; Ryan

et al., 2011). Antioxidants, defined as "a substance in foods that significantly decreases the adverse effects of reactive oxygen and nitrogen species on normal physiological functions" or "compounds which are able to delay or prevent autooxidation processes" have been employed to control oxidation (Di Bernardini et al., 2011). They play important roles in human health and nutrition because they protect the body from the destructive effects of reactive oxygen species (ROS) molecules (Martinez-Maqueda et al., 2012). A number of enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase help to regulate oxidative processes in the human body. However, the ability of these endogenous enzymes to regulate oxidative stress is often weakened when excess free radicals are formed beyond their antioxidant capacity (Erdmann et al., 2008; Wu et al., 2005; Young & Woodside, 2001).

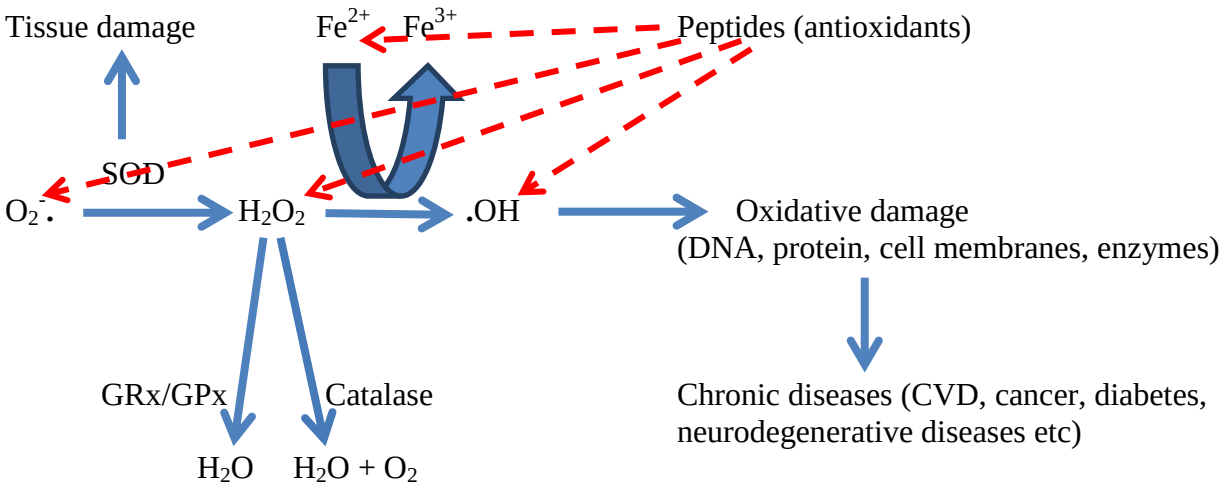
Many synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) have been used in food products to control these oxidative processes with great success. However, the use of natural antioxidants as an alternative to synthetic antioxidants in foods is generating interest because of the potential health risk of these synthetic antioxidants (Centenaro et al., 2011; Di Bernardini et al., 2011; Ryan et al., 2011). Clinical and epidemiological studies have shown an inverse association between the consumption of fruits and vegetables and the occurrence of inflammatory, cardiovascular, cancer and age-related diseases (Huang et al., 2005). Dietary antioxidants have also been reported to enhance the endogenous antioxidant defense system against the damaging effects of oxidative stress (Kim et al, 2013). Apart from the dietary intake of antioxidants such as vitamin C, vitamin E, polyphenols, flavonoids, isoflavones and carotenoids to boost the body's defense mechanisms in the fight against oxidative stress, food derived bioactive peptides with antioxidant properties have also been reported (Di Bernardini et

al., 2011; Erdmann et al., 2008; Kim et al., 2013). This could offer potentially huge beneficial effects both in health and in functional food systems applications (Di Bernardini et al., 2011).

2.4.2.1 Mechanisms of action of antioxidant peptides

As had been previously stated, though ROS is generated as a by-product of cellular metabolism, uncontrolled production of ROS can lead to deleterious consequences due to oxidative stress and inflammation, which consequently cause damages to cellular biomolecules such as proteins, lipids, carbohydrates, enzymes and DNA (Kim et al., 2013). This can also lead to several physiological disorders and terminal degenerative diseases if not adequately controlled by the use of antioxidant systems either derived endogeneously or from external sources and in particular, food derived peptides. For instance, oxidative damage to DNA often results in uncontrolled cell proliferation as well increased rate of cell death while protein oxidation causes protein aggregation and neuronal death in several types of aging-related neurodegenerative diseases (Kregel and Zhang, 2007). This is further complicated by a modification of transcription factors and activation of signaling pathways and altered gene expressions for many physiological processes. These damaged macromolecules in turn can regulate cell-signaling pathways to potentiate the destructive effects of oxidative stress (Ceriello, 2008; Kregel and Zhang, 2007). A peptide obtained from loach was effective in improving the endogenous cellular antioxidant defense system (SOD, CAT, and GSH-Px enzymes) and thereby ROS in mice with high levels of oxidative stress (You et al., 2011). Another peptide purified from *Myritus coruscus* was found to increase the SOD activity in mice and a concurrent reduction in oxidative stress (Kim et al., 2013). Girgih et al (2014) also reported imcreased SOD and CAT activity in the blood of SHRs

Fig. 2.2 Mechanism of action of antioxidant peptides (adapted from Young and Woodside, 2001)



fed hemp meal hydrolysates (HMH) thereby downregulating oxidative stress and the risk and severity of associated cardiovascular diseases, especially hypertension.

Although the exact mechanisms of actions for the antioxidant activities of bioactive peptides and protein hydrolysates are not clearly known, it has been observed that they depend on the enzyme specificity, degree of hydrolysis and nature of the released peptides as well as the molecular weight, amino acid composition and hydrophobicity of the amino acids (Erdmann et al., 2008; Girgih et al., 2011b; Kim et al 2013). Antioxidant activity also depends on the presence of free amino acids within the hydrolysates as well as their ability to chelate metal ions, scavenge free radicals, quench singlet oxygen, inhibit linoleic acid oxidation and also act as reducing agents (Centenaro et al., 2011; Kitts & Weiler, 2003; Tang et al., 2009). The antioxidant property is also due to the presence and sequence of certain amino acids, especially,

those with high amounts of histidine and some other hydrophobic amino acids such as tyrosine, methionine, lysine, tryptophan and proline (Erdmann et al., 2008; Wu et al., 2005). Histidine-containing dipeptides carnosine and anserine found in chicken meat have been shown to exhibit antioxidant and free radical scavenging activities with helpful vasodilatory and antihypertensive effects in animals (Wu et al., 2005). It is believed that histidine containing peptides act by either donating hydrogen, trapping lipid peroxyl radicals or chelating metal ions. Hydrophobicity of peptides is also thought to increase antioxidant ability due to increased accessibility to hydrophobic targets (Erdmann et al., 2008). It is however important to note that none of these properties alone can be said to be responsible for the antioxidant properties of peptides but most likely due to synergistic and cooperative effects of these mechanisms (Chen et al., 1998).

2.4.2.2 Antioxidant peptides and protein hydrolysates

Food-derived bioactive peptides with antioxidant properties against both enzymatic and non-enzymatic peroxidation of lipids and their essential fatty acids have been discovered in several foods such as milk, eggs, wheat, potato, fungi, and until recently hydrolysates of fish and a few domestic animal muscles and bones (Di Bernardini et al., 2011; Erdmann et al., 2008; Girgih et al., 2011b; Hernandez-Ledesma et al., 2005; Jamdar et al., 2012; Li et al., 2008; Naqash & Nazeer, 2011; Pownall et al., 2010; Ryan et al., 2011). The free radical scavenging activities of chickpea protein hydrolysates (CPH) and their various gel-filtration fractions was measured in order to determine its antioxidant potentials (Li et al., 2008). The fractions were reported to possess antioxidant activities with good reducing power, DPPH, hydroxyl, and superoxide radical scavenging activities as well as excellent inhibition of linoleic acid peroxidation. Fraction IV was observed to have the highest antioxidant activities, which were also comparable to BHT and α-tocopherol. The ability of CPH to exhibit excellent antioxidant

activities was attributed to their concentration, their ability to donate electrons, the amino acid composition (Arg, Phe, Lys, Leu, Ala and Asp) of the fractions as well as their molecular weights. The antioxidant activities of protein hydrolysates is known to be influenced by the content of hydrophobic amino acids and the total hydrophobic amino acids (THAA) as increase in hydrophobicity will likely increase their solubility in lipid systems as well as improve their antioxidant capacity (Girgih et al., 2011; He et al., 2013; Li et al., 2008).

Similarly, pea seed protein hydrolysates obtained from the enzymatic hydrolysis of pea protein isolate with thermolysin and its HPLC fractions have been investigated for their amino acid composition and antioxidant properties (Pownall et al., 2010). HPLC fractionation using C12 reverse phase HPLC yielded five different fractions (F1 – F5), with the fractions eluting earlier (F1 – F2) having lower content of hydrophobic and aromatic amino acids than the fractions that eluted later (F3 –F5). Contrary to the observation above, hydrophobicity of the amino acids (Leu, Phe, Val and Trp) had no positive influence on the reducing power of the samples compared to glutathione. However, the samples (F3 – F5) exhibited good DPPH, hydroxyl, superoxide, hydrogen peroxide scavenging activities and inhibition of linoleic acid oxidation which also depended on the hydrophobicity of the amino acids, the presence of some specific amino acids as well as the molecular weight of the peptides. Fractionation of the hydrolysates also resulted in the concentration of the active peptides, and consequently higher potency in the fractions F4 and F5 (Pownall et al., 2010).

The antioxidant activity of two whey proteins hydrolysates, α-lactalbumin (α-La) and β-lactoglobulin A (β-Lg A) hydrolysed using different enzymes (pepsin, trypsin, chymotrypsin, thermolysin and Corolase PP) and the RP-HPLC fractions has been investigated (Hernandez-Ledesma et al., 2005). Corolase PP was reported to give the most potent antioxidant scavenging

activity with ORAC values of 2.315 and 2.151 μmol of Trolox equivalent/mg of protein for α-La and β-Lg A respectively. This was attributed to the high degradation of whey proteins by Corolase PP (a complex mixture of enzymes) which has synergistic hydrolytic action on whey proteins and the low enzyme specificity of thermolysin. These hydrolytic processes also led to the generation of small molecular sized peptides (between 390 and 855 kDa). The differences observed in the radical scavenging activity by the hydrolysates were also attributed to differences in the molecular size and the amino acid sequence of the peptides (Chen et al., 1998; Hernandez-Ledesma et al., 2005). RP-HPLC purification and characterisation of the 3 kDa ultrafiltration membrane permeate obtained from Corolase PP β-Lg A hydrolysate yielded 5 fractions (F1 – F5) with F1, F2 and F4 exhibiting better antioxidant activity in terms of ORAC values (2.327, 3.384, 3.449 μmol Trolox equivalent/mg of protein respectively) than the permeate (1.897 μmol Trolox equivalent/mg of protein). The fractions were subjected to amino acid sequencing and a total of 42 peptides were identified with various amino acid sequences. However, an analysis of one of the sequences (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile) revealed strong antioxidant radical scavenging activity with ORAC value of 2.621 μmol Trolox equivalent/mg of protein, which was higher than BHA (2.43 μmol Trolox equivalent/mg of protein).

The antioxidant properties of peptides isolated from the backbone of the tropical two-winged flying fish (*Exocoetus volitans*) hydrolysed with papain, pepsin and trypsin have been determined (Naqash & Nazeer, 2011). Proximate composition analyses showed the protein content of the hydrolysates to be high (>60%) due likely to the solubilisation of the protein during the enzyme digestion process, removal of the insoluble non-protein and lipid components that were not hydrolyzed. Though all the hydrolysates significantly inhibited linoleic acid oxidation, the peptic hydrolysate exhibited the highest inhibition probably due to amino acid

composition and sequence (Je et al., 2004; Naqash & Nazeer, 2011). Scavenging of free radicals activities also revealed that the peptic hydrolysate exhibited the highest scavenging abilities and therefore, it was subsequently subjected to fast protein liquid chromatography (FPLC) using DEAE XK 26/20 anionic column. The fractions were collected, pooled, lyophilised and assayed for antioxidant potencies. The fractions exhibited strong antioxidant potentials with fraction III showing the highest potency, resulting in further fractionation using G-25 gel permeation column. Further analysis revealed fraction IIIb to be most potent in quenching DPPH (47.7%), hydroxyl (50.2%), and superoxide (44.2%). Analysis of the amino acid profile of fraction IIIb showed that it contains both essential and non-essential amino acids necessary for adults. Fraction IIIb contained high contents of glutamic acid, lysine, glycine and threonine and a minor content of histidine and methionine; these amino acids are known to possess strong antioxidant activities (Chen et al., 1998; He et al., 2013; Naqash & Nazeer, 2011).

2.4.2.3 Antioxidant protein hydrolysates from poultry and chicken by-products

Very limited research has been done in the area of antioxidant properties of domestic animal muscles, and especially chicken. Most of these studies however, reported that chicken products significantly suppressed the development of oxidative products (Centenaro et al., 2011; Jamdar et al., 2012; Wu et al., 2005). The antioxidant activities of chicken essence has been demonstrated by determining the inhibition of linoleic acid oxidation, DPPH radical scavenging activity, reducing power, and metal ion chelating activity (Wu et al., 2005). It was observed that the antioxidant activity of the chicken essence increased with increasing concentration. Amino acid analyses revealed that chicken essence contained an appreciable quantity of free amino acids with taurine as the predominating compound. Other low molecular weight peptides were also present in a large quantity. The chicken essence was reported to contain high levels of

potent antioxidants such as anserine and carnosine. Separation by size exclusion chromatography revealed that the peptide with molecular weight of approximately 1400 Da possessed the strongest antioxidant activity, followed by peptides with 900 and 500 Da. Further isolation produced two antioxidant peptides that were identified to have the following amino acid sequences: His-Val-Thr-Glu-Glu and Pro-Val-Pro-Ala-Glu-Gly-Val, respectively.

The antioxidant activities of Argentine croaker (fish) and chicken bones hydrolysed with flavourzyme, α-chymotrypsin and trypsin enzymes has been determined (Centenaro et al., 2011). The hydrolysates exhibited different degrees of hydrolysis and antioxidant activities with the flavourzyme hydrolysates having high activity against lipid peroxidation (77.3 and 61.6% for fish and chicken respectively). The hydrolysates also exhibited moderate DPPH free radical scavenging, ABTS scavenging and hydroxyl radical scavenging activities. The study concluded that the antioxidant activity of peptides depends on the enzyme used for hydrolysis, the degree of hydrolysis as well as the system in which the antioxidants are tested. Based on the results obtained, it was suggested that the hydrolysates could therefore, be used as antioxidant substances in food systems to control lipid peroxidation as well functional ingredient for the control of hypertension.

The antioxidant and ACE-inhibitory properties of poultry viscera protein hydrolysate (PVPH) and its peptide fractions were also determined (Jamdar et al., 2012). PVPH displayed excellent antioxidant activity in terms of radical scavenging activities as well as total antioxidant activity at peptide concentrations of 0.2 and 2.0 mg/ml respectively, increasing with increasing concentration. The reducing power was reportedly high for the samples at peptide concentrations of 9 and 30 mg/ml respectively. It was observed that the reducing power of the PVPH samples increased with increasing peptide concentration for all the samples with the >10 kDa fraction

having the highest reducing power compared to the 3-10 kDa and <3 kDa fractions, suggesting that the peptide size plays a critical role in determining the reducing power. The ABTS and hydroxyl radical scavenging activities for the fraction III was reported to be significantly higher than the other fractions. However, the superoxide radical scavenging activity was observed to be independent of peptide size.

2.5 Metabolomics and metabolites profiling

Metabolomics is primarily concerned with the study of low molecular weight compounds in bio-fluids and other matrices (Bruce et al., 2009; Kristensen et al., 2012). It is a systematic approach to the study of *in vivo* metabolic profiles that will provide information on drug toxicity, disease processes and gene function at several stages in the discovery and development process (Law et al., 2008; Lu et al., 2008). Metabolomics has also been defined as "the measurement of the metabolome or the full set of low molecular weight endogeneous compounds (or metabolites) present within cells, tissues, organisms or bio-fluids that can reflect genetic modifications, exposure to pathogens, toxic agents, pharmaceuticals and nutritional and environmental changes" (Aa et al., 2010; Theodoridis et al., 2008). The small molecules are diverse endogenous and exogenous chemical entities ranging from peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, alkaloids, minerals and several other chemicals that can be metabolized, or synthesized in the cell or an organism (Wishart, 2008). These metabolites play key roles within *in vivo* physiological and metabolic pathways, and as such, metabolomics approach is very vital in exploring the various mechanisms underlying disease conditions, especially hypertension and their complications.

Several analytical techniques are currently being used for metabolomics studies as its success depends on a high quality data set that gives an overview of the condition of an organism

through the metabolites profiles (Law et al., 2008). However, it is vital that whatever technique that is used permits the interrogation of samples with minimal sample preparation as well as been able to reliably analyze a wide range of metabolites (Theodoridis et al., 2008). The techniques used includes nuclear magnetic resonance (NMR), liquid chromatography coupled to mass spectrometry (LC-MS), gas chromatography coupled to mass spectrometry (GC-MS), capillary electrophoresis coupled to mass spectrometry (CE-MS), fourier transform infrared spectrometry (FT-IR) and direct infusion mass spectrometry (MS) (Theodoridis et al., 2008). The detection and characterization of metabolites is normally done using electronic databases that contain descriptive and spectral information on the metabolites being investigated. Multivariate statistical analysis and pattern recognition (chemometrics) tools such as principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) are used to compare and identify features that distinguish the various categories of complex metabolic profiles (Law et al., 2008; Theodoridis et al., 2008; Wishart, 2008). Computer-aided software packages using special algorithms are applied to the raw data set to filter, bin, align and normalize the features detected in order to identify investigated ions as likely biomarkers. Biomarker identifications are usually made by MS data based either on the exact mass, fragmentation pattern or database searches (METLIN, Lipid Maps, NIST, KEGG). Where identification is made, the metabolite is subsequently confirmed using a standard, if available and possible pathway analysis done using the KEGG compound database (Doshi & Day, 2009; Theodoris et al., 2008).

2.5.1 Application of metabolomics profiling

According to Wishart (2008), the application of metabolomics has previously been focused on clinical and pharmaceutical studies, drug discovery and assessment, clinical toxicology and chemistry. However, owing to its potential for simultaneous characterization of

diverse chemical compounds in biological systems, the application to metabolomics to food and nutrition has received increasing attention. This will likely aid our understanding of the complex molecular nature of foods and most especially identify bioactive food ingredients and their potential usefulness in the development of nutraceuticals and functional food products that could be used in the prevention and management of diseases such as atherosclerosis, heart disease, cancer and arthritis (Wishart, 2008). As a result, metabolomics profile investigations are being increasingly used to determine small molecules or metabolites that can be considered as potential biomarkers of oxidative stress, inflammation, CVD and a host of other conditions (Llorach et al., 2009; Wishart, 2008). One of the ways by which this is done is diet intervention trials either on a short-term or long-term basis which measures quantitative molecular biomarkers and physiological stress response in biofluids, especially urine and plasma (Kristensen et al., 2012; Wishart, 2008).

Several metabolomics studies have been conducted to establish the metabolites that can be considered either as biomarkers of food intake or disease conditions (Bruce et al., 2009; Kristensen et al., 2012; Law et al., 2008; Llorach et al., 2009). The effects of fresh apple and apple-pectin on the urinary metabolome of rats were investigated in order to establish the exposure markers of fruit and fruit fibre intake (Kristensen et al., 2012). Three groups of 24 Fisher 344 male rats were fed a standard diet supplemented with either 7% apple pectin or 10 g raw apple for 24 days. Metabolomics analysis of the metabolites revealed the presence of quinic acid, m-coumaric acid and (-) epicatechin as exposure markers of apple intake while hippuric acid was identified as effect marker. With regards to pectin, pyrrole-2-carboxylic acid and 2-furoylglycine were identified as exposure markers whereas 2-piperidinone was identified as effect marker of pectin intake. They reported that most of the metabolites are related to different

metabolic pathways such as phase II glucuronidation, glycine conjugation or microbial metabolism and as such, can help our understanding of the health implications of fruit consumption (Kristensen et al., 2012).

Cocoa and cocoa products are known to be associated with improved antioxidant status, antiplatelet effects, regulate immune system, and cause vasodilation and as such, the health benefits of cocoa consumption due to its phytochemicals contents have been studied using metabolomics profiling to characterize the intake and effects on metabolism (Llorach et al., 2009). Urinary metabolites of ten human subjects given a single dose of cocoa powder with milk or water, or milk with cocoa powder in a 24 h randomized, cross-over and placebo-controlled trial were examined. Although the results revealed 27 significant metabolites that were related to cocoa phytochemicals such as alkaloid derivatives, polyphenol metabolites, and products of cocoa processing, the changes were not influenced by the matrix. They concluded that the results support the theory that food-derived metabolites could be related to some anticipated health effects (Llorach et al., 2009).

Tea, which is similar to cocoa in phytochemicals contents, is reported to have beneficial health effects due to its rich contents of polyphenolic compounds (especially catechins) (Law et al., 2008). Human urinary metabolic profiles of 8 male adults 3 h following consumption of green tea containing 12 g of green tea solids in 300 mL of water without milk and sugar was investigated using GC-MS, LC-MS and ^{1}H NMR in order to determine biomarkers of tea intake. The results revealed changes in a number of metabolites associated with glucose metabolism, citric acid cycle and amino acid metabolism following green tea consumption. The GC-MS methodology was reported to give a different set of metabolites for characterizing changes in human urines compared to the LC-MS and ^{1}H NMR. However, the data from the LC-MS and ^{1}H

NMR showed close agreements suggesting that there is no significance in ion suppression of the LC-MS for metabolites that were eluted early. Though there were differences in metabolites between the GC-MS and LC-MS/^{1}H NMR methodologies, this multiple analytical method provided useful information of the metabolic profiles following ingestion of plants with bioactive and medicinal properties (Law et al., 2008).

Metabolomics profiling has also been applied to whole grain products since whole grain consumption is reported to have beneficial health effects by reducing incidences of CVD, diabetes and other diet-related conditions (Bondia-Pons, Barri, Hanhineva, Juntunen, Dragsted, Mykkanen, et al., 2013). Urinary metabolites profiles of 20 subjects consuming rye bread versus wheat bread from a 2 x 4 weeks crossover diet intervention trial were investigated for potential biomarkers of whole grain consumption in an untargeted metabolomics study. The results showed 16 metabolites as main potential biomarkers of whole grain consumption, with 3-(3,5-dihydroxyphenyl)-1-propanoic acid sulphate as the most discriminative metabolite identified. Other metabolites identified are alkylresorcinol metabolites, enterolactone glucuronide, azelaic acid, 2-aminophenol sulphate and 2,4-dihydroxy-1,4-benzoxanzin-3-one. Though, the study was considered as a preliminary work, they suggested further validation in order to make these markers indices of compliance in healthy Nordic diets (Bondia-Pons et al., 2013).

2.5.2 Metabolites profiling in hypertension and oxidative stress

Metabolomics approach has also been applied to the study of biological perturbations responsible for hypertension and oxidative stress. Most of these studies used the SHR model since its pathophysiological processes closely resemble those of human essential hypertension (Aa et al., 2010; Akira et al., 2012; Lu et al., 2008). The SHR progressively develops hypertension with age, involving several pathological complications such as cardiovascular,

cerebrovascular and renal failures when compared to its normotensive (control) WKY rats (Akira et al., 2012; Lu et al., 2008). The BP may steadily rise with age to 200 – 250 mmHg, and as such, understanding the metabolic changes responsible for these BP elevation is highly paramount (Akira et al., 2012). Although several works have been reported with regards to metabolites profile changes in hypertension, the pioneering work in this area was done by Brindle et al. (2003). Using the serum profiles of patients with normal (≤ 130 mmHg), borderline (131 – 149 mmHg) and high BP (≥ 150 mmHg) obtained by ^{1}H NMR, they were able to determine the relationship between metabolic profiles of serum and hypertension. Using this application, they were able to differentiate the serum samples of normal SBP from borderline and high SBP samples. However, 'normal' borderline and the high SBP samples were not different from each other in their serum metabolic profiles. The results also demonstrated that the relationship between serum metabolic profiles and BP were partly due to differences in lipoprotein particle composition between the samples. Also, metabolic changes in serum related to BP were observed in NMR profile even before the SBP reached the defined hypertension values, suggesting that the current definition for hypertension may actually be too high (Brindle et al., 2003).

The development of hypertension and age-related changes has also been characterized using plasma samples from the SHRs and its normotensive WKY (Lu et al., 2008). Several compounds observed to change significantly are free fatty acids (FFA) (hexadecanoic acid, linoleic acid, and stearic acid), amino acids (threonic acid, tyrosine, tryptophan, threonine, phenylalanine, serine, ornithine, methionine, and 3-hydroxyproline), 3-hydroxybutyric acid, citric acid, creatinine, erythrose, myo-inositol, D-methylglucopyranoside, tocopherol, sitosterol, and nonesterified cholesterol. While FFA were found to be significantly increased in SHR when

compared to WKY rats, their levels also increased with age from 10 – 18 weeks in the SHRs, suggesting that FFA are potential biomarkers for hypertension.

Metabolic characteristics of SHRs were similarly investigated for all ages, beginning with the prehypertensive as well as the hypertensive stage in comparison to WKY rats using ^{1}H NMR urinary metabolites (Akira et al., 2008; Akira et al., 2012). In the first study by Akira et al. (2008) to compare the urinary metabolic profiles of SHR with that of their age-matched WKY rats, many metabolites such as citrate, α-ketoglutarate and hippurate were observed to be significantly changing and their levels were lower in SHR urine when compared to WKY urine. The decreased level of citrate was reported to be probably due to mild acidosis which is an indication of the pathogenesis of BP elevation as well as impairment of Krebs cycle (Akira et al., 2008). This may also explain the decrease in the excretion of α-ketoglutarate since it is a decarboxylate intermediate of the Krebs cycle. However, hippurate and trimethylamine-N-oxide were reported to be products of the intestinal microflora. They concluded that changes in these metabolites indicate that they may be associated with BP regulation in the SHR (Akira et al., 2008). However, in the second study to investigate the urinary metabolic profiles of the SHR across different stages of hypertension, urinary excreted metabolites shown to be characteristic of the SHR are citrate, α-ketoglutarate, succinate, hyppurate, phenylacetylglycine, p-cresol glucuronide, creatine, taurine, and medium chain dicarboxylates (Akira et al., 2012). Decrease in Krebs cycle intermediates, especially citrate may be due to acidosis in the SHR at the prehypertensive stage and also during early stage of development of hypertension, suggesting an impairment of the renal energy metabolism in the rats. However, metabolic changes in hippurate, p-cresol and phenylacetylglycine give an indication of a change in SHR gut microflora due to genetic and metabolic factors (Akira et al., 2012).

2.5.3 Metabolomics profiles of bioactives in the control of hypertension and oxidative stress

As previously discussed, the use of natural bioactive ingredients as alternatives to synthetic drugs for hypertension prevention and management of is generating attention owing to the fact that they are deemed safe and are naturally derived. However, the molecular basis for the regulatory control of these bioactives on BP and perturbed metabolism has not been fully described (Aa et al., 2010; Akira et al., 2013; Jiang et al., 2012). A proper characterization of the metabolites involved in these regulatory effects will assist our understanding of the molecular nature of hypertension and oxidative stress especially the pathways involved as well as better approaches to the control and management of the condition and other associated health conditions. Ping Gan prescription, a traditional Chinese medicine was used in an intervention study in comparison with captopril to characterize effects on SHR metabolic profiles (Jiang et al., 2012). There were significant differences between the SHR group and the WKY group in their metabolite profiles prior to the intervention treatment. Seven metabolites that were significantly changed between the rat groups and identified as potential biomarkers of hypertension are LysoPC(22:6), LysoPC(20:4), LysoPC(18:1), cholyglycine, PE(P-16:0e/0:0), sphingosine-1-phosphate and 2-oxo-4-methylbutanoic acid. However, after treatment with Pin Gan prescription, these biomarkers were reverted to normal while the untreated group remained unaffected. These biomarkers were associated with sphingolipid and fat metabolism and invariably oxidation of lipoproteins resulting in the formation of oxidized low density lipoprotein (ox-LDL) which has been reported to be an independent risk factor for hypertension. Lyso-PC is known to be the chief component of ox-LDL, which is associated with reduction of synthesis of endothelial NO and leads to loss of diastolic function (Jiang et al., 2012).

The regulatory effects of total ginsenosides in comparison to known conventional antihypertensive drugs on BP and perturbed metabolism in SHRs was also investigated in order to understand the cause-effect relationships between high BP and hypertension-induced metabolic disorders (Aa et al., 2010). Male SHRs and WKY rats were administered either with the vehicle (0.5% carboxymethylcellulose sodium aqueous solution) as control or the treatments (30 or 3 mg/kg per day ginsenosides; 30 mg/kg captopril per day; 5 mg/kg per day amlodipine; 5 mg/kg per day terazosin per day or 10 mg/kg per day hydrochlorothiazide) suspended in 1 mL of the vehicle for 8 weeks. BP was correlated with plasma metabolites profiles analyzed using a GC/TOF/MS with pattern recognition tools to identify potential biomarkers of hypertension regulation by the treatments. Their results showed that the treatments had different regulatory effects on BP and perturbed metabolisms. The antihypertensive agents reduced BP with only little effects on perturbed metabolism in SHRs, suggesting that lowering of hypertension did not necessarily remedy perturbed metabolism and that metabolic perturbation may not be necessarily due to hypertension. However, total ginsenosides on the other hand regulated perturbed metabolism with a prolonged BP-lowering effects, suggesting that perturbed metabolism may be responsible for hypertension. There was a positive correlation between BP and FFAs (oleic acid, 9-(Z)-hexadecanoic acid, and palmitic acid), suggesting that FFA metabolism may likely be associated with development as well as control of hypertension (Aa et al., 2010). Contrary to the above observation, administration of antihypertensive agents to hypertensive patients remarkably altered lipid metabolism, suggesting a modifying effect on lipid parameters (PC and TG) and as such on metabolism perturbation (Hu et al., 2011).

The molecular mechanism for the efficacy and toxicity of taurine in the prevention of CVD and hypertension was recently investigated (Akira et al., 2013). Twenty-four h urine

samples from SHRs chronically administered 3% taurine in drinking water from age 4 – 14 weeks were subjected to ^{1}H NMR spectroscopy. They reported metabolite changes from 6 weeks of age with inter-individual variations and a concurrent BP reduction. There were decreases in citrates, α-ketoglutarate, and succinate, and increases in phenylacetylglycine and p-cresol sulfate, suggesting that chronic taurine administration to SHR resulted in accelerated metabolic acidosis which likely caused perturbation in the tricarboxylic acid cycle (TCA) as well as a change in metabolism by intestinal microflora. According to Akira et al. (2013), citrate reabsorption and metabolism in the renal proximal tubules are accelerated under the condition of metabolic acidosis, thereby inducing depletion of intracellular citrates and further reabsorption. Though metabolic acidosis is related to genetic hypertension in SHR, the results however, are in conflict with this observation, suggesting that other mechanisms may be involved in the BP-lowering action of taurine. The decreases in α-ketoglutarate and succinate may also be due to improved renal tubular reabsorption occasioned by their depletion following depletion of citrate, a precursor for these metabolites (Akira et al., 2013; Akira et al., 2012). The increase in phenylacetylglycine and p-cresol sulfate suggests that taurine may also possibly reduce BP by modulating the metabolism of intestinal microflora (Akira et al., 2013).

2.3 References

Aa, J. Y., Wang, G. J., Hao, H. P., Huang, Q., Lu, Y. H., Yan, B., Zha, W. B., Liu, L. S., & Kang, A. (2010). Differential regulations of blood pressure and perturbed metabolism by total ginsenosides and conventional antihypertensive agents in spontaneously hypertensive rats. *Acta Pharmacologica Sinica, 31*(8), 930-937.

Adlernissen, J. (1979). Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenzenesulfonic Acid. *Journal of Agricultural and Food Chemistry, 27*(6), 1256-1262.

Ahhmed, A. M., & Muguruma, M. (2010). A review of meat protein hydrolysates and hypertension. *Meat Science, 86*(1), 110-118.

Akira, K., Hichiya, H., Morita, M., Shimizu, A., & Mitome, H. (2013). Metabonomic study on the biochemical response of spontaneously hypertensive rats to chronic taurine supplementation using H-1 NMR spectroscopic urinalysis. *Journal of Pharmaceutical and Biomedical Analysis, 85*, 155-161.

Akira, K., Masu, S., Imachi, M., Mitome, H., Hashimoto, M., & Hashimoto, T. (2008). H-1 NMR-based metabonomic analysis of urine from young spontaneously hypertensive rats. *Journal of Pharmaceutical and Biomedical Analysis, 46*(3), 550-556.

Akira, K., Masu, S., Imachi, M., Mitome, H., & Hashimoto, T. (2012). A metabonomic study of biochemical changes characteristic of genetically hypertensive rats based on H-1 NMR spectroscopic urinalysis. *Hypertension Research, 35*(4), 404-412.

Aluko, R. E. (2008a). Antihypertensive properties of plant-derived inhibitors of angiotensin I-converting enzyme activity: a review. *Recent Prog Med Plants, 22*, 21.

Aluko, R. E. (2008b). Determination of nutritional and bioactive properties of peptides in enzymatic pea, chickpea, and mung bean protein hydrolysates. *Journal of Aoac International, 91*(4), 947-956.

Aluko, R. E., & Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. *Journal of Food Science, 68*(4), 1254-1258.

Babji, A. S., Chin, S. Y., Chempaka, M. Y. S., & Alina, A. R. (1998). Quality of mechanically deboned chicken meat frankfurter incorporated with chicken skin. *International Journal of Food Sciences and Nutrition, 49*(5), 319-326.

Badr, H. M. (2005). Chemical properties of chicken muscles and skin as affected by gamma irradiation and refrigerated storage. *Journal of Food Technology, 3*(1), 9.

Barbana, C., & Boye, J. I. (2011). Angiotensin I-converting enzyme inhibitory properties of lentil protein hydrolysates: Determination of the kinetics of inhibition. *Food Chemistry, 127*, 8.

Barbut, S. (2012). Convenience breaded poultry meat products - New developments. *Trends in Food Science & Technology, 26*(1), 14-20.

Bhat, Z. F., Kumar, P., & Kumar, S. (2013). Effect of skin, enrobing and refrigerated storage on the quality characteristics of chicken meat balls. *Journal of Food Science and Technology-Mysore, 50*(5), 890-899.

Biswas, S., Chakraborty, A., Sarkar, S., Barpuzari, R. N., & Barpuzari, T. (2007). Effect of incorporation of chicken fat and skin on the quality of chicken sausages. *The Journal of Poultry Science, 44*, 5.

Bondia-Pons, I., Barri, T., Hanhineva, K., Juntunen, K., Dragsted, L. O., Mykkanen, H., & Poutanen, K. (2013). UPLC-QTOF/MS metabolic profiling unveils urinary changes in humans after a whole grain rye versus refined wheat bread intervention. *Molecular Nutrition & Food Research, 57*(3), 412-422.

Bonifer, L. B., & Froning, G. W. (1996). Chicken skin composition as affected by aqueous washing. *Journal of Food Science, 61*(5), 895-898.

Brindle, J. T., Nicholson, J. K., Schofield, P. M., Grainger, D. J., & Holmes, E. (2003). Application of chemometrics to 1H NMR spectroscopic data to investigatea relationship between human serum metabolic profiles and hypertension. *Analyst, 128*, 5.

Bruce, S. J., Tavazzi, I., Parisod, V., Rezzi, S., Kochhar, S., & Guy, P. A. (2009). Investigation of human blood plasma sample preparation for performing metabolomics using ultrahigh performance liquid chromatography/mass spectrometry. *Analytical Chemistry, 81*(9), 3285-3296.

Centenaro, G. S., Mellado, M. S., & Prentice-Hernandez, C. (2011). Antioxidant activity of protein hydrolysates of fish and chicken bones. *Adv. J. Food Sci. Technol, 3*(4), 9.

Ceriello, A. (2008). Possible Role of Oxidative Stress in the Pathogenesis of Hypertension. *Diabetes Care, 31*, S181-S184.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry, 46*(1), 49-53.

Cheng, F. Y., Liu, Y. T., Wan, T. C., Lin, L. C., & Sakata, R. (2008a). The development of angiotensin I-converting enzyme inhibitor derived from chicken bone protein. *Animal Science Journal, 79*(1), 122-128.

Cheng, F. Y., Wan, T. C., Liu, Y. T., Lai, K. M., Lin, L. C., & Sakata, R. (2008b). A study of in vitro antihypertensive properties of enzymatic hydrolysate from chicken leg bone protein. . *An. Sci. Journal, 79*, 6.

Cheng, F. Y., Wan, T. C., Liu, Y. T., Lin, L. C., & Sakata, R. (2008c). A study of *in vivo* antihypertensive properties of enzymatic hydrolysate from chicken leg bone protein. *Animal Science Journal, 79*, 6.

Cheng, F. Y., Wan, T. C., Liu, Y. T., Chen, C. M., Lin, L. C., & Sakata, R. (2009). Determination of angiotensin-I converting enzyme inhibitory peptides in chicken leg bone protein hydrolysate with alcalase. *Animal Science Journal, 80*(1), 91-97.

Cliche, S., Amiot, J., Avezard, C., & Gariepy, C. (2003). Extraction and characterization of collagen with or without telopeptides from chicken skin. *Poultry Science, 82*(3), 503-509.

Di Bernardini, R., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., & Hayes, M. (2011). Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. *Food Chemistry, 124*(4), 1296-1307.

Doshi, R., & Day, P. J. R. (2009). Metabolomics of Serum Peptides. *Protein and Peptide Letters, 16*(5), 460-466.

Erdmann, K., Cheung, B. W. Y., & Schroder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry, 19*(10), 643-654.

Feddern, V., Kupski, L., Cipolatti, E. P., Giacobbo, G., Mendes, G. L., Badiale-Furlong, E., & de Souza-Soares, L. A. (2010). Physico-chemical composition, fractionated glycerides and fatty acid profile of chicken skin fat. *European Journal of Lipid Science and Technology, 112*(11), 1277-1284.

FitzGerald, R. J., Murray, B. A., & Walsh, D. J. (2004). Hypotensive peptides from milk proteins. *Journal of Nutrition, 134*(4), 980s-988s.

Fujita, H., Yokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science, 65*(4), 564-569.

Girgih, A. T., Alashi, A., He, R., Malomo, S. A., & Aluko, R. E. (2013). Preventive and treatment effects of a hemp seed (*Cannabis sativa* L.) meal protein hydrolysate against high blood pressure in spontaneously hypertensive rats. *European Journal of Nutrition*.

Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2011). In Vitro Antioxidant Properties of Hemp Seed (*Cannabis sativa* L.) Protein Hydrolysate Fractions. *Journal of the American Oil Chemists Society, 88*(3), 381-389.

Girgih, A. T., Udenigwe, C. C., Li, H., Adebiyi, A. P., & Aluko, R. E. (2011). Kinetics of Enzyme Inhibition and Antihypertensive Effects of Hemp Seed (Cannabis sativa L.) Protein Hydrolysates. *Journal of the American Oil Chemists Society*, *88*(11), 1767-1774.

Girgih, A. T., Alashi, A. M., He, R., Malomo, S. A., Raj, P., Netticadan, T and Aluko, R. E. (2014). Hemp Seed Meal Protein Hydrolysate Reduces Oxidative Stress Factors in Spontaneously Hypertensive Rats. *Nutrients* 2014, *6*, 5652 – 5666.

He, R., Girgih, A. T., Malomo, S. A., Ju, X. R., & Aluko, R. E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods, 5*(1), 219-227.

Hernandez-Ledesma, B., Davalos, A., Bartolome, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from (alpha-lactalbumin and beta-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, *53*(3), 588-593.

Hu, C. X., Kong, H. W., Qu, F. X., Li, Y., Yu, Z. Q., Gao, P., Peng, S. Q., & Xu, G. W. (2011). Application of plasma lipidomics in studying the response of patients with essential hypertension to antihypertensive drug therapy. *Molecular Biosystems, 7*(12), 3271-3279.

Huang, D. J., Ou, B. X., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*(6), 1841-1856.

Jamdar, S. N., & Harikumar, P. (2008). A rapid autolytic-method for the preparation of protein hydrolysate from poultry viscera. *Bioresource Technology, 99*(15), 6934-6940.

Jamdar, S. N., Rajalakshmi, V., & Sharma, A. (2012). Antioxidant and Ace Inhibitory Properties of Poultry Viscera Protein Hydrolysate and Its Peptide Fractions. *Journal of Food Biochemistry, 36*(4), 494-501.

Je, J. Y., Park, P. J., Kwon, J. Y., & Kim, S. K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (Theragra chalcogramma) frame protein hydrolysate. *Journal of Agricultural and Food Chemistry, 52*(26), 7842-7845.

Jiang, H. Q., Nie, L., Li, Y. L., & Xie, J. (2012). Application of ultra-performance liquid chromatography coupled with mass spectrometry to metabonomic study on spontaneously hypertensive rats and intervention effects of Ping Gan prescription. *Journal of Separation Science, 35*(4), 483-489.

Kim, E. K., Oh, H. J., Kim, Y. S., Hwang, J. W., Ahn, C. B., Lee, J. S., Jeon, Y. J., Moon, S. H., Sung, S. H., Jeon, B. T., & Park, P. J. (2013). Purification of a novel peptide derived from Mytilus coruscus and in vitro/in vivo evaluation of its bioactive properties Fish & Shellfish Immunology, 34, 1078 – 1084.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design, 9*(16), 1309-1323.

Kregel, K. C., & Zhang, H. J. (2007). An integrated view of oxidative stress in aging: Basic mechanisms, functional effects, and pathological considerations. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, *292*, 18–36.

Kristensen, M., Engelsen, S. B., & Dragsted, L. O. (2012). LC-MS metabolomics top-down approach reveals new exposure and effect biomarkers of apple and apple-pectin intake. *Metabolomics*, *8*(1), 64-73.

Lasekan, A., Abu Bakar, F., & Hashim, D. (2013). Potential of chicken by-products as sources of useful biological resources. *Waste Management*, *33*(3), 552-565.

Law, W. S., Huang, P. Y., Ong, E. S., Ong, C. N., Li, S. F. Y., Pasikanti, K. K., & Chan, E. C. Y. (2008). Metabonomics investigation of human urine after ingestion of green tea with gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry and H-1 NMR spectroscopy. *Rapid Communications in Mass Spectrometry*, *22*(16), 2436-2446.

Lee, S. H., Qian, Z. J., & Kim, S. K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, *118*(1), 96-102.

Li, Y. H., Jiang, B., Zhang, T., Mu, W. M., & Liu, J. (2008). Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). *Food Chemistry*, *106*(2), 444-450.

Llorach, R., Urpi-Sarda, M., Jauregui, O., Monagas, M., & Andres-Lacueva, C. (2009). An LC-MS-based metabolomics approach for exploring urinary metabolome modifications after cocoa consumption. *Journal of Proteome Research*, *8*(11), 5060-5068.

Lu, Y., A, J., Wang, G., Hao, H., Huang, Q., Yan, B., Zha, W., Gu, S., Ren, H., Zhang, Y., Fan, X., Zhang, M., & Hao, K. (2008). Gas chromatography/time-of-flight mass spectrometry

based metabonomic approach to differentiation hypertension- and age-related metabolic variation in spontaneously hypertensive rats. *Rapid Communications in Mass Spectrometry, 22*, 7.

Martinez-Maqueda, D., Miralles, B., Recio, I., & Hernandez-Ledesma, B. (2012). Antihypertensive peptides from food proteins: a review. *Food & Function, 3*(4), 350-361.

Nabha, L., Garbern, J. C., Buller, C. L., & Charpie, J. R. (2005). Vascular oxidative stress precedes high blood pressure in spontaneously hypertensive rats. *Clin. Exp. Hypertens., 27*, 71– 82.

Nakade, K., Kamishima, R., Inoue, Y., Ahhmed, A., Kawahara, S., Nakayama, T., Maruyama, M., Numata, M., Ohta, K., Aoki, T., & Muguruma, M. (2008). Identification of an antihypertensive peptide derived from chicken bone extract. *Animal Science Journal, 79*(6), 710-715.

Naqash, S. Y., & Nazeer, R. A. (2011). Evaluation of bioactive peptides isolated from Exocoetus volitans backbone. . *Inter. J. Food Sci. Technol, 46*, 7.

Ovelgonne, E. A. S., Muir, W. I., & Scott, T. A. (2007). Meat and bone meal, future nutraceuticals for poultry? A review. *Aust. Poult. Sci. Symp.*(19), 4.

Piette, G., Hundt, M., Jacques, L., & Lapointe, M. (2001). Influence of extraction temperature on amounts and quality of rendered chicken fat recovered from ground or homogenized skin. *Poultry Science, 80*(4), 496-500.

Pownall, T. L., Udenigwe, C. C., & Aluko, R. E. (2010). Amino Acid Composition and Antioxidant Properties of Pea Seed (*Pisum sativum* L.) Enzymatic Protein Hydrolysate Fractions. *Journal of Agricultural and Food Chemistry, 58*(8), 4712-4718.

Qian, Z. J., Je, J. Y., & Kim, S. K. (2007). Antihypertensive effect of angiotensin I converting enzyme-inhibitory peptide from hydrolysates of big eye tuna dark muscle, Thunnus obesus. *Journal of Agricultural and Food Chemistry, 55*, 6.

Raghavan, S., & Kristinsson, H. G. (2009). ACE-inhibitory activity of tilapia protein hydrolysates. *Food Chemistry, 117*(4), 582-588.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive Peptides from Muscle Sources: Meat and Fish. *Nutrients, 3*(9), 765-791.

Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T., & Morimatsu, F. (2008). Angiotensin I-Converting Enzyme-inhibitory Peptides Obtained from Chicken Collagen Hydrolysate. *Journal of Agricultural and Food Chemistry, 56*(20), 9586-9591.

Saiga, A., Okumura, T., Makihara, T., Katsuda, S. I., Morimatsu, F., & Nishimura, T. (2006). Action mechanism of an angiotensin I-converting enzyme inhibitory peptide derived from chicken breast muscle. *Journal of Agricultural and Food Chemistry*, *54*(3), 942-945.

Saiga, A., Okumura, T., Makihara, T., Katsuta, S., Shimizu, T., Yamada, R., & Nishimura, T. (2003). Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. *Journal of Agricultural and Food Chemistry*, *51*(6), 1741-1745.

Samanarayaka, A., G. P., Kitts, D. D., & Li-Chan, E. C. Y. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a pacific hake (Merluccius productus) fish protein hydrolysate subjected to simulated gastrointestinal digestion and caco-2 cell permeation. *J. Agric. Food Chem, 58*, 8.

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of Functional Foods, 3*(4), 229-254.

Schmieder, R. E., Hilgers, K. F., Schlaich, M. P., & Schmidt, B. M. W. (2007). Renin-angiotensin system and cardiovascular risk. *Lancet, 369*(9568), 1208-1219.

Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International, 91*(4), 914-931.

Simão, S., Gomes, P., Pinto, V., Silva, E., Amaral, J. S., Igreja, B., Alfonso, J., Serrão, M. P., Pinho, M. J., Soares-da-Silva, P. (2011). Age-related changes in renal expression of oxidant and antioxidant enzymes and oxidative stress markers in male SHR and WKY rats. *Exp. Gerontol., 46*, 468–474.

Tang, C. H., Wang, X. S., & Yang, X. Q. (2009). Enzymatic hydrolysis of hemp (Cannabis sativa L.) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry, 114*(4), 1484-1490.

Theodoridis, G., Gika, H. G., & Wilson, I. D. (2008). LC-MS-based methodology for global metabolite profiling in metabonomics/metabolomics. *Trac-Trends in Analytical Chemistry, 27*(3), 251-260.

Udenigwe, C. C., Lin, Y. S., Hou, W. C., & Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*(2), 199-207.

Vaziri, N. D. (2008). Causal Link Between Oxidative Stress, Inflammation, and Hypertension. *Iranian Journal of Kidney diseases, 2*(1), 10.

Vercruysse, L., Van Camp, J., & Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. *Journal of Agricultural and Food Chemistry, 53*(21), 8106-8115.

Vermeirssen, V., Camp, J. V., & Verstraete, W. (2004). Bioavailabilty of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*, 10.

Wishart, D. S. (2008). Metabolomics: applications to food science and nutrition research. *Trends in Food Science & Technology*, *19*(9), 482-493.

Wu, H. C., Pan, B. S., Chang, C. L., & Shiau, C. Y. (2005). Low-molecular-weight peptides as related to antioxidant properties of chicken essence. *Journal of Food and Drug Analysis*, *13*(2), 176-183.

You, L., Zhao, M., Liu, R. H., & Regenstein, J. M. (2011). Antioxidant and antiproliferative activities of loach (*Misgurnus anguillicaudatus*) peptides prepared by papain digestion. *J. Agric. Food Chem.*, *59*, 7948 – 7953.

Young, I. S., & Woodside, J. V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology*, *54*(3), 176-186.

CHAPTER 3

MANUSCRIPT ONE

INHIBITIONS OF RENIN AND ANGIOTENSIN CONVERTING ENZYME ACTIVITIES BY ENZYMATIC CHICKEN SKIN HYDROLYSATES

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3.0 Abstract

Enzymatic hydrolysates from chicken skin protein were investigated for their in vitro inhibitions of angiotensin converting enzyme (ACE) and renin activities. Enzyme hydrolysis of the chicken skin protein from the thigh and breast muscles was done using alcalase or a combination of pepsin/pancreatin (PP) at enzyme concentrations of 1–4%. The chicken skin protein hydrolysates (CSPH) were then fractionated by membrane ultrafiltration into different molecular weight peptides (<1, 1–3, 3–5 and 5–10 kDa). Results showed that degree of hydrolysis (DH) of the hydrolysates increased significantly with protease concentration for all the samples (72.61–81.88%) and correlated positively with peptide yield. The alcalase hydrolysates generally had significantly higher ($p < 0.05$) ACE-inhibitory activity when compared to PP hydrolysates. ACE inhibition was inversely related to size of ultrafiltration membrane peptides. A moderate renin-inhibitory activity was observed (15–36%), which was dependent on the type of protease; the PP hydrolysates showed significantly higher ($p < 0.05$) inhibition than alcalase hydrolysates. These results suggest that CSPH can be considered a potential ingredient for the development of functional foods and nutraceuticals that can attenuate catalytic activities of ACE and renin.

Keywords: Chicken skin, Membrane ultrafiltration, Protein hydrolysates, Peptide profile, Angiotensin converting enzyme, Renin, Enzyme inhibition

3.1 Introduction

Hypertension is a chronic condition in which blood pressure in the arteries becomes elevated and usually is persistently at or above 140 mm Hg for the systolic blood pressure (SBP) or 90 mm Hg diastolic blood pressure (DBP) or both (Ahhmed & Muguruma, 2010). It is a leading cause of death, affecting about 24% of the adult population in the developed world especially the US and is a major problem in developing countries (Daien et al., 2012). Globally, hypertension is a leading risk factor for cardiovascular disease, stroke, end stage renal disease and premature death (Erdmann, Cheung, & Schroder, 2008; Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011; Lee, Qian, & Kim, 2010). Among the many approaches that are currently being used to address this problem is increased consumption of proteins, which has been reported to lower the risk of hypertension and heart diseases probably due to the presence of biologically active peptide sequences within the primary structure (Vercruysse, Van Camp, & Smagghe, 2005; Erdmann et al., 2008; Samanarayaka, Kitts, & Li-Chan, 2010). Antihypertensive peptides are the best known and researched among the different groups of bioactive peptides, notably, the inhibitors of angiotensin converting enzyme (ACE).

The renin–angiotensin system (RAS) has long been known to play a very crucial physiological role in maintaining blood pressure and associated cardiovascular diseases (CVD) such as congestive heart failure and hypertension (Erdmann et al., 2008; Fujita, Yokoyama, & Yoshikawa, 2000; Qian, Je, & Kim, 2007; Raghavan & Kristinsson, 2009). RAS mainly controlled by 2 enzymes, renin and ACE, plays vital roles in the maintenance of blood pressure homeostasis, fluid and electrolyte balance in the human body (Girgih et al., 2011; Lee et al., 2010). Renin, an aspartyl protease, is responsible for catalyzing the initial step, converting angiotensinogen to angiotensin-I for the subsequent conversion by ACE (Udenigwe, Lin, Hou, &

Aluko, 2009). ACE (EC 3.4.15.1) belongs to the class of zinc proteases located in the vascular endothelial lining of the lungs and is present in several body tissues where it serves as an integral part in the moderation of blood pressure as well as the normal functioning of the heart (Je, Park, Kwon, & Kim, 2004; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). ACE is a decapeptidylcarboxylase that cleaves dipeptides from the C- terminus of oligopeptides, and in the process, catalyzing the hydrolysis (conversion) of the inactive decapeptide, angiotensin I into the potent vasoconstrictor, angiotensin II (Martinez-Maqueda, Miralles, Recio, & Hernandez-Ledesma, 2012). ACE also inactivates bradykinin (a vasodilator), which leads to blood pressure elevation (Vercruysse et al., 2005; Je et al., 2004; Raghavan & Kristinsson, 2009).

The mechanism by which ACE inhibitors lower blood pressure involves maintenance of a balance between the vasoconstrictive plus salt retentive abilities of angiotensin II and the vasodilatory property of bradykinin. This is achieved by decreasing the production of angiotensin II while at the same time reducing the hydrolytic degradation of bradykinin (Ryan et al., 2011). Inhibition of these processes has long been the basis for the use of synthetic ACE inhibitors such as captopril, enalapril, lisinopril, and fosinopril in the treatment of hypertension, congestive heart failure and myocardial infarction (Erdmann et al., 2008; Fujita et al., 2000). In this respect, naturally occurring bioactive peptides are becoming attractive as potential antihypertensive agents because they are not known to exhibit negative side effects such as cough, skin rash and pain that are associated with synthetic drugs (Je et al., 2004).

ACE-inhibitory peptides with demonstrated activities have been isolated and extensively studied from several foods such as milk, whey, eggs, meat, soybean, fish and animal by-products (Aluko & Monu, 2003; Erdmann et al., 2008; Martinez-Maqueda et al., 2012; Samanarayaka et al., 2010; Vercruysse et al., 2005). They have also been isolated from chicken bone protein

extracts (Cheng, Liu, Wan, Lin, & Sakata, 2008; Cheng,Wan, et al., 2008; Nakade et al., 2008), chicken breast muscle extracts (Fujita et al., 2000; Saiga et al., 2003, 2006) as well as chicken collagen (Saiga et al., 2008). Renin inhibitory activities had equally been studied in hemp protein hydrolysates (Girgih et al., 2011) and flaxseed protein hydrolysates (Udenigwe et al., 2009).

Chicken skin is a by-product derived from chicken meat processing, is highly underutilized and constitutes a huge waste disposal burden and danger to the environment (Feddern et al., 2010). Several attempts have previously been made at developing novel products such as meat balls (Bhat, Kumar, & Kumar, 2011), collagen (Bonifer & Froning, 1996; Cliché, Amiot, & Gariepy, 2003), sausages (Biswas, Chakraborty, Sarkar, Barpuzari, & Barpuzari, 2007), and chicken meat frankfurter (Babji, Chin, Chempaka, & Alina, 1998) in order to diversify chicken skin utilization and reduce environmental waste burden. However, an area of research that is yet to be explored is the development of chicken skin based products with functional and health promoting values. The high protein content (dry weight basis) of chicken skins could, in addition to contributing to nutrition, also serve as a very active source of value-added products, including bioactive peptide-containing hydrolysates. According to Badr (2005), differences in the proximate composition of chicken skins may be due to differences in age and diet. However the chicken breast skin was shown to have a much lower fat content and higher protein content than the leg muscle skin. In this study, we produced enzymatic hydrolysates from chicken thigh and breast muscle skin proteins using alcalase and a combination of pepsin and pancreatin at concentrations of 1–4%. After fractionation using ultrafiltration membranes of 1, 3, 5 and 10 kDa molecular weight cut-offs (MWCO), the hydrolysates were then analyzed for their in vitro antihypertensive activities. Therefore, the objective of this study is to determine the

potential antihypertensive effect of chicken skin protein hydrolysates by measuring their in vitro inhibitory activities against renin and ACE.

3.2 Materials and methods

3.2.1. Materials

Chicken skins from the thigh and breast muscles used for this study were supplied by Granny's Poultry (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas), alcalase (from fermentation of Bacillus licheniformis, 3.4.21.62), ACE (from rabbit lung, EC 3.4.15.1), trinitrobenzene sulfonic acid (TNBS), N-(3-[2-furyl] acryloyl)-phenylalanylglycylglycine (FAPGG) and sodium dodecyl sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). All other analytical grade reagents and ultrafiltration membranes (1, 3, 5 and 10 kDa molecular weight cut-offs) were purchased from Fisher Scientific (Oakville, ON, Canada).

3.2.2. Preparation of chicken skin protein hydrolysates (CSPH)

Fresh thigh or breast chicken skins (approximately 250 g) were packed in freeze drying plates, frozen at –20 °C for 24 h and transferred to –80 °C for 6 h prior to freeze drying. The freeze dried samples were thereafter manually shredded and defatted repeatedly by mixing ~1 g with 10 mL of food grade acetone. The defatted skin samples were then air dried overnight in the fume hood chamber at room temperature and subsequently milled with a Waring blender to produce a fine powder that was stored at −20 °C. Proximate compositions of the defatted samples were determined using standard methods of analysis (AOAC, 1990). For the initial screening test to optimize and select the best enzyme concentration, dried chicken skin powder from the thigh or breast muscles were mixed with water to give 5% (w/w protein basis) slurries.

For the alcalase hydrolysis, the slurry was heated to 55 °C, adjusted to pH 8.0 using 2 M NaOH and hydrolysis initiated by addition of enzyme (1–4% w/v, skin protein basis); each mixture was stirred continuously for 4 h. For the pepsin + pancreatin (PP) hydrolysis, the slurry was heated to 37 °C, adjusted to pH 2.0 using 2 M HCl and the reaction initiated with the addition of pepsin enzyme (1–4% w/v, skin protein basis); the mixture was then stirred continuously for 2 h. After peptic hydrolysis, the reaction mixture was adjusted to pH 7.5 with 2 M NaOH, pancreatin was added (1–4% w/v, skin protein basis) and incubated at 37 °C for 4 h with continuous stirring. At the end of the incubation period, the enzyme reactions were terminated by heating the slurries to 95 °C for 15 min. The mixtures were thereafter centrifuged (7000 ×g at 4 °C) for 1 h and the resulting supernatant lyophilized and stored at –20 °C until needed for further analysis. The most active hydrolysate from each enzyme treatment was subsequently fractionated by sequentially passing the supernatant through ultrafiltration membranes with molecular weight cut-offs (MWCO) of 1, 3, 5 and 10 kDa in an amicon stirred ultrafiltration cell. Starting with 1 kDa MWCO, the retentate from each membrane was passed through the next higher MWCO membrane while the permeate from each membrane (1, 3, 5, and 10 kDa MWCO) was collected, lyophilized and stored at–20 °C as b1, 1–3, 3–5, and 5–10 kDa fractions, respectively. Protein content of the lyophilized CSPHs was determined by the modified Lowry method (Markwell, Haas, Biebar, & Tolbert, 1978). The above digestion and fractionation protocols were performed in triplicates and the lyophilized samples combined, analyzed for protein content and used for the renin and ACE inhibition assays.

3.2.3. Determination of degree of hydrolysis

The percent degree of hydrolysis (DH) of CSPHs was determined according to the trinitrobenzene sulfonic acid (TNBS) method described by Adler-Nissen (1979). Defatted skin

samples were digested under vacuum with 6 M HCl for 24 h and the digest used to determine total amino groups as L-leucine equivalent. The DH was calculated as percentage ratio of the leucine equivalent of CSPHs to that of defatted skin.

3.2.4. Peptide yield of CSPH and membrane fractions

The peptide yields (%) of CSPH and membrane fractions were calculated as the ratio of protein content of lyophilized CSPH to the protein content of unhydrolysed dried chicken skin samples. Also, the peptide yields (%) of the ultrafiltration membrane fractions were calculated as the ratio of the protein content of the lyophilized permeate to the protein content of the original chicken skin hydrolysates as described by Girgih et al. (2011).

3.2.5. Reverse-Phase HPLC peptide mapping

The hydrolysates and membrane fractions were dissolved in distilled water (containing 0.1% TFA) at a concentration of 10 mg/mL and 1 mL of the fraction loaded on a VYDAC HPLC C18 analytical reverse-phase column (250x4.6 mm, 5 μm, Grace, INC., Deerfield, IL) attached to a Shimadzu UPLC UV/VIS detector (SPD-20A) system. The column was eluted with a gradient from 80 to 100% methanol (containing 0.1% TFA) within 60 min at a flow rate of 1.5 mL/min; absorbance of eluted peptides was monitored at 215 nm.

3.2.6. ACE-inhibitory activity of CSPH and membrane fractions

The ACE-inhibitory activity of CSPH and membrane fractions was determined as previously reported (Udenigwe et al., 2009). A 1 mL aliquot of 0.5 mM N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 μL ACE (final activity of 20 mU) and 200 μL of 1 mg/mL sample (CSPH or membrane fractions in 50 mM Tris–HCl buffer). The rate of decrease in absorbance at 345 nm due to ACE-catalysed cleavage of the Phe-Gly bond of the FAPGG was

recorded for 2 min at room temperature using a spectrophotometer. Tris–HCl buffer was used instead of peptide sample in the blank experiment. ACE activity was expressed as rate of disappearance of FAPGG (ΔA/min) and inhibitory activity was calculated as:

ACE inhibition (%) = [$\Delta Amin^{-1}$ (blank) – $\Delta Amin^{-1}$ (sample)/$\Delta Amin^{-1}$ (blank)] x 100

Where ($\Delta Amin^{-1}$ (sample) and ($\Delta Amin^{-1}$ (blank) are ACE activity in the presence and absence of CSPH or membrane fractions, respectively.

3.2.7. Renin-inhibitory activity of CSPH and membrane fractions

Renin inhibition assay was carried out according to the method of Li and Aluko (2010) using the Renin Inhibitor Screening Assay Kit. Renin buffer was diluted with 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl prior to the determinations. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37 °C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37 °C. Before the reaction, (1) 20 μL substrate, 160 μL assay buffer, and 10 μL Milli-Q water were added to the background wells; (2) 20 μL substrate, 150 μL assay buffer, and 10 μL Milli-Q water were added to the blank wells; and (3) 20 μL substrate, 150 μL assay buffer, and 10 μL 1 mg/mL sample were added to the inhibitor wells. The reaction was initiated by adding 10 μL renin to the control and sample wells. The microplate was shaken for 10 s to mix, incubated at 37 °C for 15 min, and fluorescence intensity (FI) recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. The percentage inhibition was calculated as follows:

Renin inhibition (%) = [(FI of blank well−FI of sample well)/FI of blank well] x 100

3.2.8. Statistical analyses

Data were collected as means of 3 separate determinations on each sample. For screening analysis, 3-way analysis of variance (ANOVA) using a model that includedmuscle type (Mu), enzyme type (Et) and enzyme concentration (Ec) as fixed variables. All 2-way interactions such as muscle type by enzyme type, muscle type by enzyme concentration and enzyme type by enzyme concentration were analysed. For membrane fractions analysis, 2-way ANOVA was conducted with muscle type and membrane size as fixed variables. Tukey's multiple comparisons test was used to determine mean treatment differences and significance accepted at $p < 0.05$. For proximate composition analysis of chicken breast and thigh skins, a student t-test was used to determine significant differences ($p < 0.05$). An IBM SPSS Statistical package (version 20) was used for all statistical analyses.

3.3 Results and discussion

3.3.1. Proximate composition of defatted chicken skin samples

Prior to enzyme hydrolysis, the defatted chicken skin samples were analysed for proximate composition as shown in Table 3.1. While moisture content was similar in both samples, the chicken thigh skin sample was significantly ($p < 0.05$) higher in protein and ash contents but lower in lipid content when compared to the chicken breast skin sample. According to Bonifer and Froning (1996), chicken skins contain about 53% moisture; thus the present results suggest that over 80% of the moisture was removed during freeze-drying. The 25–29% lipid contents of the defatted chicken skin samples represent about 60% reduction based on the fact that regular chicken skin contains ~78% (dry weight basis) as previously reported by Bonifer and Froning (1996). The high residual lipid contents show that the defatting procedure was not very effective and could not remove some of the native lipids. Defatting also led to about 300%

increase in protein content from regular value of ~20% (Bonifer & Froning, 1996) to the ~70% (dry weight basis) obtained in this work. The sum total of the nutrients was slightly above 100 by about 3–4%, which may be due to overestimation of some of the parameters. This is because during moisture determination, some of the volatiles may have been lost while the nitrogen to protein conversion factor used (6.25) may not be exact. Typical of animal samples, especially skins that contain mostly lipids and collagen (proteins), the sum total analysed nutrients showed absence of carbohydrates.

Table 3.1: Proximate composition of defatted unhydrolyzed chicken skin samples

Samples	**CTS**	**CBS**
Moisture	5.84 ± 0.13	6.64 ± 0.14
Dry Matter	94.17 ± 0.13	93.37 ±0.14
Protein	68.84 ± 0.73[a]	65.07 ± 0.08[b]
Fat	25.71 ± 0.44[b]	29.21 ± 0.74[a]
Ash	4.06 ± 0.34[a]	2.43 ± 0.44[b]

[a, b] Mean intensity values (followed in brackets by the standard error of the mean) within the same variable with the same letter within the same row (parameter) are not significantly different ($P < 0.05$)

3.3.2. HPLC peptide profiles

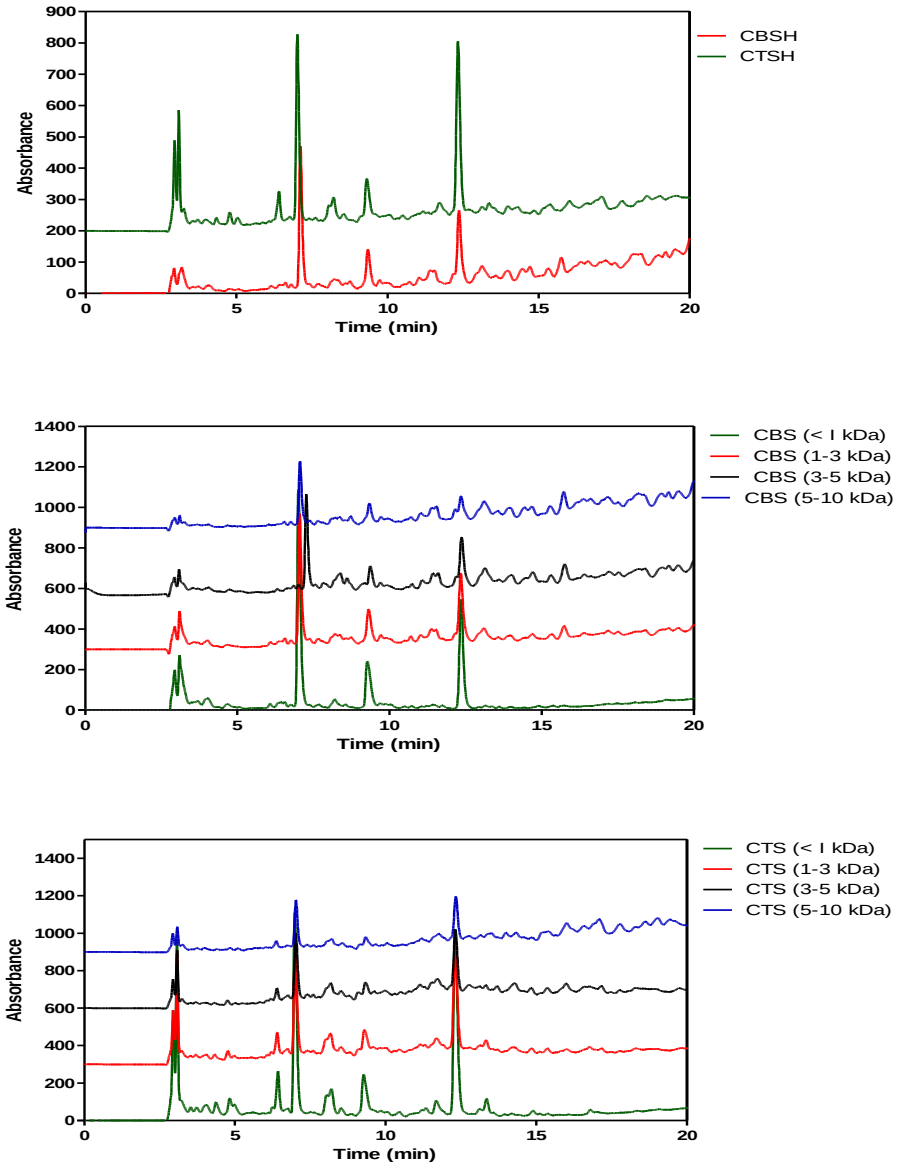
The CBSH from 1% PP hydrolysis and CTSH from 3% alcalase hydrolysis were chosen for membrane fractionation and subsequent RP-HPLC analysis because they showed the highest inhibitions of ACE and renin. The RP-HPLC separation showed that there was slightly less variation in peptide profile within the membrane ultrafiltration samples whereas the protein hydrolysates (CBSH and CTSH) had more variation (Fig. 3.1). For example the CBSH had three

eluted peaks at 4.7, 6.4 and 8.2 min (indicated by arrows) that were absent in the CTSH (Fig. 3.1A). The peak height at 12.4 min was also less for CTSH than the CBSH. Thus the CBSH has greater diversity of peptides than the CTSH, which probably indicates that the breast and thigh skin proteins have different susceptibility to digestive enzymes used in this work. For the CBSH membrane fractions, two eluted peptide peaks at 13.0 and 15.7 min were found in the >1 kDa samples but not in the < 1 kDa sample (Fig. 3.1B). Since the 13.0 and 15.7 min peaks eluted late on the hydrophobic column, the data suggests the presence of peptides with mostly hydrophobic amino acids. The shorter length of peptides in the <1 kDa sample may have reduced the additive effect of hydrophobic amino acids and hence absence of late-eluting peptides when compared to the >1 kDa samples that have longer peptides. For the CBSH membrane fractions, the peak height at 12.4 min decreased as the peptide size increased from <1 to 5–10 kDa, indicating that most of the peptides in this peak are of lower molecular weights (Fig. 3.1B). The peptide elution profiles for the CTSH membrane fractions were similar with the exception that the <1 kDa sample had a minor peak at 13.0 min, which was less prominent in the >1 kDa samples (Fig. 3.1C). But the peak heights at 7.1 and 12.4 min for the CTSH membrane fractions tended to be less at peptide sizes >3 kDa. The results suggest that the >1 kDa CBSH membrane fractions may be slightly more hydrophobic due to the 13.0 and 15.7 min peaks when compared to the CTSH membrane fractions. However, the two main peaks at 7.1 and 12.4 min were common to all the samples, which indicates that each peak is composed of peptides that differ in molecular size but similar in overall hydrophobicity.

3.3.3. Degree of hydrolysis (DH)

The DH of the hydrolysates was significantly affected by the muscle skin types, enzyme

Fig. 3.1. Reverse-phase HPLC peptide profiles for A: chicken breast skin protein hydrolysate (CBSH) from 1% pepsin–pancreatin and chicken thigh skin protein hydrolysate (CTSH) from 3% alcalase; B: ultrafiltration membrane fractions for the CBSH; and C: ultrafiltration membrane fractions for the CTSH. For each plot, arrows indicate positions where eluted peaks differ between the samples.



types and enzyme concentrations as well as in the interactions between muscle type and enzyme type, muscle type and enzyme concentration as well as enzyme type and enzyme concentration (Table 3.2). An investigation of the plots produced for the three 2-way interactions studied in our statistical model revealed that the three main factors (muscle, enzyme type and enzyme concentration) affected the degree of hydrolysis with a combination of CBSH, and 4% concentration of alcalase giving the highest DH values. This reflects a difference in the magnitude of the sample intensities between the muscle types, enzymes and enzyme concentration. The plot of the interaction data also revealed that generally, the DH values for the chicken breast skin hydrolysates (CBSH) was significantly ($p < 0.05$) and consistently higher than the values obtained for chicken thigh skin hydrolysates (CTSH) at all enzyme types and enzyme concentrations. The CBSH, alcalase hydrolysate, and 4% concentration were also significantly higher than the CTSH, pepsin-pancreatin and 1% enzyme concentration respectively. This may be due to the lower protein content (and consequently, fewer substrates for proteolysis) of the CBSH (65.07%) than the CTSH (68.84%). The DH of hydrolysis increased with increasing enzyme concentration and the 4% enzyme concentration significantly ($p < 0.05$) had the highest value. The DH of hydrolysates is a parameter generally used to monitor proteolysis and it is the most widely used indicator for comparing different protein hydrolysates (Cheng, Liu, et al., 2008; Cheng,Wan, et al., 2008; Guerard, Guimas, & Binet, 2002). Peptides are typically generated during protein hydrolysis by protease enzymes due to the cleavage of peptide bonds. Therefore, it will be expected that the higher the proteolytic activity of the enzyme or enzyme concentration, the more peptides that will be produced. The high DH values obtained for the CSPH suggest that chicken skin proteins are very susceptible to

Table 3.2: Results from 3-way ANOVA and Tukey's test (triplicate analyses) of the effects of muscle type, Mu; enzyme type, Et; enzyme concentration, Ec; thigh, T; breast, B; pepsin-pancreatin, PP; alcalase, A on the degree of hydrolysis, protein, peptide yield, ACE and renin inhibition of chicken skin hydrolysates.

	Source of variation						**Mean intensity for muscle types**		**Mean intensity for enzyme types**		**Mean intensity for enzyme concentration**			
Parameter	Mu	Et	Ec	Mu x Et	Mu x Ec	Et x Ec	T	B	PP	A	1	2	3	4
Degree of hydrolysis	2650 ***	16.86 ***	111.82 ***	8.35 **	6.71 **	10.43 ***	68.51^{b} (0.30)	88.75^{a} (0.30)	77.82^{b} (0.30)	79.43^{a} (0.30)	72.61^{c} (0.40)	79.27^{b} (0.40)	80.75^{b} (0.40)	81.88^{a} (0.40)
Protein	0.25 NS	1.54 NS	0.38 NS	11.13 **	5.62 **	2.97 *	65.79 (0.69)	65.31 (0.69)	66.18 (0.69)	64.95 (0.69)	65.54 (0.98)	65.58 (0.98)	64.82 (0.98)	66.29 (0.98)
Peptide yield	53.59 ***	105.79 ***	35.28 ***	2.66 NS	4.05 *	0.67 NS	65.35^{b} (0.78)	73.47^{a} (0.78)	75.11^{a} (0.78)	63.71^{b} (0.78)	60.54^{c} (1.10)	68.69^{b} (1.10)	72.67^{ab} (1.10)	75.60^{a} (1.10)
ACE inhibition	0.19 NS	6.65 *	2.21 NS	18.14 ***	10.23 ***	7.33 **	68.28 (1.14)	67.57 (1.14)	65.85^{b} (1.14)	70.00^{a} (1.14)	66.33 (1.61)	71.32 (1.61)	67.91 (1.61)	66.14 (1.61)

Renin	0.50	195.87	19.79	8.77	26.24	10.74	28.87	27.33	43.43[a]	12.78[b]	37.16[a]	33.57[ab]	26.65[b]	15.03[c]
inhibition	NS	***	***	**	***	***	(1.55)	(1.55)	(1.55)	(1.55)	(2.19)	(2.19)	(2.19)	(2.19)

[1]NS, not significant at P ≥ 0.05, Significant at *P < 0.05, **P < 0.01, ***P < 0.001

Mean intensity values (followed in brackets by the standard error of the mean) within the same variable (muscle type, enzyme type, and enzyme concentration with the same letter within the same row (parameter) are not significantly different (P < 0.05)

proteolysis under the reaction conditions used in this work. The DH results are similar to those reported for hemp seed protein hydrolysates obtained from PP hydrolysis (Girgih et al., 2011).

3.3.4. Protein content

The protein content of the chicken skin protein hydrolysates did not show any significant differences between the muscle skin type, enzyme type and enzyme concentrations (Table 3.2). However, significant interactions between muscle type and enzyme type, muscle type and enzyme concentration and enzyme type and enzyme concentration in the protein content indicated that the magnitude of the sample intensities differed between muscle types and enzymes. Investigation of the interaction plots revealed that PP at 1% concentration gave higher protein content for CTSH while alcalase at 4% concentration was more effective for CBSH protein content. The muscle type and membrane size showed significant differences in protein content of the CSPH and the membrane fractions with CBSH being significantly higher than CTSH (Table 3.3). The protein content of the membrane fractions also significantly increased from 1 kDa to 3 kDa but showed non-significant increases with increasing membrane size of the peptides afterwards, with the 10 kDa sized peptides being significantly higher in protein content. This trend is similar to what was reported by Girgih, Udenigwe, and Aluko (2010) in hemp seed protein hydrolysates and the low content of protein observed here especially for the <1 kDa fraction may be attributed to the presence of low molecular weight non proteinous components especially salts (formed during acid-base neutralization) and soluble sugars. The presence of high level of salts in the <1 kDa permeate is due to the fact that this membrane was the first to be used during ultrafiltration. However, no significant interactions existed between the muscle types and membrane types.

3.3.5. Peptide yield

The percent yield reflects the efficiency of proteolytic process since it leads to the generation of peptides. The only significant interaction obtained was for muscle type and enzyme concentration. The interaction plot showed that for 1 and 2% concentrations an increase in peptide yield was obtained for both CBSH and CTSH with higher values for CBSH. However, at higher enzyme concentrations (3 and 4%), only CBSH showed an increase in peptide yield (Table 3.2). In addition, the peptide yield of the hydrolysates was significantly affected by enzyme type as PP gave significantly higher values compared to alcalase as shown in Table 3.2. The peptide yield of the hydrolysates was significantly affected by the muscle skin type, enzyme type and enzyme concentration; however, with the exception of muscle type and enzyme concentration, there were no significant interactions effect observed between muscle type and enzyme type or enzyme type and enzyme concentration (Table 3.2). Analysis of mean intensities revealed that CBSH had significantly higher peptide content than CTSH. Also, pepsin–pancreatin hydrolysis yielded more peptides than alcalase while peptide yield equally increases with enzyme concentration. This may be a reflection of the combination effect of pepsin and pancreatin as pre-digestion with pepsin may have led to the exposure of susceptible peptide bonds for a more efficient hydrolysis by pancreatin (Girgih et al., 2011). Fractionation of the peptides into different membrane sizes and the muscle type gave a significant interaction with a higher peptide yield value with 1 and 3 kDa cut-off membranes for CTSH compared to CBSH. Our results show a direct relationship between peptide yield and DH of CSPH, which is consistent with previously reported studies (Cheng, Liu, et al., 2008; Cheng, Wan, et al., 2008; Shahidi, Han, & Synowiecki, 1995). The yield is also an indication of the economic viability

Table 3.3: Results from 2-way ANOVA and Tukey's test (triplicate analyses) of the effects of muscle type, Mu; Membrane type, Me; thigh, T; breast, B; hydrolysate, H; 1, 3, 5, and 10 kDa on protein content, peptide yield, ACE and renin inhibition of chicken skin protein hydrolysates and membrane fractions.

	Source of variation			Mean intensity for muscle type		Mean intensities for membrane types				
Parameters	Mu	Me	Mu x Me	T	B	Hydrolysate	1 kDa	3 kDa	5 kDa	10 kDa
Protein	9.81	43.88	2.49	63.92^{b}	67.76^{a}	63.12^{b}	51.05^{c}	70.62^{a}	72.34^{a}	72.08^{a}
	**	***	NS	(0.87)	(0.87)	(1.37)	(1.37)	(1.37)	(1.37)	(1.37)
Peptide	303.97	10200	301.62	20.47^{b}	24.38^{a}	67.69^{a}	8.16^{e}	14.06^{b}	11.66^{c}	10.55^{d}
yield	***	***	***	(0.16)	(0.16)	(0.25)	(0.25)	(0.25)	(0.25)	(0.25)
ACE	3.57	10.68	5.64	78.10	75.13	72.28^{bc}	81.51^{a}	82.07^{a}	78.17^{ab}	69.05^{c}
inhibition	NS	***	**	(1.11)	(1.11)	(1.76)	(1.76)	(1.76)	(1.76)	(1.76)
Renin	107.90	7.64	9.37	37.28^{a}	15.59^{b}	25.13^{b}	19.72^{b}	21.98^{b}	35.99^{a}	29.35^{ab}
inhibition	***	***	***	(1.48)	(1.48)	(2.33)	(2.33)	(2.33)	(2.33)	(2.33)

[1]NS, not significant at P ≥ 0.05, Significant at *P < 0.05, **P < 0.01, ***P < 0.001

Mean intensity values (followed in brackets by the standard error of the mean) within the same variable with the same letter within the same row (muscle type and membrane type) are not significantly different (P < 0.05)

when considerations for commercialization of the protein hydrolysates as a functional and nutraceutical ingredient are to be made since higher yields are more beneficial to processing and marketing of new products (Girgih et al., 2011). The current results (Table 3.3) showed that with the exception of b1 kDa sized peptides, the hydrolysates contain higher amounts of low molecular weight peptides (1–3 kDa) when compared to bigger peptides (3–5 kDa and 5–10 kDa). This may be a useful indication of their bioavailability and physiological efficiency during oral administration as short chain peptides are usually more resistant to gastrointestinal proteolysis and are absorbed in more intact form into the circulatory system than long chain peptides (Fujita et al., 2000; Nakade et al., 2008). The low yield of the <1 kDa peptides could be due to peptide-peptide interactions that produced aggregates with sizes greater than 1 kDa and would probably end up in the 1–3 kDa fraction.

3.3.6. ACE-inhibitory activity

An investigation of the plots produced for the three 2 way interactions studied in our statistical model revealed that the three main factors (muscle, enzyme type and enzyme concentration) affected the ACE-inhibitory activity of hydrolysates. While a combination of 2% of alcalase gave the highest ACE-inhibitory activity in CBSH, a combination of 1% of PP worked best for CTSH (Table 3.2). This has significant implications for commercialization when CBSH is used as alcalase is cheap and readily available. In Fig. 3.2, CBSH-P hydrolysate at the 4% enzyme concentration had the lowest reported value of 49.55% and the CTSH-A hydrolysate at the 3% enzyme concentration with the highest inhibitory value of 79.62%. With the exception of CTSA hydrolysate at the 1% enzyme concentration, the alcalase hydrolysates showed higher ACE inhibitory activity ($p < 0.05$) than the pepsin-pancreatin hydrolysates. Similar findings for

Fig. 3.2. Percentage (mean + standard deviation) ACE-inhibitory activity of chicken skin protein hydrolysates (CSPH) at different concentrations of enzymes and 1 mg/mL sample concentration. CTSH-P, chicken thigh skin protein hydrolysed with pepsin–pancreatin; CBSH-P, chicken breast skin protein hydrolysed with pepsin–pancreatin; CTSH-A, chicken thigh skin protein hydrolysed with alcalase; CBSH-A, chicken breast skin protein hydrolysed with alcalase. Bars with different alphabets have mean values that are significantly different ($p < 0.05$).

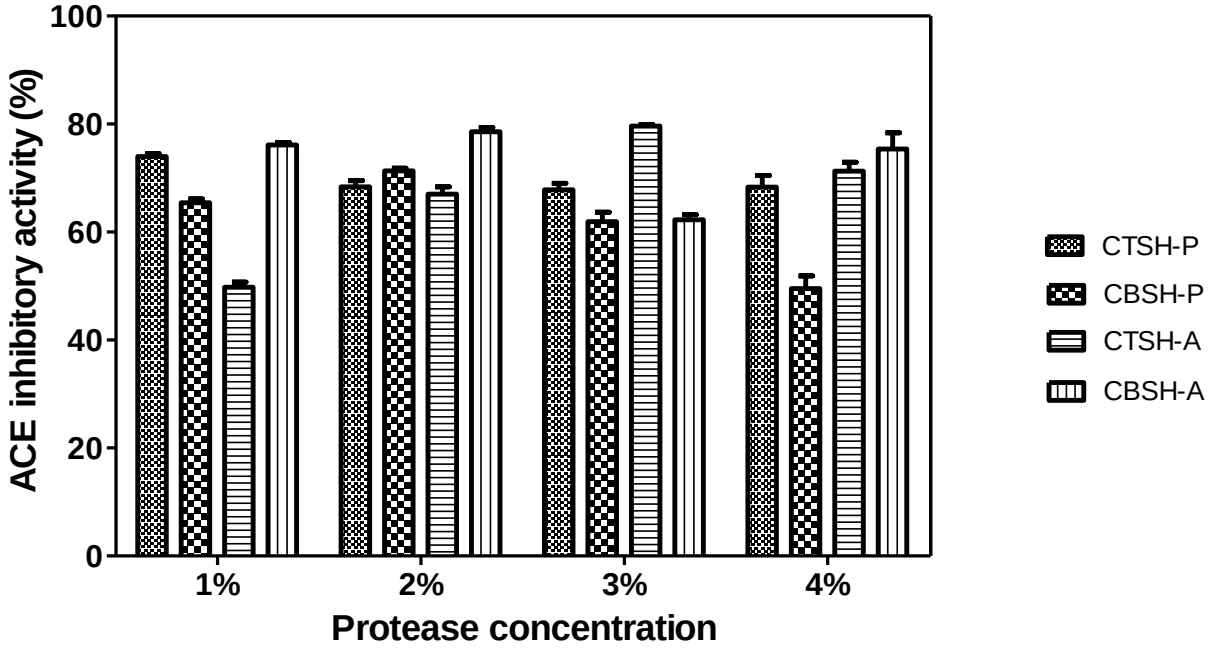
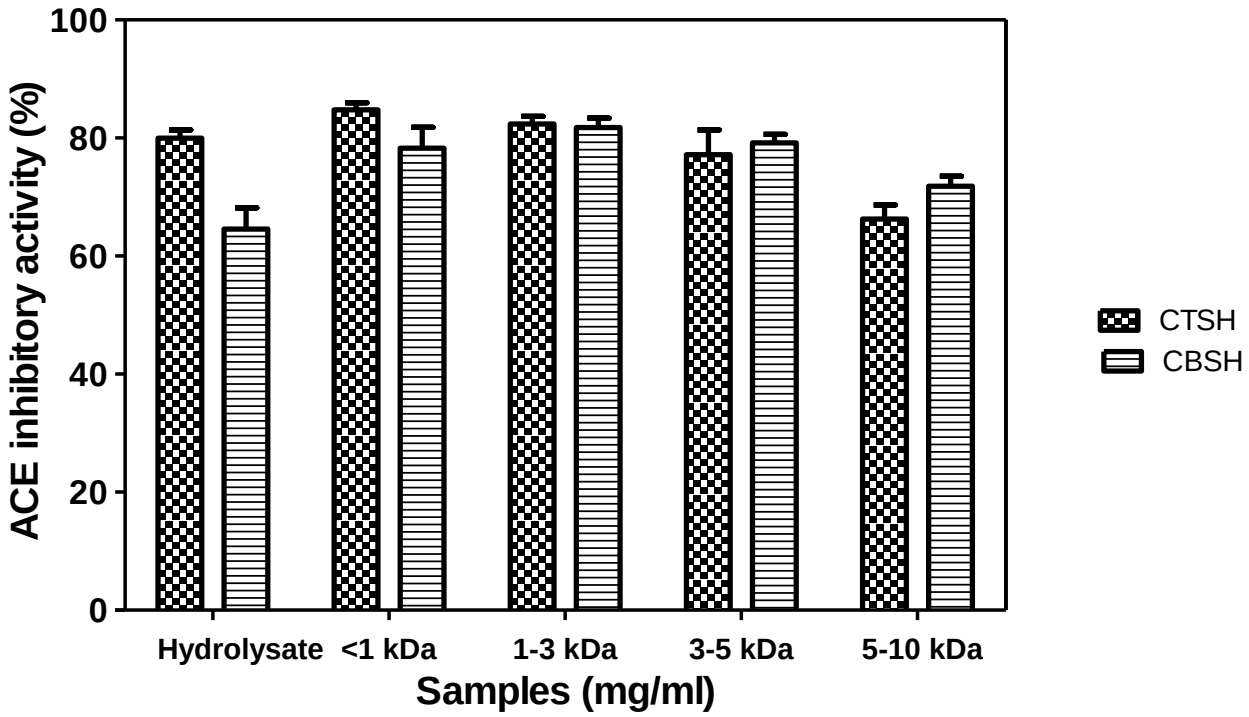


Fig. 3.3. Percentage (mean + standard deviation) ACE-inhibitory activity of chicken skin protein hydrolysates andmembrane fractions determined at 1 mg/mL sample concentration. CTSH—chicken thigh skin protein hydrolysates from 3% alcalase; CBSH—chicken breast skin protein hydrolysates from1% pepsin–pancreatin. Barswith different alphabets have mean values that are significantly different ($p < 0.05$).



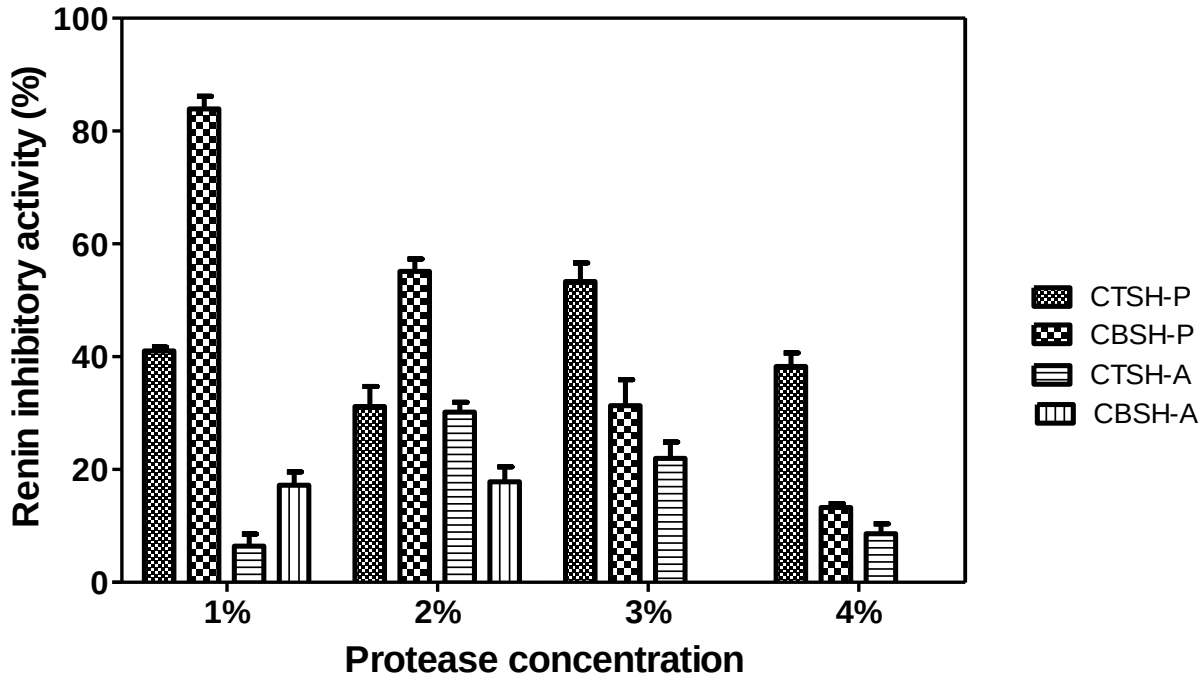
the ACE inhibition of protein hydrolysates were reported by Cheng, Liu, et al. (2008) for chicken leg bone protein hydrolysates produced with alcalase for 4 h. As shown in Table 3.3 a significant interaction was observed between the membrane fractionation of the CSPH and the muscle type in their ACE inhibition activity with a higher values obtained at 1 kDa and in hydrolysate in CTSH while higher values were obtained at 5 and 10 kDa for CBSH and no differences at 3 kDa cut-off membranes. Saiga et al. (2008) reported that low molecular (<3 kDa) peptide fractions from chicken collagen hydrolysate had higher antihypertensive activity than high molecular (≥3 kDa) peptide fractions. On the contrary, Raghavan and Kristinsson (2009) reported a lower ACE activity for membrane fractions when compared to the hydrolysates and attributed it to a possible loss of the characteristic synergistic effect that takes place when peptides are present together in the whole hydrolysate. Jamdar, Rajalakshmi, and Sharma (2012) however, reported that the ACE-inhibitory activity is not dependent on the size of the peptide but rather on their amino acid composition. It has been previously observed that many of the ACE-inhibitory peptides usually consist of 2–12 amino acid residues and that these short chain peptides are usually more resistant to gastrointestinal proteolysis and are also readily absorbed in more intact form into the circulatory system than long chain peptides (Fujita et al., 2000; Nakade et al., 2008). The results from this study (Fig. 3.3) showed a higher ACE-inhibitory activity than that obtained from the protein hydrolysates of pacific hake (Samanarayaka et al., 2010), tilapia (Raghavan & Kristinsson, 2009), and tuna dark muscle (Qian et al., 2007) but lower than the values reported for tuna frame (Lee et al., 2010).

3.3.7. Renin inhibitory activity

Renin is involved in catalyzing the hydrolysis of angiotensinogen to angiotensin I. Though, angiotensin I has no detrimental effect physiologically, it acts as a substrate for

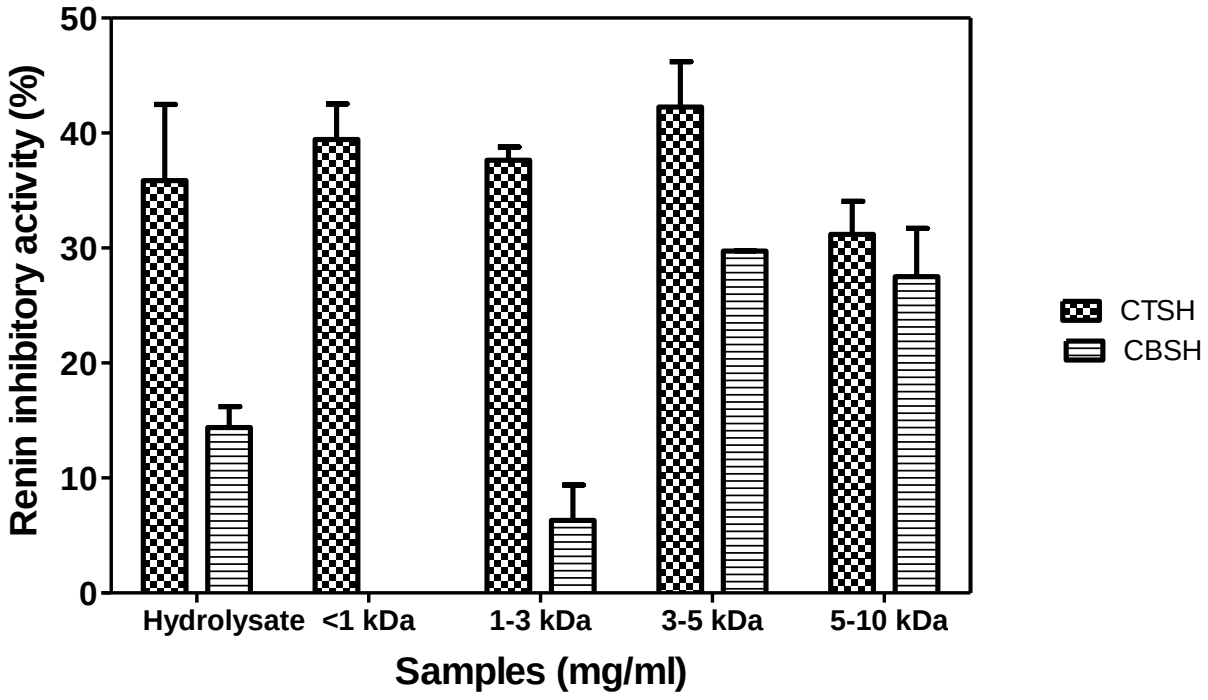
hydrolysis to angiotensin II by ACE. Direct inhibition of renin offers better control of hypertension than the inhibition of ACE since it prevents angiotensin I production which could also be converted to angiotensin II by pathways other than ACE such as the chymase catalytic pathway (Udenigwe & Aluko, 2012; Udenigwe et al., 2009). Inhibition of renin therefore, produces a highly selective inhibition of RAS with an improved side-effect profile for therapeutic agents (Girgih et al., 2011). Very few studies have been done regarding the inhibition of renin activity with enzymatic hydrolysates derived from food proteins. An investigation of the plots produced for the three 2 way interactions studied in our statistical model revealed that the three main factors (muscle, enzyme type and enzyme concentration) affected the renin-inhibitory activity of hydrolysates. While a combination of 1% of PP gave the highest renin-inhibitory activity in CBSH, a combination of 3 or 4% of PP worked best for CTSH. The effects of muscle type, enzyme type and enzyme concentration on renin inhibitory activity of the CSPH are shown in Table 3.2 and Fig. 3.4. The renin inhibition activity of the hydrolysates did not vary significantly with the muscle skin type but varied with enzyme type and enzyme concentration. Also there is an interaction effect between the muscle skin type and enzyme type, muscle skin type and enzyme concentration as well as enzyme type and enzyme concentration. The intensities analysis for the enzyme type showed that CSPH hydrolysed with pepsin pancreatin had a significantly higher renin inhibition than the CSPH hydrolysed with alcalase, indicating that the renin-inhibitory activity of food protein hydrolysates is dependent on type of proteolytic treatment (Udenigwe et al., 2009). The mean intensities for the enzyme concentration also showed that the renin inhibition significantly decreased with increasing protease concentration. The membrane fractions also exhibited similar moderate renin inhibitory activity which varied

Fig. 3.4. Percentage (mean + standard deviation) inhibition of human recombinant renin activity by chicken skin protein hydrolysates (CSPH) at differents enzyme concentrations and 1 mg/mL sample concentration. CTSH-P, chicken thigh skin protein hydrolysed with pepsin–pancreatin; CBSH-P, chicken breast skin protein hydrolysed with pepsin-pancreatin; CTSH-A, chicken thigh skin protein hydrolysed with alcalase; CBSH-A, chicken breast skin protein hydrolysed with alcalase. Bars with different alphabets have mean values that are significantly different ($p < 0.05$).



significantly with the muscle skin type and membrane size as well as in the interaction effect between muscle skin type and membrane size as shown in Table 3 and Fig. 5. An investigation of the plot produced for the 2 way interaction studied in our statistical model revealed that the two main factors membrane cut-off size) affected the renin inhibitory activity of hydrolysates. The cut-off size did not affect both CBSH and CTSH in the same way. Although, CTSH gave the highest renin-inhibitory activity for all cut-off sizes studied here, CBSH gave similar renin-activities only for 10 kDa cut-off size. The CTSH was significantly higher than the CBSH and this also increased significantly with increasing membrane size. Within the fractions, membrane fractions from the CTSH also showed comparatively higher inhibition of renin than the fractions from the CBSH (Fig. 3.5). Similar results have also been reported in several other studies. Girgih et al. (2011) reported that peptide samples from hemp seed protein hydrolysates and fractions moderately inhibited renin activity with IC50 values of 0.81, 2.52 and 1.89 mg/mL for the hydrolysate, <1 and 1–3 kDa membrane fractions respectively. Moderate renin inhibition was also achieved with flaxseed protein hydrolysates and membrane fractions by Udenigwe et al. (2009) with an IC50 of 1.22 to 2.81 mg/mL sample. Li and Aluko (2010) isolated 3 di-peptides from pea protein hydrolysate that also moderately inhibited renin activity with an IC50 of 9.2, 17.8 and 22.5 mM respectively. According to Udenigwe et al. (2009), inhibition of ACE is easier than renin inhibition and as a result, food-derived ACE inhibitory peptides are more commonly available than renin inhibitory peptides. This is due to the difficulty in the down regulation of renin activitiesboth in vitro and in vivo (Girgih et al., 2011). Comparatively, CSPH had better ACE inhibitory activity than renin inhibitory activity.

Fig. 3.5. Percentage (mean + standard deviation) inhibition of human recombinant renin activity by chicken skin protein hydrolysates and membrane fractions determined at 1 mg/mL sample concentration. CTSH—chicken thigh skin protein hydrolysates from 3% alcalase; CBSH—chicken breast skin protein hydrolysates from 1% pepsin–pancreatin. Bars with different alphabets have mean values that are significantly different ($p < 0.05$).



3.4. Conclusions

Chicken skin protein hydrolysates produced through alcalase or simulated gastrointestinal digestions were shown to exhibit inhibitory activities against ACE and renin during in vitro tests. Fractionation of the hydrolysates with ultrafiltration membranes also produced peptide of different sizes with strong ACE and moderate renin inhibitory activities. This work has shown that poultry skin is a suitable raw material for the production of potentially bioactive peptides that can be used as ingredients to formulate antihypertensive functional foods and nutraceuticals. The high yield exhibited by the hydrolysates is a good indication of potential viability for future commercialization of the bioactive chicken skin products. Further research is needed to characterize the molecular structure of active peptides present in the CSPH and to determine potential blood pressure lowering effects in an animal model of hypertension.

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3.5 References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. Journal of Agricultural and Food Chemistry, 27, 1256–1262.

Ahhmed, A. M., & Muguruma, M. (2010). A review of meat protein hydrolysates and hypertension. *Meat Science, 86,* 110 – 118.

Aluko, R. E., & Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. Journal of Food Science, 68, 1254–1258.

AOAC (1990). (15th ed.). Official Methods of Analysis, Vols. I and II, Washington, DC: Association of Official Analytical Chemists, Inc.

Babji, A. S., Chin, S. Y., Chempaka, M. Y. S., & Alina, A. R. (1998). Quality of mechanically deboned chicken meat frankfurter incorporated with chicken skin. International Journal of Food Science and Nutrition, 49, 19–326.

Badr, H. M. (2005). Chemical properties of chicken muscles and skin as affected by gamma irradiation and refrigerated storage. Journal of Food Technology, 3, 1–9.

Bhat, Z. F., Kumar, P., & Kumar, S. (2011). Effect of skin, enrobing and refrigerated storage on the quality characteristics of chicken meat balls. Journal of Food Science and Technology, 1–10.

Biswas, S., Chakraborty, A., Sarkar, S., Barpuzari, R. N., & Barpuzari, T. (2007). Effect of incorporation of chicken fat and skin on the quality of chicken sausages. The Journal of Poultry Science, 44, 111–115.

Bonifer, L. B., & Froning, G. W. (1996). Chicken skin composition as affected by aqueous washing. Journal of Food Science, 61, 895–898. Cheng, F. Y., Liu, Y. T., Wan, T. C., Lin, L. C., & Sakata, R. (2008). The development of angiotensin I converting enzyme inhibitor derived from chicken bone protein. Animal Science Journal, 79, 122–128.

Cheng, F. Y., Wan, T. C., Liu, Y. T., Lai, K. M., Lin, L. C., & Sakata, R. (2008). A study of in vitro antihypertensive properties of enzymatic hydrolysate from chicken leg bone protein. Animal Science Journal, 79, 614–619.

Cliché, S., Amiot, J., & Gariepy, C. (2003). Extraction and characterization of collagen with or without telopeptides from chicken skin. Poultry Science, 82, 503–509.

Daien, V., Duny, Y., Ribstein, J., du Cailar, G.,Mimran, V., Villain,M., Daures, J. P., & Fesler, P. (2012). Treatment of hypertension with renin–angiotensin system inhibitors and renal dysfunction: a systematic review and meta-analysis. American Journal of Hypertension, 25, 126–132.

Erdmann, K., Cheung, B. W. Y., & Schroder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. The Journal of Nutritional Biochemistry, 19, 643–654.

Feddern, V., Kupski, L., Cipolatti, E. P., Giacobbo, G., Mendes, G. L., Furlong, E. B., & Soares, L. A. S. (2010). Physico-chemical composition, fractionated glycerides and fatty acid profile of chicken fat. European Journal Lipid Science and Technology, 112, 1277–1284.

Fujita, H., Yokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. Journal of Food Science, 65, 564–569.

Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2010). In vitro antioxidant properties of hemp seed (Cannabis sativa L.) protein hydrolysate fractions. Journal of the American Oil Chemists' Society, 88, 381–389.

Girgih, A. T., Udenigwe, C. C., Li, H., Adebiyi, A. P., & Aluko, R. E. (2011). Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (Cannabis sativa L.) protein hydrolysates. Journal of the American Oil Chemists' Society, 88, 1767–1774.

Guerard, F., Guimas, L., & Binet, A. (2002). Production of tuna waste hydrolysates by a commercial neutral protease preparation. Journal of Molecular Catalysis B: Enzymatic, 19–20, 489–498.

Jamdar, S. N., Rajalakshmi, V., & Sharma, A. (2012). Antioxidant and ACE inhibitory properties of poultry viscera protein hydrolysate and its peptide fractions. Journal of Food Biochemistry, 36, 494–501.

Je, J. Y., Park, P. J., Kwon, J. Y., & Kim, S. K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska Pollack (Theragra chalcogramma) frame protein hydrolysate. Journal of Agricultural and Food Chemistry, 52, 7842–7845.

Lee, S. H., Qian, Z. J., & Kim, S. K. (2010). A novel Angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chemistry, 118, 96–102.

Li, H., & Aluko, R. E. (2010). Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. Journal of Agricultural and Food Chemistry, 58, 11471–11476.

Markwell, M. A. C., Haas, S. M., Biebar, L. L., & Tolbert, N. E. (1978). A modification of Lowry procedure to simplify protein determination in membrane and protein samples. Analytical Biochemistry, 87, 206–211.

Martinez-Maqueda, D., Miralles, B., Recio, I., & Hernandez-Ledesma, B. (2012). Antihypertensive peptides from food proteins: a review. Food and Function, 3, 350–361.

Nakade, K., Kamishima, R., Inoue, Y., Ahhmed, A., Kawahara, S., Nakayama, T., Maruyama, M., Numata, M., Ohta, K., Aoki, T., & Muguruma, M. (2008). Identification of an antihypertensive peptide derived from chicken bone extract. Animal Science Journal, 79, 710–715.

Qian, Z. J., Je, J. Y., & Kim, S. K. (2007). Antihypertensive effect of angiotensin I converting

enzyme inhibitory peptide from hydrolysates of big eye tuna dark muscle, (Thunnus obesus). Journal of Agricultural and Food Chemistry, 55, 8398–8403.

Raghavan, S., & Kristinsson, H. G. (2009). ACE-inhibitory activity of Tilapia hydrolysates. Food Chemistry, 117, 582–588.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: meat and fish. Nutrients, 3, 765–791.

Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T., & Morimatsu, F. (2008). Angiotensin I-converting enzyme–enzyme inhibitory peptides obtained from chicken collagen hydrolysate. Journal of Agricultural and Food Chemistry, 56, 9586–9591.

Saiga, A., Okumura, T., Makihara, T., Katsuda, S., Morimatsu, F., & Nishimura, T. (2006). Action mechanism of an angiotensin I converting enzyme inhibitory peptide derived from chicken breast muscle. Journal of Agricultural and Food Chemistry, 54, 942–945.

Saiga, A., Okumura, T., Makihara, T., Katsuda, S., Shimizu, T., Yamada, R., & Nishimura, T. (2003). Angiotensin I converting enzyme inhibitory peptides in a hydrolysed chicken breast muscle extract. Journal of Agricultural and Food Chemistry, 51, 1741–1745.

Samanarayaka, A. G. P., Kitts, D. D., & Li-Chan, E. C. Y. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a pacific hake (Merluccius productus) fish protein hydrolysate subjected to simulated gastrointestinal digestion and caco-2 cell permeation. Journal of Agricultural and Food Chemistry, 58, 1535–1542.

Shahidi, F., Han, X. Q., & Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (Mallotus villosus). Food Chemistry, 53, 285–293.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production, processing and potential health benefits. Journal of Food Science, 71, R11–R24.

Udenigwe, C. C., Lin, Y. S., Hou,W. C., & Aluko, R. A. (2009). Kinetics of inhibition of renin and angiotensin-I-converting by flaxseed protein hydrolysate fractions. Journal of Functional Foods, 1, 199–207.

Vercruysse, L., Van Camp, J., & Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: a review. Journal of Agricultural and Food Chemistry, 53, 8106–8115.

TRANSITION STATEMENT ONE

In order to address the general objective of this work, which is to produce bioactive peptides from the enzymatic hydrolysis of chicken skin proteins that could be used in the prevention and treatment of hypertension, oxidative stress and associated health conditions using a metabolomics approach, each manuscript is designed to address a specific objective. In the first manuscript to determine the potential antihypertensive effect of chicken skin protein hydrolysates by measuring their *in vitro* inhibitory activities against renin and ACE, we optimized enzymatic hydrolysate production from chicken thigh and breast muscle skin proteins using alcalase and a combination of pepsin and pancreatin at concentrations of 1–4%. After fractionation using ultrafiltration membranes of 1, 3, 5 and 10 kDa molecular weight cut-offs (MWCO), the hydrolysates were then analyzed for their *in vitro* antihypertensive activities. The hydrolysates produced through alcalase or simulated gastrointestinal digestions and their ultrafiltration membrane fractions exhibited strong *in vitro* inhibitory activities against ACE and renin, suggesting that chicken skin is a suitable raw material for the production of potentially bioactive peptides that can be used as ingredients to formulate antihypertensive functional foods and nutraceuticals. The second manuscript is to evaluate the ability of chicken skin protein hydrolysates and ultrafiltration membrane fractions to be used as potential antioxidant agents in the scavenging of free radicals of oxidation. This will form the basis for subsequent *in vivo* experiments to determine their bioactive potentials in the management of oxidative stress and hypertension.

CHAPTER 4

MANUSCRIPT TWO

IN VITRO ANTIOXIDANT PROPERTIES OF CHICKEN SKIN ENZYMATIC PROTEIN HYDROLYSATES AND MEMBRANE FRACTIONS

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4.0 Abstract

Chicken thigh and breast skin proteins were hydrolysed using alcalase or a combination of pepsin and pancreatin (PP), each at concentrations of 1–4%. The chicken skin protein hydrolysates (CSPHs) were then fractionated by membrane ultrafiltration into different molecular weight peptides (<1, 1–3, 3–5 and 5–10 kDa) and analysed for antioxidant properties. Results showed that the CSPHs had a significantly ($p < 0.05$) lower scavenging activity against DPPH radicals when compared to reduced glutathione. The chicken breast skin hydrolysates had significantly higher DPPH scavenging activity than the chicken thigh skin hydrolysates. DPPH scavenging and metal ion chelation increased significantly ($p < 0.05$) from 29–40% to 86–89%, respectively with increasing proteolytic enzyme concentration. In contrast, the antioxidant properties decreased as peptide size increased. We conclude that CSPHs and their peptide fractions may be used as ingredients in the formulation of functional foods and nutraceuticals for the control and management of oxidative stress-related diseases.

Keywords: Chicken skin, Protein hydrolysates, Peptides, Free radical scavenging, Functional, foods, Oxidative stress

4.1 Introduction

Erdmann, Cheung, and Schroder (2008) defined biologically active peptides as ‘‘food-derived peptides that exert, beyond their nutritional value, a physiological, hormone-like effect in humans’’. Bioactive peptides consist of natural amino acid sequences (often 2–20 residues) encrypted in the parent or natural protein molecule, and are usually inactive within the sequence of the protein. They are, however, released during gastrointestinal digestion or in vitro protein hydrolysis with proteases and play important roles in the regulation and modulation of metabolism during digestion of food in the intestine. Thus, bioactive peptides have the potential of being metabolic aid supplements, in the form of nutraceuticals and functional food ingredients for the promotion of health and prevention of diseases (Bernardini et al., 2011).

Bioactive peptides have been isolated from various food sources such as milk and whey (Erdmann, Cheung, & Schroder, 2008), meat and fish (Martinez-Maqueda, Miralles, Recio, & Hernandez-Ledesma, 2012; Samanarayaka, Kitts, & Li-Chan, 2010) and quinoa seeds (Aluko & Monu, 2003). The potential metabolic regulatory effects of bioactive peptides relate to nutrient uptake, antihypertensive, antioxidant, anticancer, antithrombotic, opioid or antiproliferative as well as antimicrobial activities (Erdmann et al., 2008; Samanarayaka et al., 2010; Udenigwe & Aluko, 2012). Many of the known bioactive peptides exhibit multifunctional properties, are easily absorbed and could be used to reduce symptoms of oxidative stress, hypertension and dyslipidemia, which are all risk factors of coronary heart disease (Erdmann et al., 2008; Lee, Qian, & Kim, 2010; Samanarayaka et al., 2010). Of particular interest to human health is the uncontrolled production of free radicals (superoxide, hydroxyl, singlet oxygen, peroxyl) during cellular metabolism/oxidation, which leads to oxidative stress. Oxidative stress has been implicated in the initiation or progression of many vascular diseases due to extensive damage of

critically important biological polymers such as DNA, proteins and lipids (Erdmann et al., 2008). The toxic free radicals can also modify low density lipoprotein (LDL), which may lead to increased atherogenicity of oxidized LDL (Erdmann et al., 2008). This in turn can be a causative factor in many terminal degenerative diseases such as cardiovascular disease, diabetes, cancer, Alzheimer's disease and a host of other conditions (Bernardini et al., 2011; Erdmann et al., 2008; Naqash & Nazeer, 2011; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011).

Antioxidants play an important role in human health and nutrition as they are known to protect the body against reactive oxygen species (ROS) (Martinez-Maqueda et al., 2012; Ryan et al., 2011). The use of bioactive peptides as antioxidative agents is generating interest not only as natural alternatives to synthetic antioxidants, but for their beneficial effects in terms of health implications, non-residual side effects and their functionality in food systems (Bernardini et al., 2011; Erdmann et al., 2008; Girgih, Udenigwe, & Aluko, 2010; Ryan et al., 2011). The ability of endogenous enzymatic antioxidants (catalase, superoxide dismutase and glutathione peroxidase) to regulate this process is often weakened when excess free radicals are produced beyond cellular antioxidant capacity.

Food-derived bioactive peptides with antioxidant properties have been reported in several foods, such as milk and eggs (Erdmann et al., 2008), fish and a few domestic animal muscles (Bernardini et al., 2011; Ryan et al., 2011). They have also been isolated from poultry viscera protein hydrolysate (Jamdar, Rajalakshmi & Sharma, 2012) and flying fish backbone (Naqash & Nazeer, 2011). However, information on the antioxidant properties of chicken skin protein hydrolysates is scanty. Chicken skin is a byproduct derived from chicken meat processing which is highly underutilized, constituting huge cost for waste disposal and danger to the environment as well as the loss of nutritional value (Feddern et al., 2010). Several attempts have previously

been made at developing novel chicken skin based products in order to diversify the utilisation of chicken skin as well as reduce waste, such as chicken meat balls (Bhat, Kumar, & Kumar, 2011), collagen (Bonifer & Froning, 1996; Cliché, Amiot, & Gariepy, 2003), sausages (Biswas, Chakraborty, Sarkar, Barpuzari, & Barpuzari, 2007) and chicken meat frankfurter (Babji, Chin, Chempaka, & Alina, 1998). However, an area of research that is yet to be explored is the development of chicken skin based products with functional and health promoting values. The high protein content (dry weight basis) could, in addition to contributing to nutrition, also serve as a very active source of value-added products, including bioactive peptide-containing hydrolysates. Therefore, the objective of this study was to determine the effects of muscle source as well as type and level of protease on the in vitro antioxidative properties of chicken skin enzymatic hydrolysates and their ultrafiltration membrane peptide fractions.

4.2 Materials and methods

4.2.1. Materials

Chicken skins from the thigh and breast muscles used for this study were supplied by Granny's poultry (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas, EC 232-468-9), alcalase (from fermentation of Bacillus licheniformis, 3.4.21.62), trinitrobenzene sulfonic acid (TNBS), Triton X-100, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H2O2), potassium ferricyanide, ferrous sulphate, ferrous chloride, 1,10-phenanthroline, reduced glutathione (GSH) and ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2.4-tiazine- 40,400-disulfide acid sodium salt were purchased from Sigma–Aldrich (St. Louis, MO). All other analytical grade reagents and ultrafiltration membranes (1, 3, 5 and 10 kDa molecular weight cut-offs) were purchased from Fisher Scientific (Oakville, ON, Canada).

4.2.2. Preparation of chicken skin protein hydrolysates (CSPH)

Fresh thigh or breast chicken skins (approximately 250 g) were packed in freeze drying plates, frozen at – 20 ^{o}C for 24 h and transferred to – 80 ^{o}C for 6 h prior to freeze drying. The freeze dried samples were shredded manually and defatted repeatedly by mixing ~1 g of sample with 10 ml acetone (Fisher Scientific, Oakville, ON, Canada). The mixture was stirred in the fume hood for 3 h and decanted manually followed by two additional consecutive extractions of the residue. The defatted skin samples were then air dried overnight in the fume hood chamber at room temperature and subsequently milled with a Waring blender to produce a fine powder that was stored at – 20 ^{o}C. For the initial screening test to optimize and select the best enzyme concentration, dried chicken skin powder from the thigh or breast muscles was mixed with water to give 5% (w/v, protein basis) slurries. Two different enzyme treatments (alcalase and pepsin + pancreatin) were separately used for sample hydrolysis. For the alcalase hydrolysis, the slurry was heated to 55 ^{o}C, adjusted to pH 8.0 using 2 M NaOH and the hydrolysis initiated by the addition of alcalase enzyme (1–4% w/w, skin protein basis). Each mixture was stirred continuously for 4 h. For the pepsin + pancreatin (PP) hydrolysis, the slurry was heated to 37 ^{o}C, adjusted to pH 2.0 using 2 M HCl and the reaction initiated with the addition of pepsin enzyme (1–4% w/w, skin protein basis). The mixture then stirred continuously for 2 h. After the peptic hydrolysis, the reaction mixture was adjusted to a pH of 7.5 with 2 MNaOH, pancreatin was added (1–4% w/w, skin protein basis) and incubated at 37 ^{o}C for 4 h with continuous stirring. At the end of the incubation periods, both the alcalase and PP reactions were terminated by heating at 95 ^{o}C for 15 min to ensure complete denaturation of residual enzymes. The mixtures were thereafter centrifuged (7000g at 4 ^{o}C) for 1 h and the resulting supernatant lyophilized and stored at – 20 ^{o}C as the chicken thigh skin hydrolysate (CTSH) or chicken breast skin hydrolysate

(CBSH) until needed for further analysis. The most active hydrolysate from each enzymatic treatment was subsequently fractionated by sequentially passing the supernatant through ultrafiltration membranes with molecular cut-offs (MWCO) of 1, 3, 5 and 10 kDa in an Amicon stirred ultrafiltration cell. Starting with 1 kDa MWCO, the retentate from each membrane was passed through the next higher MWCO membrane while permeates from each membrane (1, 3, 5 and 10 kDa MWCO) was collected, lyophilized and stored at – 20 ^{o}C as <1, 1–3, 3–5 and 5–10 kDa fractions respectively, until required for analysis. Protein content of the lyophilized CSPHs was determined by the modified Lowry method (Markwell Haas, Biebar, & Tolbert, 1978). The above digestion and fractionation protocols were performed in triplicates and the lyophilized samples combined, analyzed for protein content and used for the antioxidative assays.

4.2.3. Amino acid composition analysis

The amino acid profiles of the defatted chicken thigh skin (CTS), chicken breast skin (CBS) and chicken skin protein hydrolysates from the thigh and breast muscles (CTSH and CBSH) samples were determined using the HPLC system after samples were hydrolysed with 6 M HCl according to the method of Bidlingmeyer, Cohen, and Tarvin (1984). The cysteine and methionine contents were determined after performic acid oxidation according to the method of Gehrke, Wall, Absheer, Kaiser, and Zumwalt (1985). The tryptophan content however, was determined after alkaline hydrolysis by the method of Landryl and Delhaye (1992).

4.2.4. DPPH radical scavenging assay

The scavenging activity of CSPHs and membrane ultrafiltration fractions against DPPH radical was determined according to the method described by Aluko and Monu (2003) with slight modifications for a 96-well clear flat-bottom plate. Peptide samples were dissolved in 0.1 M sodium phosphate buffer, pH7.0 containing 1% (w/v) Triton X-100. DPPH was dissolved in

methanol to a final concentration of 100 μM. Peptide samples (100 μl) were mixed (final assay concentration of 1 mg/ml) with 100 μl of the DPPH solution in the 96-well plate and incubated at room temperature in the dark for 30 min. The absorbance values of the blank (Ab) and samples (As) were measured at 517 nm. The blank consisted of sodium phosphate buffer in place of the peptide sample while GSH was used as a positive control. The percent DPPH radical scavenging activity of the samples was determined using the following equation:

DPPH radical scavenging activity (%) = [(Ab – As)/Ab] x 100

4.2.5. Chelation of metal ions

The metal chelating activity of the CSPH and its fractions was measured using a modified method of Xie, Huang, Xu, and Jin (2008). Peptide sample solution or GSH (final assay concentration of 1 mg) was combined with 0.05 ml of 2 mM FeCl2 and 1.85 ml double distiled water in a reaction tube. Ferrozine solution (0.1 ml of 5 mM) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 10 min from which an aliquot of 200 μl was removed and added to a clear bottom 96-well plate. A blank experiment was also conducted by replacing the sample with 1 ml of double distilled water. The absorbance values of blank (Ab) and sample (As) at 562 nm were measured using a spectrophotometer and the metal chelating activity of the sample was compared to that of GSH. The percentage chelating effect (%) was calculated using the following equation:

Metal chelating effect (%) = [(Ab – As)/Ab] x 100

4.2.6. Hydroxyl radical scavenging

The hydroxyl radical scavenging activity of CSPHs and membrane fractions was determined according to the method reported by de Avellar et al. (2004). Peptide samples and 3 mM of 1,10-phenanthroline were separately dissolved in 0.1 M sodium phosphate buffer (pH

7.4). FeSO4 (3 mM) and 0.01% hydrogen peroxide were both separately dissolved in distilled water. An aliquot (50 µl) of peptide samples (equivalent to a final assay concentration of 1 mg/ml) or buffer (blank) was first added to a clear, flat bottom 96-well plate followed by 50 µl of 1,10-phenanthroline and then 50 µl of FeSO4. To initiate the Fenton reaction in the wells, 50 µl of hydrogen peroxide was added to the mixture, covered and incubated at 37 ^{o}C for 1 h with shaking. The absorbance was measured using a spectrophotometer at 536 nm at 10 min intervals for 1 h. The hydroxyl radical scavenging activity was calculated using the reaction rate (ΔA/min) equation below:

Hydroxyl radical scavenging activity (%) = $\{[(\Delta A/min_b) – (\Delta A/mins]/(\Delta A/min)_b\} \times 100$

4.2.7. Superoxide scavenging activity

The superoxide scavenging activity of the CSPH and its peptide fractions was measured according to previous method by Xie et al. (2008). An aliquot of peptide samples (80 µl in 50 mM Tris–HCl buffer containing 1 mM EDTA, pH 8.3, at a final concentration of 1 mg/ml) was mixed with 80 µl of the buffer directly into a clear bottom 96-well plate in darkness. Then, 40 µl of 1.5 mM pyrogallol dissolved in 10 mM HCl was added to each well. The reaction rate (ΔA/min) was measured immediately at 420 nm for 4 min at room temperature using the buffer as blank. The superoxide scavenging activity was calculated using the following equation:

Superoxide scavenging activity = $\{[(\Delta A/min)_b – (\Delta A/min)_s]/(\Delta A/min_b\} \times 100$

4.2.8. Determination of oxygen radical absorbance capacity (ORAC)

The ORAC assay of the CSPH and membrane fractions was done according to the procedures reported by You, Udenigwe, Aluko, and Wu (2010) with the following modifications. Briefly, a microplate fluorescence reader model FLx800 (Bio-Tek Instruments, Inc., Winooski, VT) with fluorescence filters (excitation wavelength 485/20 nm, emission wavelength 528/20

nm) was used and run using the Gen5™ software. Potassium phosphate buffer (75 mM, pH 7.4) was used to prepare all reagents, standards, samples and the control. Five Trolox concentrations, between 6.25 and 100 μM, were used for the calibration curve. Fluorescein (0.082 μM) and AAPH (0.15 M) were prepared immediately before use. Rutin trihydrate (10 μM) was used as the antioxidant control. Two concentrations, 150.0 and 300 μg/ml water–methanol extract, and 13.0 and 26.0 μg/ml for ethyl acetate extracts, were used in triplicate. Fluorescein (120 μl) was transferred into the wells followed by addition of 20 μl of samples or blank (buffer). The mixture was incubated for 20 min at 37 ^{o}C in the built-in incubator, and subsequently APPH solution (60 μl) was added to each well. Data were collected every min for a total of 50 min. The ORAC values were expressed as Trolox equivalents (TE) using the standard curve.

4.2.9. Statistical analysis

For enzyme screening analysis, a 3-way analysis of variance (ANOVA) was used involving a model that included muscle type (Mu), enzyme type (Et) and enzyme concentration (Ec) as fixed variables. All 2-way interactions such as muscle type by enzyme type, muscle type by enzyme concentration and enzyme type by enzyme concentration were also analysed. For membrane fractions analysis with interactions, 2-way ANOVA was conducted with muscle type and membrane size as fixed variables. Tukey's multiple comparison tests was used to determine mean treatment differences for all main variables ($p < 0.05$). An IBM SPSS Statistical package (version 20) was used for all statistical analyses.

4.3 Results and discussion

4.3.1. Amino acid composition

The CBSH from 1% PP hydrolysis and CTSH from 3% alcalase hydrolysis were chosen for membrane fractionation and amino acid analysis, based on these exhibiting the best overall

antioxidant activity. The amino acid composition of proteins and peptides has a great influence on functionality (He, Girgih, Malomo, Ju, & Aluko, 2013). The hydrophobic properties of peptides have been reported to contribute to their antioxidants properties, as they are known to enhance interactions with lipids as well as facilitate their entry into target organs (He et al., 2013). The samples contained high amounts of hydrophobic amino acids (HAA), though the CSPHs tended to contain higher amounts of HAA than the defatted chicken skin samples (Table 4.1), suggesting that the hydrolysates may possess potential antioxidant activities. The negatively charged amino acids (NCAA), such as aspartic and glutamic acids, are reported to exhibit strong antioxidant properties as they have the ability to donate their excess electrons during free radical reactions (He et al., 2013; Samanarayaka et al., 2010; Udenigwe & Aluko, 2011). The amino acid composition of the samples showed that they were considerably high in Asx and Glx, both of which include aspartic and glutamic acids, respectively (Table 4.1). Another factor which has been observed to contribute to the potency of antioxidant peptides is the presence of tyrosine, methionine, histidine and lysine. Lysine and tyrosine are reported to act as hydrogen donors while histidine has been shown to possess strong radical scavenging activity as a result of the chelating, lipid trapping and decomposition of the imidazole ring (He et al., 2013; Naqash & Nazeer, 2011; Samaranayaka & Li-Chan, 2011; Udenigwe & Aluko, 2011). The defatted chicken skins and CSPHs are rich in lysine but, low in tyrosine, methionine and histidine. The CSPHs exhibited slightly higher essential amino acids (EAA) content than the defatted chicken skins

Table 4.1: Percentage amino acid composition of defatted chicken thigh skin (CTS), chicken thigh skin protein hydrolysates (CTSH)[a], chicken breast skin (CBS), and chicken breast skin protein hydrolysates (CBSH)[b].

	CTS	CTSH	CBS	CBSH
Amino Acids (%)*				
ASX	9.12	9.05	9.02	9.16
THR	3.42	3.47	3.19	3.34
SER	4.31	4.26	4.19	4.09
GLX	13.13	13.21	12.97	13.27
PRO	10.72	10.52	11.59	11.27
GLY	15.02	14.61	16.52	11.27
ALA	7.49	7.39	7.86	7.76
CYS	0.94	0.97	0.77	0.73
VAL	3.56	3.80	2.99	3.64
MET	1.57	1.68	1.49	1.29
ILE	2.39	2.65	2.06	2.61
LEU	5.69	5.81	5.12	5.45
TYR	2.15	2.33	2.32	2.11
PHE	3.19	3.25	3.12	3.15
HIS	2.88	2.83	2.54	2.22
LYS	5.79	5.79	5.63	5.67
ARG	8.14	7.89	8.06	7.92
TRP	0.49	0.48	0.56	0.37

PCAA	16.81	16.51	16.23	15.81
NCAA	22.25	22.27	21.99	22.43
AAA	5.84	6.05	6.00	5.64
EAA	29.53	30.39	27.36	28.87

***ASX aspartic acid and asparagine; GLX glutamic acid and glutamine. Combined total of hydrophobic amino acids-alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine, and cysteine (HAA). Positively charged amino acids-arginine, histidine, lysine (PCAA). Negatively charged amino acids -ASX and GLX (NCAA). Aromatic amino acids-phenylalanine, tryptophan, and tyrosine (AAA). Essential amino acids-phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine and lysine (EAA). [a]CTSH: from 3% alcalase hydrolysis, [b]CBSH: from 1% pepsin + pancreatin hydrolysis.**

samples suggesting that the hydrolysates may have better nutritional value (He et al., 2013). Overall, the protein hydrolysis protocols did not lead to any substantial loss in amino acid content of the CSPHs.

4.3.2. DPPH radical scavenging and metal ion chelation activities

When antioxidants react with free radicals formed during oxidative reactions, stable products are formed that lead to termination of the oxidation process (Jamdar et al., 2012). DPPH has been reported to be a stable free radical with a maximum absorbance at 517 nm in methanol and as such has been widely used to test reducing substances, especially natural compounds (Girgih et al., 2010; Huang, Ou, & Prior, 2005; Jamdar et al., 2012). DPPH radicals are scavenged when they encounter a proton-donating substance, such as an antioxidant, leading to a change in colour from purple to yellow as well as a reduction in absorbance. It is therefore a very useful method for comparing the antioxidant potentials of protein hydrolysates (Girgih et al., 2010). Table 4.2 shows results from the analysis of the DPPH radical scavenging activity of all the CSPHs that were produced under different hydrolysis conditions. The DPPH radical scavenging activity of the samples was significantly affected by the muscle skin type and enzyme concentration but not enzyme type, with the CBSH (B) having significantly higher DPPH activity than the CTSH (T). In our previous study (Onuh, Girgih, Aluko, & Aliani, 2013), the CBSH was shown to have a higher degree of hydrolysis (DH) and peptide content (yield) than CTSH. Since DH is inversely related to peptide size, the CBSH may have smaller size peptides than the CTSH, which could have contributed to observed differences in DPPH radical scavenging activities. Generally, DPPH activities of the hydrolysates increased with increasing enzyme concentration, though the increases from 2% to 4% were not significantly different from each other (Table 4.2). Thus the data suggests that above 1% enzyme concentration more

antioxidant peptides were produced, which is an indication of higher levels of protein hydrolysis. GSH had significantly ($p < 0.05$) higher DPPH scavenging activity (55.10%) than all the samples. The DPPH radical scavenging activity of the membrane ultrafiltration fractions are shown in Table 4.3 and Fig. 4.1A. The results show that the DPPH radical scavenging activities of the membrane fractions are significantly affected by the muscle skin type as well as the membrane ultrafiltration, with the membrane fractions from the CTSH (T) having significantly ($p < 0.05$) better scavenging activity against DPPH radical than the CBSH (B) membrane fractions. The DPPH radical scavenging activities of membrane fractions decreased with increasing peptide size (Table 4.3 and Fig. 4.1A), suggesting that lower molecular weight peptides are more effective as potent DPPH radical scavengers than high molecular weight peptides. Similar findings showing higher DPPH scavenging activity of low molecular weight peptides have been previously reported for quinoa protein hydrolysates fractions (Aluko & Monu, 2003), black scabbard fish protein hydrolysates (Batista, Ramos, Coutinho, Bandarra, & Nunes, 2009) and hemp seed protein hydrolysates fractions (Girgih et al., 2010). However, results obtained from poultry viscera protein hydrolysates, flaxseed protein-derived peptide fractions and peptides derived from mackerel protein hydrolysates are in contrast to the current observation (Jamdar et al., 2012; Udenigwe, Lu, Han, Hou, & Aluko, 2009; Wu, Chen, & Shiau, 2003). Metal chelating ability of a compound may serve as a very important indicator of the potential antioxidant activity of that compound as it acts to stabilize oxidized form of metal ions by reducing their redox potential (Girgih et al., 2010). An analysis of all the CSPHs produced under different hydrolysis conditions for their ability to chelate metal ions is shown in Table 4.2. The samples exhibited very high metal chelating ability which was significantly affected by the muscle skin type, enzyme type and enzyme concentration. The CTSH (T) had significantly

higher metal chelating ability than the CBSH (B), which suggests differences in the susceptibility of each type of skin protein to proteolytic degradation. The alcalase hydrolysates had significantly higher metal chelation activity than the pepsin-pancreation hydrolysates which may also be due to differences in the types of peptides produced by each enzyme. The metal chelating activity of the hydrolysates also increased with increasing enzyme concentration from 86.43% to 93.09%, which is an indication of liberation of greater number of peptides as enzyme level increased. The metal chelating activity of CSPHs were significantly ($p < 0.05$) higher than that of GSH. Thus the CSPHs contain metal chelating peptides that could potentially provide similar benefit as GSH, the natural cellular antioxidant molecule. The metal chelating activity for the membrane fractions is shown in Table 4.3 and Fig. 4.1B. Membrane fractionation significantly reduced the metal chelation of the hydrolysates suggesting a reduced additive and synergistic effect by the membrane fractions. The metal chelation activities of the membrane fractions were also significantly ($p < 0.05$) affected by the muscle skin type and membrane size with the CTSH (T) membrane fractions having significantly better metal chelating activity than the CBSH (B) membrane fractions. Differences in susceptibility of the muscles to proteolytic degradation may be responsible for these observed differences. The metal chelating activities of the membrane fractions also decreased with increasing peptide size suggesting also that low molecular sized peptides exhibits better metal chelation. The significantly ($p < 0.05$) higher metal chelation activity of the hydrolysate when compared to the membrane fractions is in agreement with those from a previous study (Girgih et al., 2010). Thus, an additive and synergistic effect may have

Table 4.2: Results from 3-way ANOVA and Tukey's test of the effects of muscle type, Mu [number of replicates, n = 3]; enzyme type, Et [n = 3]; enzyme concentration, Ec [n = 3]; thigh, T [n = 3]; breast, B [n = 3]; pepsin-pancreatin, PP [n = 3]; alcalase, A [n = 3] on the DPPH radical, metal chelation and hydroxyl radical scavenging activity of chicken skin protein hydrolysates

	Source of variation (F-Values)						Mean intensity for muscle type			Mean intensity for enzyme type			Mean intensity for enzyme concentration				
Parameters	Mu	Et	Ec	Mu x Et	Mu x Ec	Et x Ec	T	B	GSH	PP	A	GSH	1	2	3	4	GSH
DPPH Radical	14.38 **	0.004 NS[1]	18.35 ***	16.6 9 ***	20.27 ***	9.71 ***	33.32[c] (0.75)	37.36[b] (0.75)	55.10[a] (2.13)	35.30 (0.75)	35.37 (0.75)	55.10 (2.13)	29.39[c] (1.07)	35.03[b] (1.07)	40.39[b] (1.07)	36.54[b] (1.07)	55.10[a] (2.13)
Metal chelation	10.21 **	91.37 ***	127.95 ***	37.3 7 ***	19.78 ***	196.7 4 ***	91.12[a] (0.19)	90.26[b] (0.19)	84.80[c] (0.54)	89.41[b] (0.19)	91.98[a] (0.19)	84.80[c] (0.54)	86.43[c] (0.27)	90.59[b] (0.27)	93.09[a] (0.27)	92.65[a] (0.27)	84.80[d] (0.54)
Hydroxyl Radical	42.09 ***	53.92 ***	6.34 **	9.43 ***	68.90 ***	5.48 **	3.17[c] (1.68)	18.58[b] (1.68)	74.30[a] (4.75)	2.15[c] (1.68)	19.59[b] (1.68)	74.29[a] (4.75)	14.95[b] (2.38)	2.03[c] (2.38)	12.48[bc] (2.38)	14.03[bc] (2.38)	74.30[a] (4.75)

[a, b, c, d] Mean intensity values (followed in brackets by the standard error of the mean) within the same variable "muscle", "enzyme type" and "enzyme concentration" with the same letter within the same row (parameter) are not significantly different (P < 0.05)

[1]NS: Not significant at P ≥ 0.05, *P < 0.05, **P < 0.01, ***P < 0.001

Table 4.3: Results from 2-way ANOVA and Tukey's test of the effects of muscle type, Mu [number of replicates, n = 3]; membrane type, Me [n = 3]; thigh, T [n = 3]; breast, B [n = 3]; pepsin-pancreatin, PP [n = 3]; alcalase, A [n = 3] on the DPPH radical, metal chelation, hydroxyl radical scavenging, superoxide radical and oxygen radical scavenging activity of chicken skin hydrolysate membrane fractions

	Source of variation (F values)			Mean intensity for muscle type			Mean intensity for membrane cut-off size (KDa)					
Parameters	Mu	Me	Mu x Me	T	B	GSH	Hydrolysate	1 kDa	3 kDa	5 kDa	10 kDa	GSH
DPPH	19.33	11.83	1.79	33.45^{b}	27.42^{c}	55.10^{a}	33.09^{bc}	38.52^{b}	27.50^{c}	26.44^{c}	26.62^{c}	55.10^{a}
radical	***	***	NS[1]	(0.97)	(0.97)	(2.17)	(1.54)	(1.54)	(1.54)	(1.54)	(1.54)	(2.17)
Metal	1298	141.89	69.66	87.03^{a}	62.20^{b}	84.80^{a}	87.70^{a}	79.94^{b}	70.36^{c}	70.65^{c}	64.43^{d}	84.80^{a}
chelation	***	***	***	(0.49)	(0.49)	(1.09)	(0.77)	(0.77)	(0.77)	().77)	(0.77)	(1.09)
Hydroxyl	18.14	24.56	16.41	9.37^{b}	3.50^{c}	74.30^{a}	0.00^{d}	17.46^{b}	10.56^{bc}	4.13^{cd}	0.00^{d}	74.30^{a}
radical	***	***	***	(0.97)	90.97)	92.16)	(1.53)	(1.53)	(1.53)	(1.53)	(1.53)	(2.16)
Superoxide	0.001	25.69	0.91	17.55	17.59	71.37	0.00^{c}	24.38^{b}	21.06^{b}	22.31^{b}	20.13^{b}	71.37^{a}
Radical	NS	***	NS	(1.24)	(1.24)	(2.78)	(1.96)	(1.96)	(1.96)	(1.96)	(1.96)	(2.78)

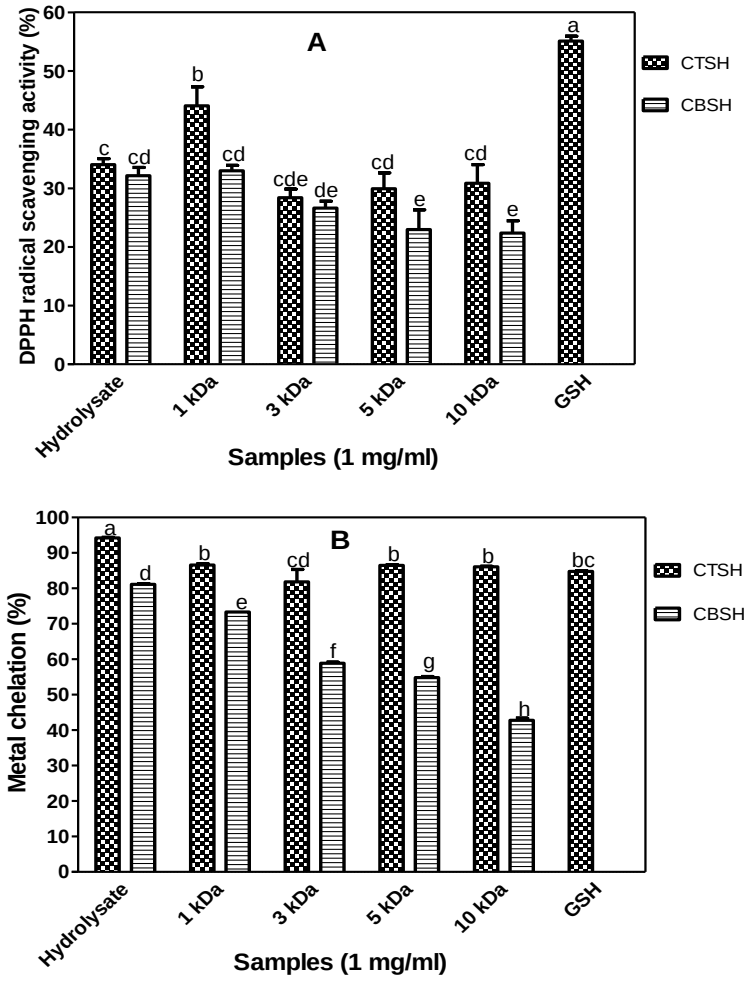
ORAC	28.14	145.41	3.95	3497[a]	3180[b]	535[c]	3440[b]	4386[a]	3632[b]	30.43[c]	2190[d]	535[e]
	***	***	*	(42.28)	(42.28)	(94.54)	(66.85)	(66.85)	(66.85)	(66.85)	(66.85)	(94.54)

[a,b,c]Mean intensity values (followed in brackets by the standard error of the mean) within the same variable "muscle type" and " membrane cut-off size in KDa" with the same letter within the same row (parameter) are not significantly different (P < 0.05)

GSH: Glutathione

[1]NS: Not significant at P ≥ 0.05, *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 4.1. Percentage (mean ± standard deviation) DPPH radical scavenging activity (A) and metal ion chelating activity (B) of chicken skin protein hydrolysates and membrane fractions determined at 1 mg/ml sample concentration. CTSH: chicken thigh skin protein hydrolysate from 3% alcalase digestion; CBSH: chicken breast skin protein hydrolysate from 1% pepsin + pancreatin digestion. Bars with different alphabets have mean values that are significantly different ($p < 0.05$).

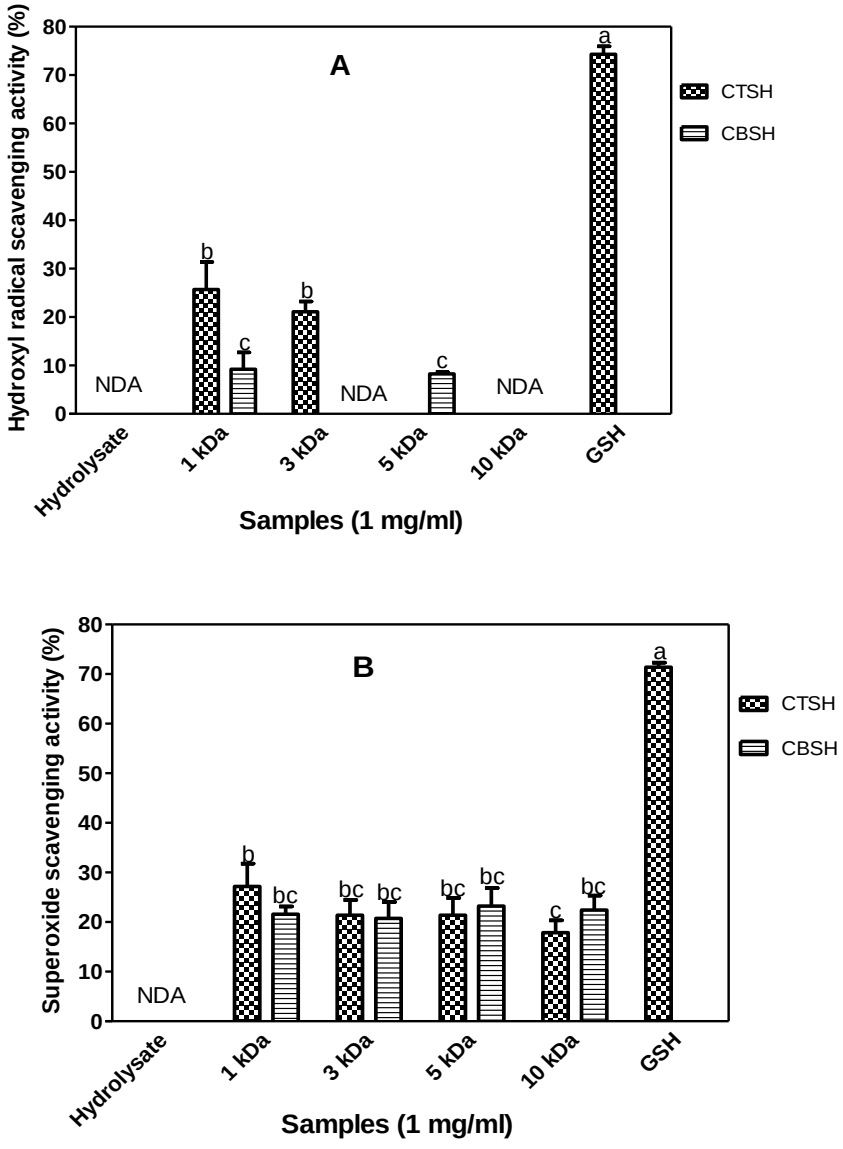


contributed to the higher activity of the hydrolysates; these effects were reduced when the peptides were separated into different fractions by membrane ultrafiltration. The results suggest that CSPH and its membrane ultrafiltration fractions have the potential to be used as antioxidants against transition metal ion-mediated oxidative stress. Metal ion chelation by peptide can also play an important role in the prevention of hydroxyl radical formation, a compound that is very reactive and destructive to cellular tissues (Huang et al., 2005).

4.3.3. Hydroxyl and superoxide radical scavenging activities

Hydroxyl radical is generated from the reaction between H_2O_2 and metal ions such as Fe (II) or Cu (II) in the Fenton reaction, and is believed to be an extremely reactive and short-lived species with the destructive ability to hydroxylate DNA, proteins, and lipids (Girgih et al., 2010; Huang et al., 2005; Jamdar et al., 2012). It can also react with aromatic compounds to form hydroxycyclohexadienyl radical which undergoes further reactions with oxygen to form peroxyl radical or decompose to phenoxyl radical (Jamdar et al., 2012). The scavenging of hydroxyl radical is therefore imperative for protection against various diseases caused by the oxidative stress induced by this radical (Girgih et al., 2010; Huang et al., 2005; Naqash & Nazeer, 2011). Hydroxyl radical scavenging activity of all the CSPHs produced under different hydrolysis conditions is shown in Table 4.2. The hydrolysates exhibited a very weak hydroxyl radical scavenging activity, which may be due to a low ratio of hydroxyl radical scavenging to non-radical scavenging peptides. The hydroxyl radical scavenging activity of CSPH was significantly affected by the muscle skin type, enzyme type as well as enzyme concentration. The CBSH (B) had significantly ($p < 0.05$) higher hydroxyl radical scavenging activity than the CTSH (T) which may be attributed to differences in susceptibility to proteolysis. The alcalase-hydrolysed samples also had significantly ($p < 0.05$) higher hydroxyl radical scavenging activity than the

Fig. 4.2. Percentage (mean ± standard deviation) hydroxyl radical scavenging activity (A) and superoxide radical scavenging activity (B) of chicken skin protein hydrolysates and membrane fractions determined at 1 mg/ml sample concentration. CTSH: chicken thigh skin protein hydrolysate from 3% alcalase digestion; CBSH: chicken breast skin protein hydrolysate from 1% pepsin + pancreatin digestion. Bars with different alphabets have mean values that are significantly different ($p < 0.05$).



PP-hydrolysed samples, suggesting that proteolytic activities of the enzymes used may have generated peptides with differences in scavenging ability. The hydroxyl radical scavenging activities of the CSPHs were also lower when compared to the activity obtained for GSH (Table 4.2). The hydroxyl radical scavenging activities of the membrane fractions are shown in Table 4.3 and Fig. 4.2A. Membrane fractionated peptides also had poor hydroxyl radical scavenging activity, which was significantly affected by the muscle type and membrane size used (Table 4.3). The results also show that the CTSH (T) membrane fractions had significantly ($p < 0.05$) higher hydroxyl radical scavenging activity than the CBSH (B) membrane fractions. The hydroxyl radical scavenging activities of CSPH membrane fractions decreased with increasing peptide size, indicating that lower molecular sized peptides are better hydroxyl radical scavengers than higher molecular sized peptides. This also suggests a very small ratio of hydroxyl radical scavenging peptides to non-scavenging peptides in the samples (Girgih et al., 2010). The results are in agreement with those reported by Jamdar et al. (2012) who showed that smaller peptides exhibited better hydroxyl radical scavenging activity than higher molecular weight peptides.

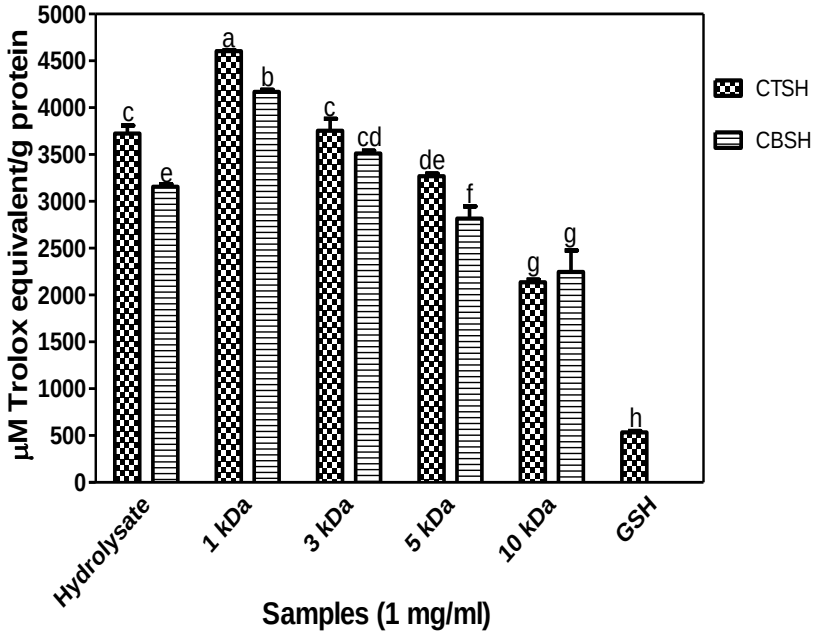
The superoxide radical is a highly toxic radical species that is generated by numerous biological reactions and even though they cannot initiate lipid peroxidation directly, they are the precursors of other highly reactive species such as hydrogen peroxide and hydroxyl radical (Jamdar et al., 2012). Living cells have an endowed biological defense mechanism of enzymatic antioxidants responsible for the conversion of reactive oxygen species/reactive nitrogen species (ROS/NOS) to harmless molecules. One such system is the conversion of superoxide anion (O2) to oxygen (O_2) and H_2O_2 by superoxide dismutase (SOD) (Huang et al., 2005). However, the body's ability to scavenge this radical may be overwhelmed by excessive production of free

radicals. The ability of CSPHs and its membrane fractions to scavenge superoxide radical is shown in Table 4.3 and Fig. 4.2B. The hydrolysates exhibited no superoxide radical scavenging activity. However, the membrane fractions showed some moderate superoxide radical scavenging activity which was significantly affected by the membrane size but not the muscle skin type. Therefore, it is possible that membrane separation of the protein hydrolysates led to the isolation of active peptides that can scavenge superoxide radical. The results also revealed that GSH had significantly ($p < 0.05$) higher superoxide radical scavenging activity than all the membrane fractions. The values obtained in this work for superoxide radical scavenging activities of the peptides are lower than the values previously reported by Jamdar et al. (2012) for poultry viscera protein hydrolysate, which were obtained from a higher peptide concentration of 2 mg/ml sample.

4.3.4. Oxygen radical absorbance capacity (ORAC)

The ORAC assay tests a compound's capacity to act as an antioxidant by quenching peroxyl radicals (ROO), which is assessed using fluorescence decay curve of the sample in comparison to a blank with no antioxidant; the results are normally reported as Trolox equivalent (Sheih, Wub, & Fang, 2009). Fig. 4.3 shows that the CSPHs had a high peroxyl radical scavenging activity against fluorescein with ORAC values of 3723 and 3155 lM TE/g for CTSH and CBSH, respectively. The CSPH ORAC values were significantly affected by the muscle skin type with the CTSH (T) having higher values than the CBSH (B) as shown in Table 4.3. Membrane fractionation showed that the smaller peptide sizes (1 and 3 kDa permeates) had significantly ($p < 0.05$) higher ORAC values than the bigger peptides present in the 5 and 10 kDa permeates (Table 4.3 and Fig. 4.3). The higher ORAC values of the small peptides may be due to

Fig. 4.3. Percentage (mean ± standard deviation) oxygen radical absorption capacity (ORAC) of chicken skin protein hydrolysates and membrane fractions determined at 1 mg/ml sample concentration. CTSH: chicken thigh skin protein hydrolysate from 3% alcalase digestion; CBSH: chicken breast skin protein hydrolysate from 1% pepsin + pancreatin digestion. Bars with different alphabets have mean values that are significantly different ($p < 0.05$).



increased ability to interact with and donate electrons to the free radical when compared to peptides that may have reduced ability to interact with the free radical. Since low molecular weight peptides are known to be more readily bioavailable and effective in the intestine (Sheih et al., 2009), the higher ORAC value of the 1 kDa peptides suggests a higher potential as an antioxidant when compared to the bigger size peptides.

Comparatively, the chicken skin protein hydrolysates differ greatly in their scavenging abilities against the different antioxidant radicals tests. The hydrolysates had very high chelating activity against metal ions, high scavenging of peroxyl radicals (ORAC) and moderate scavenging activity against DPPH radicals. These activities increased with increasing enzyme concentration. However, the scavenging activity against hydroxyl radicals by the hydrolysates was very poor while the hydrolysates exhibited no scavenging activity against superoxide radicals. For the membrane fractions, the samples exhibited very high scavenging of peroxyl radicals (ORAC) and chelating of metal ions and moderate scavenging of DPPH and superoxide radicals. These also showed a general trend of increasing activity with decreasing peptide size. The hydroxyl radical scavenging activity of the samples on the other hand was very weak. As stated earlier, these differences in the scavenging abilities of the chicken skin protein hydrolysates and its membrane fractions may be due to the peptide size, the degree of hydrolysis and the amino acid composition (He et al., 2013; Jamdar et al., 2012; Onuh et al., 2013). It may also be due to differences in substrates, reaction conditions and methods used (Huang et al., 2005). However, the samples had a general or similar pattern of increasing scavenging activities against the antioxidant radicals with increasing enzyme concentration and also decreasing peptide size.

4.4. Conclusion

This study has shown that CSPHs and its membrane ultrafiltration fractions can scavenge reactive oxygen species during in vitro studies. This is because these peptide products exhibited varying abilities to scavenge or quench DPPH, hydroxyl, peroxyl and superoxide radicals as well as chelate metal ions. The effectiveness of CSPHs was shown to be dependent on the source of the chicken skin (breast or thigh), type of protease used during chicken skin protein digestion (alcalase was better) as well as molecular size of the peptides (1 kDa permeate peptides had the best antioxidant activities). The CBSH exhibited slighted better free radical scavenging activities whereas upon membrane ultrafiltration, the CTSH fractions showed better antioxidant activities. Therefore, the CSPHs or 1 kDa permeate may be suitable ingredients for the formulation of functional foods and nutraceuticals that can potentially be used to prevent or manage diseases associated with oxidative stress. Further studies that involve peptide purification, amino sequencing of active peptides and in vivo studies, however need to be done to determine bioavailability, potency and safety.

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4.5 References

Aluko, R. E., & Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. Journal of Food Science, 68, 1254–1258.

Babji, A. S., Chin, S. Y., Chempaka, M. Y. S., & Alina, A. R. (1998). Quality of mechanically deboned chicken meat frankfurter incorporated with chicken skin. International Journal of Food Sciences and Nutrition, 49, 19–326.

Batista, I., Ramos, C., Coutinho, J., Bandarra, N. M., & Nunes, M. I. (2009). Characterisation of protein hydrolysates and lipids obtained from black scabbard fish (*Aphanopus carbo*) by-products and antioxidative activity of the hydrolysates produced. Process Biochemistry, 45, 18–24.

Bernardini, R. D., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., et al. (2011). Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. Food Chemistry, 124, 1296–1307.

Bhat, Z. F., Kumar, P., & Kumar, S. (2011). Effect of skin, enrobing and refrigerated storage on the quality characteristics of chicken meat balls. Journal of Food Science and Technology, 1–10.

Bidlingmeyer, B., Cohen, S., & Tarvin, T. (1984). Rapid analysis of amino acids using pre-column derivatization. Journal of Chromatography, 336, 93–104.

Biswas, S., Chakraborty, A., Sarkar, S., Barpuzari, R. N., & Barpuzari, T. (2007). Effect of incorporation of chicken fat and skin on the quality of chicken sausages. The Journal Poultry Science, 44, 111–115.

Bonifer, L. B., & Froning, G. W. (1996). Chicken skin composition as affected by aqueous washing. Journal of Food Science, 61, 895–898.

Cliche, S., Amiot, J., & Gariepy, C. (2003). Extraction and characterization of collagen with or without telopeptides from chicken skin. Poultry Science, 82, 503–509.

de Avellar, I. G., Magalhaes, M. M., Silva, A. B., Souza, L. L., Leitao, A. C., & Hermes- Lima, M. (2004). Reevaluating the role of 1,10-phenanthroline in oxidative reactions involving ferrous ions and DNA damage. Biochimica et Biophysica Acta, 1675, 46–53.

Erdmann, K., Cheung, B. W. Y., & Schroder, H. (2008). The possible roles of food derived bioactive peptides in reducing the risk of cardiovascular disease. Journal of Nutritional Biochemistry, 19, 643–654.

Feddern, V., Kupski, L., Cipolatti, E. P., Giacobbo, G., Mendes, G. L., Furlong, E. B., et al. (2010). Physico-chemical composition, fractionated glycerides and fatty acid profile of chicken fat. European Journal of Lipid Science and Technology, 112, 1277–1284.

Gehrke, C., Wall, L., Absheer, J., Kaiser, F., & Zumwalt, R. (1985). Sample preparation for chromatography of amino acids: Acid hydrolysis of proteins. Journal of the Association of Official Analytical Chemists, 68, 811–821.

Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2010). In vitro antioxidant properties of hemp seed (Cannabis sativa L.) protein hydrolysate fractions. Journal of the American Oil Chemists Society, 88, 381–389.

He, R., Girgih, A. T., Malomo, S. A., Ju, X., & Aluko, R. E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. Journal of Functional Foods, 5, 219–227.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, 53, 1841–1856.

Jamdar, S. N., Rajalakshmi, V., & Sharma, A. (2012). Antioxidant and ACE inhibitory properties of poultry viscera protein hydrolysate and its peptide fractions. Journal of Food Biochemistry, 36, 494–501.

Landryl, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. Journal of Agricultural and Food Chemistry, 40, 776–779.

Lee, S. H., Qian, Z. J., & Kim, S. K. (2010). A novel Angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chemistry, 118, 96–102.

Markwell, M. A. C., Haas, S. M., Biebar, L. L., & Tolbert, N. E. (1978). A modification of lowry procedure to simplify protein determination in membrane and protein samples. Anal. Biochem, 87, 206–211.

Martinez-Maqueda, D., Miralles, B., Recio, I., & Hernandez-Ledesma, B. (2012). Antihypertensive peptides from food proteins: A review. Food and Function, 3, 350–361.

Naqash, S. Y., & Nazeer, R. A. (2011). Evaluation of bioactive peptides isolated from Exocoetus volitans backbone. International Journal of Food Science and Technology, 46, 37–43.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. Food Research International, 53, 260–267.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: Meat and fish. Nutrients, 3, 765–791.

Samanarayaka, A. G. P., Kitts, D. D., & Li-Chan, E. C. Y. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a pacific hake (Merluccius productus) fish protein hydrolysate subjected to simulated gastrointestinal digestion and caco-2 cell permeation. Journal of Agricultural and Food Chemistry, 58, 1535–1542.

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. Journal of Functional Foods, 3, 229–254.

Sheih, I. C., Wub, T. K., & Fang, T. J. (2009). Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. Bioresource Technology, 100, 3419–3425.

Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. International Journal of Molecular Sciences, 12, 3148–3161.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: Production, processing and potential health benefits. Journal of Food Science, 71, R11–R24.

Udenigwe, C. C., Lu, Y. L., Han, C. H., Hou, W. C., & Aluko, R. E. (2009). Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. Food Chemistry, 116, 277–284.

Wu, H. C., Chen, H. M., & Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austiasicus). Food Research International, 36, 949–957.

Xie, Z., Huang, J., Xu, X., & Jin, Z. (2008). Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. Food Chemistry, 111, 370–376.

You, S.-J., Udenigwe, C. C., Aluko, R. E., & Wu, J. (2010). Multifunctional peptides from egg white lysozyme. Food Research International, 43, 848–855.

TRANSITION STATEMENT TWO

This study demonstrated the ability of chicken skin protein hydrolysates and its membrane ultrafiltration fractions to scavenge free radical species (DPPH, hydroxyl, peroxyl and superoxide radicals) as well as chelate metal ions during *in vitro* studies. This is an indication that they may be suitable ingredients for the formulation of functional foods and nutraceuticals for the prevention and management of oxidative stress-induced diseases. These two initial studies established the potential ability of chicken skin protein hydrolysates and their ultrafiltration membrane fractions as antihypertensive and antioxidative agents. However, the ability of the chicken skin protein hydrolysates and ultrafiltration membrane fractions to lower systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) needs to be established. Some bioactive peptides are known to undergo *in vivo* structural changes within the gastrointestinal tract after oral administration with potential change in bioactivity. Moreover, the kinetics of enzyme inhibition by the chicken skin protein hydrolysates against ACE and renin needs to be determined. The third manuscript therefore, evaluated kinetics studies of ACE and renin enzymes inhibition as well as the blood pressure lowering effects of chicken skin protein hydrolysates and their membrane fractions in SHR during acute oral gavage feeding.

CHAPTER 5

MANUSCRIPT THREE

KINETICS OF *IN VITRO* RENIN AND ANGIOTENSIN CONVERTING ENZYME INHIBITION BY CHICKEN SKIN PROTEIN HYDROLYSATES AND THEIR BLOOD PRESSURE LOWERING EFFECTS IN SPONTANEOUSLY HYPERTENSIVE RATS

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5.0 Abstract

Chicken skin protein hydrolysates (CSPHs) and their membrane ultrafiltration fractions were investigated for their blood pressure lowering effects. Chicken skins from thigh or breast muscles were hydrolyzed with 3% Alcalase or 1% pepsin/pancreatin, respectively; hydrolysates were then fractionated into <1, 1 – 3, 3 – 5, and 5 – 10 kDa peptide sizes. Chicken breast skin hydrolysate (CBSH) and peptide fractions inhibited angiotensin converting enzyme (ACE) with IC_{50} values of 0.36 – 0.64 mg/ml, which were significantly ($p<0.05$) higher than the 0.42 – 0.75 mg/ml values obtained for chicken thigh skin hydrolysate (CTSH) and peptide fractions. Results suggest that a high content of branched-chain amino acids contributed to the significantly ($p<0.05$) higher ACE-inhibition by <1 kDa peptide fraction. All the samples with exception of the CBSH 3 kDa fraction had similar renin inhibitory values. Kinetics studies revealed that enzyme inhibition pattern was mostly of the mixed-type for ACE and renin. Oral administration of 100 mg peptides/kg body weight to spontaneously hypertensive rats led to maximum systolic blood pressure reduction of –32.67 and –31.33 mmHg after 6 h for CTSH and CBSH, respectively. We conclude that the CSPHs have potential use as active ingredients to formulate antihypertensive functional foods and nutraceuticals.

Keywords: Chicken skin; protein hydrolysates; renin; angiotensin converting enzyme; antihypertensive peptides; spontaneously hypertensive rats

5.1 Introduction

Hypertension (high blood pressure) has been reported to be one of the primary risk factors for cardiovascular risk events, myocardial infarction, heart failure, stroke, vascular dementia, end stage renal disease and premature death (Erdmann, Cheung, & Schroder, 2008). It is estimated that hypertension affects one third of the world's population, especially in the developed and industrialized economies and is rapidly becoming a major problem in developing countries (Barbana & Boye, 2011; Daien et al., 2012; Onuh, Girgih, Aluko, & Aliani, 2013). Though the cause of essential (primary) hypertension remains unknown, the renin-angiotensin system (RAS) has been reported to play vital roles in the progression of cardiovascular and chronic renal diseases (Erdmann et al., 2008; Hou, Chen, & Lin, 2003; Segall, Covic, & Goldsmith, 2007; Udenigwe, Lin, Hou, & Aluko, 2009). Among the several processes and reactions involved in the RAS pathway, renin and angiotensin-I-converting enzyme (ACE) appear to be the two principal enzymes responsible for regulation of blood pressure (Barbana & Boye, 2011; Udenigwe et al., 2009). In this pathway, renin is actively involved in catalyzing the initial and rate-limiting step by converting angiotensinogen to angiotensin-I, which is subsequently converted to the potent vasoconstrictor, angiotensin-II by ACE (Barbana & Boye, 2011; Martinez-Maqueda, Miralles, Recio, & Hernandez-Ledesma, 2012; Udenigwe et al., 2009). ACE is also responsible for the degradation of the vasodilator, bradykinin (Barbana & Boye, 2011; Segall et al., 2007). It is, therefore, believed that by inhibiting ACE activity, the formation of angiotensin-II and destruction of bradykinin would have equally been blocked or suppressed, consequently contributing to lowering of blood pressure (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). More importantly is the direct inhibition of renin activity which has been reported to completely block the RAS pathway since renin is known to catalyze the rate-limiting step in RAS (Fitzgerald, 2011).

Several synthetic antihypertensive compounds (e.g., captopril, alacepril, lisinopril, enalapril and fosinopril) developed as ACE inhibitors have long been used to inhibit RAS and treat hypertension including other associated cardiovascular disease conditions (Ajibola, Eleyinmi, & Aluko, 2011; Erdmann et al., 2008; Samanarayaka, Kitts, & Li-Chan, 2010). Aliskiren is however, the only available drug therapy for use as renin inhibitor (He, Malomo, Girgih, Ju, & Auko, 2013). However, their use has generated some safety concerns owing to their undesirable side effects like cough, skin rash, edema and pain, necessitating the search for a safer alternative (Je, Park, Kwon, & Kim, 2004). In contrast, food protein-derived peptides are generating attention among the scientific community for their health promoting potentials and potentially reduced negative side effects (Barbana & Boye, 2011). This has resulted in the development of renin and ACE inhibitory peptides following enzymatic hydrolysis of proteins from several plant and animal sources such as fish (Je et al., 2004), poultry (Jamdar, Rajalakshmi, & Sharma, 2012), collagen (Saiga, Iwai, Hayakawa, Takahata, Kitamura, Nishimura, et al., 2008) , lentils (Barbana & Boye, 2011), pea (Li & Aluko, 2010), and hemp seed (Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011).

Chicken skin protein can be enzymatically hydrolysed after drying and fat extraction to yield peptides with antioxidant properties (Onuh, Girgih, Aluko, & Aliani, 2014). We also recently reported that chicken skin protein hydrolysates and membrane fractions had in vitro inhibitory activities against renin and ACE (Onuh et al., 2013). However, there is a paucity of information on the ability of the chicken skin hydrolysates and membrane fractions to lower systolic blood pressure in spontaneously hypertensive rats (SHR). Also, the kinetics of enzyme inhibition of the chicken skin protein hydrolysates against ACE and renin has not been reported. Therefore, the objective of this study was to determine *in vitro* kinetics of enzyme inhibition as well as the

blood pressure lowering effects of chicken skin protein hydrolysates and their membrane fractions after oral administration to SHR.

5.2 Materials and methods

5.2.1. Materials

Chicken skins from the thigh and breast muscles used for this study were supplied by Granny's Poultry (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas), alcalase (from fermentation of *Bacillus licheniformis, E.C.* 3.4.21.62), ACE (from rabbit lung, EC 3.4.15.1), trinitrobenzene sulfonic acid (TNBS), N-(3-[2-furyl] acryloyl)-phenylalanylglycylglycine (FAPGG) and sodium dodecyl sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). All other analytical grade reagents and ultrafiltration membranes (1, 3, 5 and 10 kDa molecular weight cut-offs) were purchased from Fisher Scientific (Oakville, ON, Canada).

5.2.2. Preparation of chicken skin protein hydrolysates (CSPH) and membrane fractions

Chicken skin protein hydrolysates were prepared as described in our previous work (Onuh et al., 2013). Fresh thigh or breast chicken skins (approximately 250g) were packed in freeze-drying plates, frozen at –20^{o}C for 24 h and transferred to – 80^{o}C for 6 h prior to freeze-drying. The freeze-dried samples were thereafter manually shredded and defatted repeatedly by mixing ~1 g with 10 mL of acetone. The mixture was stirred in the fume hood for 3 h and decanted manually followed by two additional consecutive extractions of the residue. The defatted skin samples were then air dried overnight in the fume hood chamber at room temperature and subsequently milled with a Waring blender to produce a fine powder that was stored at –20^{o}C. Dried chicken skin powder from the thigh or breast muscles was mixed with

water to give 5% (w/w protein basis) slurries. Two different enzyme treatments (3% alcalase or 1% pepsin + pancreatin) were separately used for sample hydrolysis based on our previous work (Onuh et al., 2013). For the alcalase hydrolysis, the slurry was heated to 55 ^{o}C, adjusted to pH 8.0 using 2 M NaOH and hydrolysis initiated by addition of 3% (w/w, skin protein basis) enzyme; each mixture was stirred continuously for 4 h. For the pepsin+pancreatin (PP) hydrolysis, the slurry was heated to 37^{o}C, adjusted to pH 2.0 using 2 M HCl and the reaction initiated with the addition of 1% (w/w, skin protein basis) pepsin enzyme; the mixture was then stirred continuously for 2 h. After peptic hydrolysis, the reaction mixture was adjusted to pH 7.5 with 2 M NaOH, 1% (w/w, skin protein basis) pancreatin was added and incubated at 37^{o}C for 4 h with continuous stirring. At the end of the incubation period, both the alcalase and PP reactions were terminated by heating the slurry to 95^{o}C for 15 min to ensure complete denaturation of residual enzymes. The mixtures were thereafter centrifuged (7,000 x *g* at 4^{o}C) for 1 h and the resulting supernatant lyophilized and stored at –20^{o}C until needed for further analysis. The most active hydrolysate from each enzyme treatment was subsequently fractionated by sequentially passing the supernatant through ultrafiltration membranes with molecular weight cut-offs (MWCO) of 1, 3, 5 and 10 kDa in an Amicon stirred ultrafiltration cell. Starting with 1 kDa MWCO, the retentate from each membrane was passed through the next higher MWCO membrane while permeates from each membrane (1, 3, 5, and 10 kDa MWCO) was collected, lyophilized and stored at–20^{o}C as <1, 1-3, 3-5, and 5-10 kDa fractions, respectively, until required for analysis. Protein content of the lyophilized CSPHs was determined by the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978). The above digestion and fractionation protocols were performed in triplicates and the lyophilized samples combined, analyzed for protein content and used for the renin and ACE inhibition assays.

5.2.3. Amino acid composition analysis

The amino acid profiles of the chicken skin protein hydrolysates from the thigh and breast muscles (CTSH and CBSH) as well as the various ultrafiltration fractions (<1 kDa, 1 – 3 kDa, 3 – 5 kDa and 5 – 10 kDa) samples were determined using the HPLC system after samples were hydrolyzed with 6 M HCl according to the method of (Bidlingmeyer, Cohen, & Tarvin, 1984). The cysteine and methionine contents were determined after performic acid oxidation according to the method of (Gehrke, Wall, Absheer, Kaiser, & Zumwalt, 1985). The tryptophan content however, was determined after alkaline hydrolysis by the method of (Landry & Delhaye, 1992).

5.2.4 Analysis of molecular weight distribution

Molecular weight distribution of CSPH peptides was determined according to He, Alashi, Malomo, Girgih, Chao, Ju, et al. (2013) using an AKTA FPLC system (GE Healthcare, Montreal, PQ) equipped with a Superdex Peptide12 10/300 GL column 154 (10 x 300 mm), and UV detector (λ = 214 nm). An aliquot (100 µL) of the sample (5 mg/mL in 50 mM phosphate buffer, pH 7.0 containing 0.15 M NaCl) was loaded onto the column and elution was performed at room temperature using the phosphate buffer at a flow rate of 0.5 mL/min. The column was calibrated with the following standard proteins and an amino acid: cytochrome C (12,384 Da); aprotinin (6,512 Da); vitamin B_{12} (1,855 Da); and glycine (75 Da). The molecular weight (MW) of peptide peaks in samples was estimated from a linear plot of log MW versus elution volume of standards.

5.2.5. ACE-inhibition assay

The ACE-inhibitory activity of CSPH and membrane fractions was determined as previously reported (Udenigwe et al., 2009). A 1 mL aliquot of 0.5 mM N-[3-(2-Furyl)acryloyl]-

L-phenylalanyl-glycyl-glycine (FAPGG) (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 μL ACE (final activity of 20 mU) and 200 μL of 1mg/mL sample (CSPH or membrane fractions in 50 mM Tris–HCl buffer). The rate of decrease in absorbance at 345 nm due to ACE-catalyzed cleavage of the Phe-Gly bond of the FAPGG was recorded for 2 min at room temperature on a varian 50 BIO UV-visible spectrophotometer (Varian, Australia). Tris–HCl buffer was used instead of peptide sample in the blank experiment. ACE activity was expressed as rate of disappearance of FAPGG (ΔA/min) and inhibitory activity was calculated as:

ACE inhibition (%) = [$\Delta Amin^{-1}$ (blank) – $\Delta Amin^{-1}$ (sample)/$\Delta Amin^{-1}$ (blank)] x 100

Where ($\Delta Amin^{-1}$ (sample) and ($\Delta Amin^{-1}$ (blank) are ACE activity in the presence and absence of CSPH or membrane fractions, respectively. The inhibitor concentration (IC_{50}) of peptide fractions that inhibited ACE activity by 50% was calculated using a non-linear regression from a plot of percentage ACE inhibition versus five peptide concentrations (0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml). The kinetics of ACE inhibition was determined using four concentrations (0.0625, 0.125, 0.25 and 0.5 mM) of FAPGG to obtain the Lineweaver-Burk plots. The inhibition constant (K_i) was calculated as the x-axis intercept from a plot of the slope of the Lineweaver-Burk lines against peptide concentrations.

5.2.6. Renin-inhibition assay

Renin inhibition assay was carried out according to the method of (Li & Aluko, 2010) using the Renin Inhibitor Screening Assay Kit. Renin buffer was diluted with 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl prior to the determinations. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37^{o}C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini,

Molecular Devices, Sunnyvale, CA) maintained at 37^{o}C. Before the reaction, (1) 20 µL substrate, 160 µL assay buffer, and 10 µL Milli-Q water were added to the background wells; (2) 20 µL substrate, 150 µL assay buffer, and 10 µL Milli-Q water were added to the blank wells; and (3) 20 µL substrate, 150 µL assay buffer, and 10 µL 1 mg/mL sample were added to the inhibitor wells. The reaction was initiated by adding 10 µL renin to the control and sample wells. The microplate was shaken for 10 s to mix, incubated at 37^{o}C for 15 min, and fluorescence intensity (FI) recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm on a Spectra Max Gemini microplate spectrophotometer (Sunnyvale, CA, USA). The percentage renin inhibition was calculated as follows:

Renin inhibition (%) = [(FI of blank well - FI of sample well)/FI of blank well] x 100.

The inhibitor concentration (IC_{50}) of peptide fractions that inhibited renin activity by 50% was calculated using a non-linear regression from a plot of percentage renin inhibition versus five peptide concentrations (0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml). The renin inhibition kinetics studied were determined using varying substrate concentrations (0.625, 1.25, 2.5, 5 and 10 µM substrate) in the absence and presence of peptides. The mode of inhibition and Ki values were determinedas described above for ACE.

5.2.7 Evaluation of blood pressure lowering effects in SHR

Animal studies were conducted according to the Canadian Council on Animal Care ethics guidelines with a protocol approved by the University of Manitoba Animal Protocol and Management Review Committee. Male SHRs purchased from Charles Rivers Laboratories (Montreal, PQ, Canada) were individually housed in steel cages in a room maintained at 25^{o}C and relative humidity of 50% under a 12 h day and night at the Richardson Center for Functional Foods and Nutraceuticals. The rats were fed regular laboratory diet (chow) and tap water ad

libitum. At 20 weeks of age and body weight (BW) of 330 – 370 g, the rats were divided into groups with 4 rats per treatment. CSPHs and membrane fractions as well as undigested chicken skin protein were dissolved in 1 mL phosphate buffered saline (PBS) at a dose of 100 mg/kg BW. Captopril (used for blood pressure control) was administered at a dose of 10 mg/kg BW as a positive control while PBS (1 mL) was administered as a negative control. The samples were administered to the rats by oral gavage and the systolic blood pressure (SBP) measured at 2, 4, 6, 8 and 24 h using the tail-cuff plethysmography method (Mouse Rat Tail Cuff Blood Pressure System, IITC Life Science, Woodland Hills, CA, USA) in slightly anaesthetized rats as previously described (Aukema, Gauthier, Roy, Jia, Li, & Aluko, 2011).

5.2.8. Statistical analyses

All results obtained were expressed as means of triplicate values for statistical analysis. The data were subjected to one way analysis of variance (ANOVA) while Tukey's multiple comparison tests was used to determine mean treatment differences when significant ($p < 0.05$). An IBM SPSS Statistical package (version 20) was used for all statistical analyses.

5.3 Results and discussion

5.3.1. Amino acid composition

As expected with poultry products, the hydrolysates and their membrane fractions are high in aspartic acid, glutamic acid, proline, glycine, alanine and arginine contents (Table 5.1). Aspartic acid and glutamic acid as well as their amines (negatively charged amino acids, NCAA) are known to be strong electron donors, which could facilitate ionic interactions with metal cations present at the active site of enzymes (He, Girgih, Malomo, Ju, & Aluko, 2013; Samaranayaka & Li-Chan, 2011). With the exception of the CBSH <1 kDa peptide fraction, the NCAA contents were similar in all the samples. The content of the hydrophobic amino acids

(HAA) is high in the samples and they are also known to enhance interactions with lipid especially the cell lipid layer and enhance entry of peptides into cells. The arginine content of the samples is also very high, especially in the CBSH <1 kDa fraction in which the amount is almost doubled relative to the other samples (15.57%). Arginine has been reported to play an important vasodilative role as it acts as a precursor for the production of proline and glutamate, promotes nitric oxide (NO) synthesis and reduce the risk of heart diseases (Jablecka, Checinski, Krauss, Micker, & Ast, 2004; Udenigwe, Adebiyi, Doyen, Li, Bazinet, & Aluko, 2012). The results showed substantial decreases in aromatic amino acid (AAA) content after separation of the hydrolysate into peptide fractions, which suggests that the AAAs are present in all the peptide sizes. However, the AAA content tended to decrease as peptide size increased, which indicates that the smaller size peptides (especially <1 kDa) contained more AAAs than the bigger size peptides (1-10 kDa). Positively charged amino acids (PCAA) content varied mostly for the CBSH where peptide separation led to increased content in the <1 and 1-3 kDa peptides; thus the smaller peptides were richer in PCAA when compared to the bigger peptides. With the exception of the <1 kDa peptides, the samples had essential amino acids (EAA) contents lower than the recommended ~31.4% for human nutrition (FAO/WHO, 1991). Therefore, apart from their potential use as blood pressure-reducing agents, the chicken skin protein hydrolysates and >1 kDa peptides may not be able to support adequate human growth if provided as the sole dietary source of proteins. The results are not surprising since chicken skins have a poor nutritional

Table 5.1- Percentage amino acid composition of chicken thigh skin protein hydrolysates (CTSH), chicken breast skin protein hydrolysates (CBSH) and membrane fractions.

Amino Acids	CBSH	CBSH <1 kDa	CBSH 1-3 kDa	CBSH 3-5 kDa	CBSH 5-10 kDa	CTSH	CTSH <1 kDa	CTSH 1-3 kDa	CTSH 3-5 kDa	CTSH 5-10 kDa
Asx	9.16	6.31	8.45	9.12	8.65	9.05	7.21	8.74	9.70	9.20
Thr	3.34	2.93	3.17	3.04	2.81	3.47	3.81	3.27	2.95	2.69
Ser	4.09	4.21	4.27	4.17	3.83	4.26	5.29	4.34	4.00	3.69
Glx	13.27	11.44	12.85	13.18	12.30	13.21	14.64	13.25	13.02	11.44
Pro	11.27	3.92	9.91	12.32	14.52	10.52	5.42	10.45	12.98	14.27
Gly	11.27	6.09	12.36	14.79	18.21	14.61	8.07	12.51	14.53	17.56
Ala	7.76	6.16	7.78	8.37	8.59	7.39	8.89	8.03	7.72	7.84
Cys	0.73	0.25	0.38	0.55	0.53	0.97	0.66	0.95	1.07	0.83
Val	3.64	4.13	4.33	3.76	3.64	3.80	4.86	4.22	3.78	3.50
Met	1.29	2.24	1.89	1.77	1.43	1.68	2.92	2.06	1.48	1.35
Ile	2.61	2.89	2.97	2.79	2.52	2.65	3.54	3.05	2.61	2.25
Leu	5.45	9.14	6.31	5.47	4.82	5.81	8.36	6.16	5.19	4.61
Tyr	2.11	5.82	2.75	1.83	1.40	2.33	3.81	2.52	1.92	1.50
Phe	3.15	6.87	3.75	3.09	2.57	3.25	4.75	3.44	2.94	2.65
His	2.22	2.93	2.17	1.78	1.64	2.83	3.24	2.32	1.95	1.91
Lys	5.67	8.11	6.60	5.90	5.47	5.79	6.11	6.28	6.00	5.81
Arg	7.92	15.57	9.65	7.62	6.86	7.89	7.55	7.86	7.74	8.57

Trp	0.37	0.99	0.42	0.43	0.22	0.48	0.87	0.56	0.42	0.34
HAA	37.11	40.17	38.59	38.62	38.80	37.19	41.16	39.37	38.63	37.80
PCAA	15.81	26.61	18.42	15.30	13.97	16.51	16.90	16.46	15.69	16.29
NCAA	22.43	17.75	21.30	22.30	20.95	22.27	21.85	21.99	22.73	20.63
AAA	5.64	0.99	0.42	0.43	0.22	6.05	0.87	0.56	0.42	0.34
BCAA	11.70	16.16	13.61	12.02	10.98	12.26	16.76	13.43	11.58	10.36
EAA	28.87	37.29	29.43	26.25	23.47	30.39	35.23	29.04	25.36	23.20

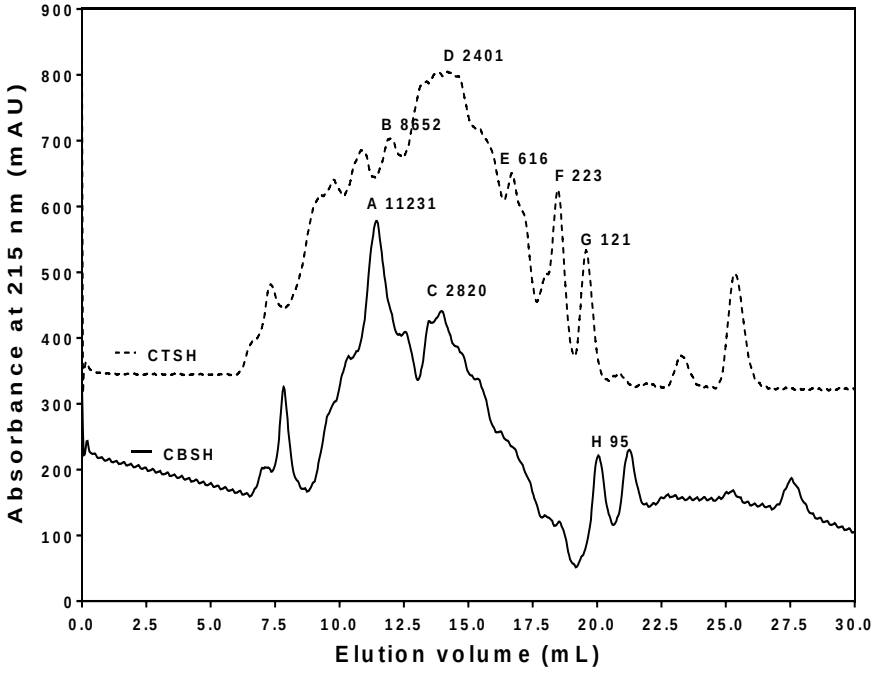
***Asx, aspartic acid + asparagine; Glx, glutamic acid + glutamine. HAA, hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine, cysteine). BCAA, branched-chain amino acids (valine, leucine, isoleucine). PCAA, positively charged amino acids (arginine, histidine, lysine). NCAA, negatively charged amino acids (Asx and Glx). AAA, aromatic amino acids (phenylalanine, tryptophan, tyrosine). EAA, essential amino acids (phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine).**

quality in terms of essential amino acids composition. Level of branched chain amino acids (BCAAs) increased when the chicken hydrolysates were fractionated into <1 kDa peptides but then decreased as peptide size increased (Table 5.1). This is important because the presence of BCAAs has been suggested to enhance ACE-inhibitory and potentially the antihypertensive properties of food protein-derived peptides (Aluko, 2008). Similar results that showed increased BCAA content in the <1 kDa fraction has been reported for kidney bean protein hydrolysates (Mundi & Aluko, 2014).

5.3.2. Molecular size distribution of chicken skin protein hydrolysates

Fig. 5.1 shows that alcalase hydrolyzed CTSH had a higher number of peaks in the 120-2400 Da than the CBSH produced from PP digestion. This differences may be because of the higher level of alcalase (3%) used for CTSH when compared to the 1% used for the CBSH. As already shown in our previous work, the respective enzyme concentrations were the optimized levels that produced protein hydrolysates with the highest bioactive potential (Onuh et al., 2013). The CTSH hydrolyzed with alcalase contained peptides in the molecular weight range of 121 – 8652 while the CBSH hydrolyzed with pepsin-pancreatin contained peptides in the molecular range of 95 – 11231 Da. It is however important to note that majority of the peptides are low molecular weight peptides, especially in the range of 220 – 3000 Da as evidenced in the peaks C, D, E and F. Low molecular weight (LMW) peptides are known to be more potent inhibitors of ACE and renin as opposed to high molecular weight peptides (Barbana & Boye, 2011; Onuh et al., 2013; Saiga, et al., 2008; Udenigwe & Aluko, 2010).

Fig. 5.1- Gel-permeation chromatograms of chicken skin protein hydrolysates after passage through a Superdex Peptide12 10/300 GL column. Column was calibrated with Cytochrome C (12,384 Da), Aprotinin (6512 Da), Vitamin B12 (1855 Da), and Glycine (75 Da).

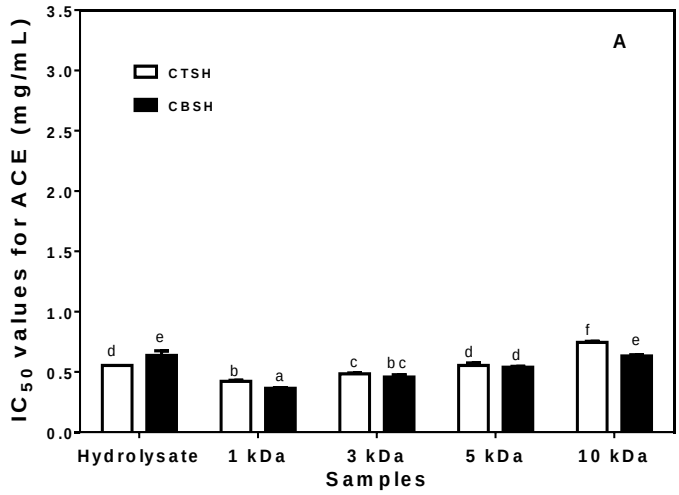


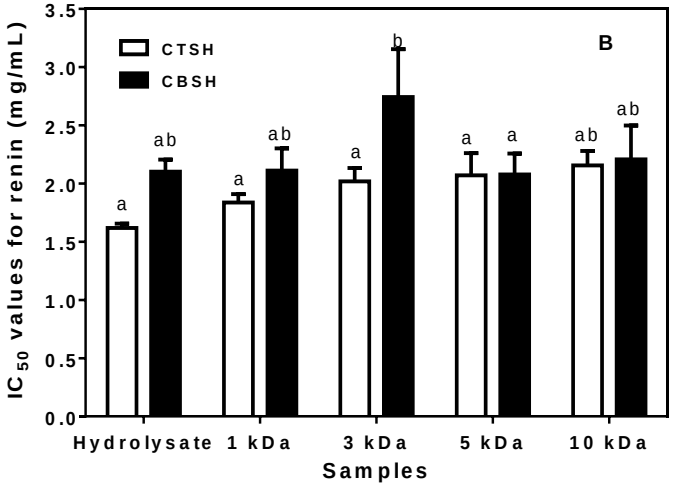
5.3.3. ACE and renin inhibitory concentrations

Fig. 5.2A shows that the IC_{50} value for ACE inhibition by CBSH is significantly higher ($p < 0.05$) than that of CTSH with a value of 0.64 mg/ml and 0.55 mg/ml for CBSH and CTSH respectively, suggesting that CTSH is slightly more potent than CBSH. This may be due to a higher degree of hydrolysis as reported in our previous study (Onuh et al., 2013), which enhanced the amount of low molecular weight peptides in CTSH than in CBSH as revealed in Fig. 5.1 by size exclusion chromatography. Fractionation using membrane ultrafiltration significantly improved the potency of the peptides as indicated by lower IC_{50} values of some of the peptide fractions, especially those that are <3 kDa in size. The IC_{50} values are 0.42, 0.48, 0.55, and 0.75 mg/ml for the CTSH <1, 1 – 3, 3 – 5 and 5 – 10 kDa fractions, respectively. For CBSH, the <1, 1 – 3, 3 – 5 and 5 – 10 kDa peptide fractions had IC_{50} values of 0.36, 0.46, 0.54, and 0.63 mg/ml, respectively. The IC_{50} values for ACE inhibition increased with increasing peptide molecular size, which is in agreement with previous studies that have suggested higher ACE-inhibitory potency of LMW peptides when compared to high molecular weight peptides (Girgih et al., 2011; He, et al., 2013; Jamdar et al., 2012; Udenigwe et al., 2009). The results are also consistent with the contents of BCAA, which increased in the <1 kDa fraction(least IC_{50} value); a gradual decrease in BCAA content as peptide molecular size increased was accompanied by increased IC_{50} values (reduced peptide potency).

There was little influence of peptide size on renin inhibition though the CTSH tended to be slightly more active than the CBSH (Fig. 5.2B). However, the CTSH with a higher content of LMW peptides had slightly higher renin-inhibitory ability when compared to the CBSH.

Fig. 5.2- Inhibitory concentrations of chicken skin protein hydrolysates and membrane fractions derived peptides that reduced 50% activity (IC_{50}) of ACE (A) and renin (B). CTSH- chicken thigh skin protein hydrolysates from 3% alcalase digestion; CBSH- chicken breast skin protein hydrolysates from 1% pepsin–pancreatin digestion. Bars with different alphabets have mean values that are significantly different ($p \leq 0.05$).



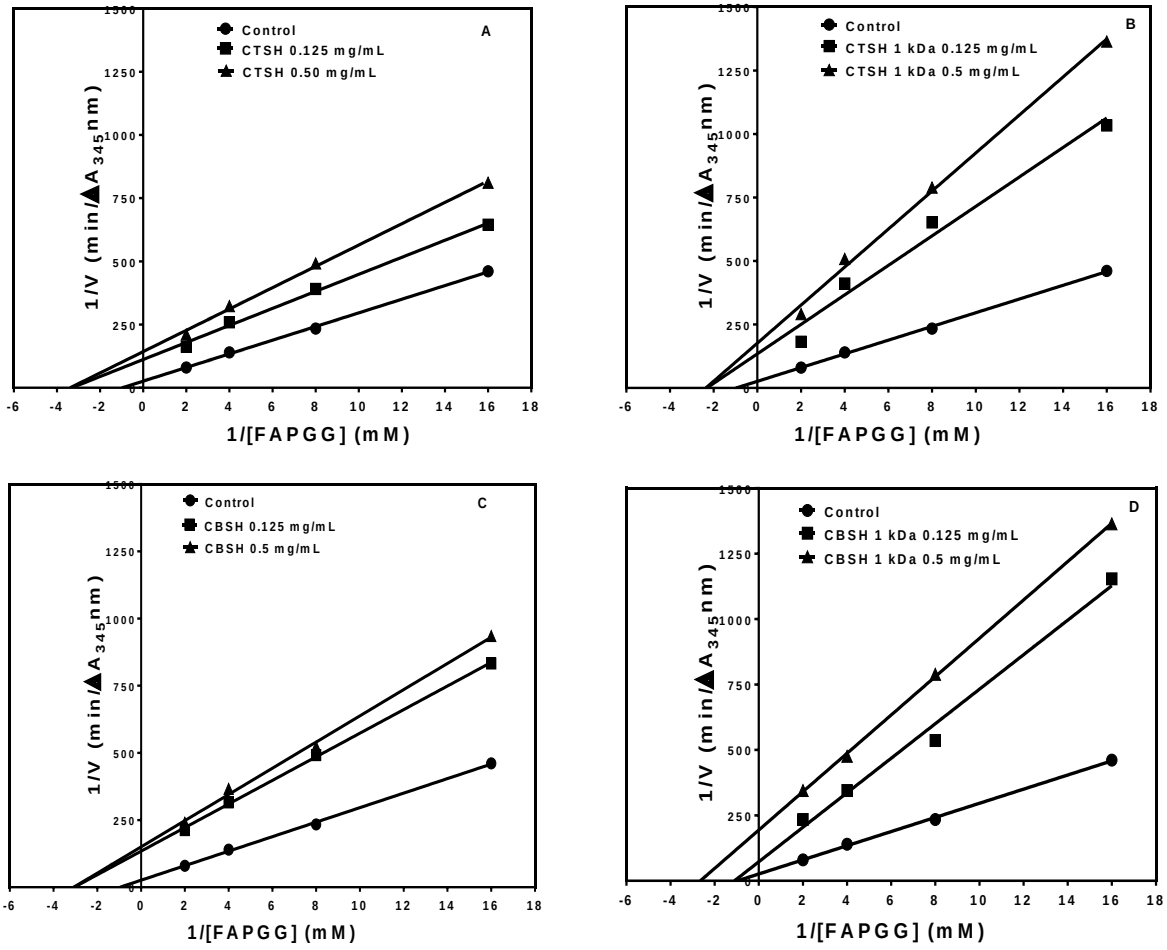


Membrane fractionation led to reduced peptide potency as shown by the increases in renin IC_{50} values. The results suggest a synergistic effect of peptides in the unfractionated hydrolysates; this synergy was reduced as a result of peptide separation as previously shown for hemp seed peptides (Girgih et al., 2011). Thus, it can be inferred that unlike ACE inhibition, the peptide-dependent inhibition of renin activity is due to the presence of small and big peptides. Overall, the peptides were more potent against ACE as shown by the lower IC_{50} values when compared to the higher values for renin inhibition. The results are consistent with previous reports that have shown that ACE activity is more readily inhibited than renin activity by most food protein hydrolysates (Girgih et al., 2011). However, pancreatin hydrolysate of canola was shown to produce similar inhibitions of ACE and renin activities (Alashi, Blanchard, Mailer, Agboola, Mawson, He, et al., 2014).

5.3.4 Kinetics of enzyme inhibition

The mode of inhibition of ACE and renin enzymes were studied based on the initial results of the inhibitory concentrations of CSPH by conducting kinetic studies of enzyme inhibition both in the absence and presence of the CSPH samples. This could provide information that will assist in understanding the ability of the inhibitor to effectively inhibit the actions of enzymes as well as the quantity of inhibitor needed either for the reaction to proceed or for the enzyme activities to be inhibited (Barbana & Boye, 2011; Girgih et al., 2011; Udenigwe et al., 2009). Lineweaver-Burk plots for ACE inhibition by different peptide concentrations are shown in Fig. 5.3 (A, B, C and D), which indicate a mostly mixed-type pattern. Therefore, the results suggest that the peptides can possibly bind to the active sites of the enzymes as well as to ACE protein molecule at sites other than the FAPGG binding site; the peptides were bound to both the free enzyme as well as the enzyme- substrate complex to reduce

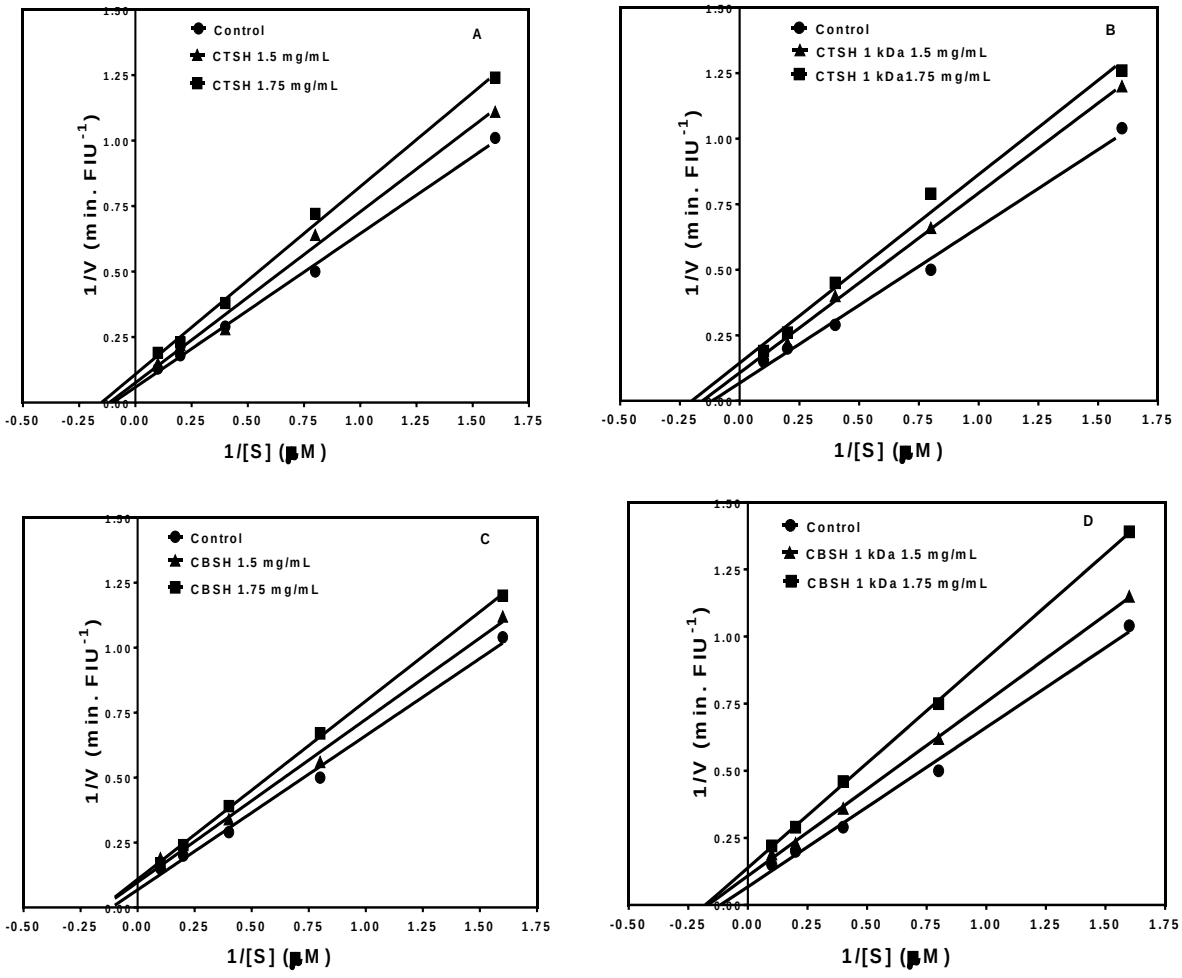
Fig. 5.3 - Lineweaver-Burk plots of the inhibition of ACE by different concentrations of: (A) chicken thigh skin hydrolysate, CTSH; (B) chicken thigh skin hydrolysate, <1 kDa peptide fraction; (C) chicken breast skin hydrolysate, CBSH; (D) chicken breast skin hydrolysate, <1 kDa peptide fraction.



catalysis rate. Peptide binding to the enzyme active site will prevent substrate binding whereas when the inhibitor binds to non-active sites, it will result in changes in the enzyme protein conformation which could reduce ACE-substrate interactions. The enzyme inhibition patterns showed increase slope of the <1 kDa peptide inhibited reactions when compared to the unfractionated hydrolysates. Higher slope values indicate reduced reaction rates, which is consistent with the higher ACE inhibitory activity values (lower IC_{50} values) shown for the <1 kDa fraction (Fig. 5.2A). The inhibition constant (K_i), is defined as the ability of the inhibitor to bind to the enzyme in other to form enzyme-inhibitor complex. The K_i values for ACE inhibition of the CTSH, CTSH <1 kDa, CBSH and CBSH <1 kDa are 0.044, 0.022, 0.077 and 0.051 mg/ml respectively. The Ki data confirms that the <1 kDa peptides bind tighter to ACE when compared to binding of and as such are more potent ACE inhibitors than the unfractionated hydrolysates (CTSH and CBSH) . The lower Ki value of CTSH is also consistent with the higher ACE-inhibitory property when compared to CBSH as shown in Fig. 5.2A.

Lineweaver-Burk plots of renin inhibition also showed mixed-type pattern (Fig. 5.4A, C, D) with the exception of the CTSH <1 kDa fraction that showed an uncompetitive mode of inhibition (Fig. 5.4B). The results suggest that <1 kDa peptides were bound only to the renin-substrate complex, which led to reduce product formation rate. In contrast the unfractionated hydrolysates and CBSH <1 kDa were bound to both the free enzyme as well as the enzyme-substrate complex. Unlike the ACE inhibition patterns, Fig. 5.4 shows minimal differences between the various peptide samples, which is consistent with data presented in Fig. 5.3B. The K_i values for renin inhibition are comparatively higher than for ACE inhibition ranging from 10.03, 7.55, 4.17 and 1.60 mg/mL for CTSH, CTSH <1 kDa, CBSH and CBSH <1 kDa

Fig. 5.4- Lineweaver-Burk plots of the inhibition of renin by different concentrations of: (A) chicken thigh skin hydrolysates, CTSH; (B) chicken thigh skin hydrolysate, <1 kDa peptide fraction; (C) chicken breast skin hydrolysate, CBSH; (D) chicken breast skin hydrolysate, <1 kDa peptide fraction.



respectively. This is also consistent with the results for the inhibitory properties of the CSPHs. This is also similar to the K_i values of 2.45 – 11.25 mg/ml reported for hempseed protein hydrolysates (Girgih et al., 2011).

5.3.5 Blood pressure lowering activity of enzymatic CSPH

The blood pressure-lowering activity of enzymatic CSPH and membrane fractions was confirmed by oral administration of the samples to SHR. Fig. 5.5 shows that CTSH significantly reduced SBP in SHR better than all the peptide fractions as well as the unhydrolyzed chicken thigh skin (CTS) at 2, 4, 6, 8 and 24 h after oral administration. The CTSH produced a decrease of –18 mmHg 2 h after administration with maximum decrease of – 30 mmHg after 4 and 8 h. At 24 h after oral administration of CTSH, the SBP lowering was –16 mmHg, which was still significant (better than saline) and similar to captopril's effect (–14 mmHg), though the drug was administered at 10 mg/kg body weight. In contrast, the maximum SBP-lowering effect for CTS was -12 mmHg after 6 h and <5 mmHg after 24 h. The CTSH also exerted better SBP-lowering effects compared to oyster protein hyrolysates which had maximum SBP-lowering effect of 20, 18, 16 and 10 mmHg at 2, 4, 6, and 8 h respectively after administration to SHRs (Wang et al., 2008).

Fig. 5.6 shows similar SBP-lowering effect of the CBSH with values of –22, –28, –31, –18 and –12 mmHg at 2, 4, 6, 8 and 24 h after oral administration. The unhydrolyzed chicken breast skin (CBS) also produced least SBP-lowering effects with a maximum of results -14 mmHg after 6 h and <5 mmHg after 24 h. The unfractionated hydrolysates showed fast acting properties considering the substantial SBP-lowering effects immediately of –18 and –22 mmHg for CTSH and CBSH, respectively just 2 h after oral administration. The results suggest that

Fig. 5.5- Systolic blood pressure lowering effects of unhydrolyzed chicken thigh skin meal, chicken thigh skin protein hydrolysate and membrane fractions in spontaneously hypertensive rats after oral administration of 100 mg/kg body weight. CTS – defatted chicken thigh skin meal, and CTSH- chicken thigh skin protein hydrolysate from 3% alcalase digestion. Bars with different alphabets have mean values that are significantly different ($p \leq 0.05$).

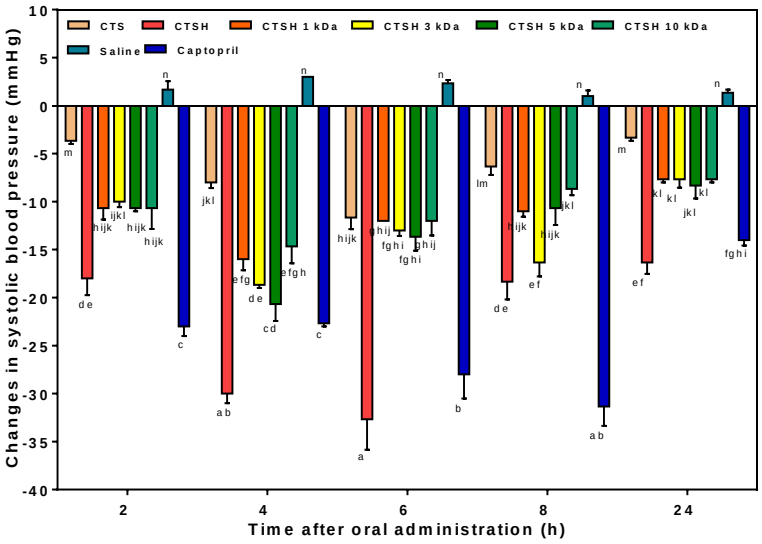
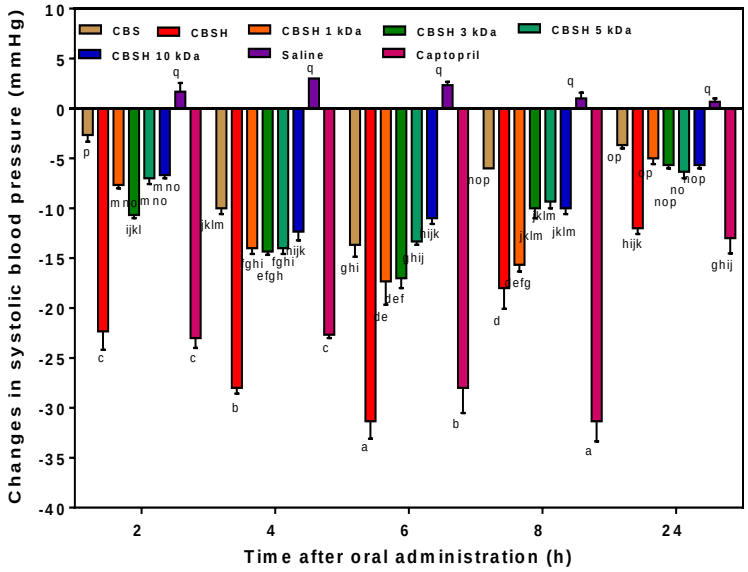


Fig. 5.6- Systolic blood pressure lowering effects of unhydrolyzed chicken breast skin meal, chicken breast skin protein hydrolysate and membrane fractions in spontaneously hypertensive rats after oral administration of 100 mg/kg body weight. CBS – defatted chicken breast skin meal, and CBSH- chicken breast skin protein hydrolysate from 1% pepsin+pancreatin digestion. Bars with different alphabets have mean values that are significantly different ($p \leq 0.05$).



CSPH may be used on a short-term basis to offer fast and efficient remedy in hypertensive individuals as previously suggested for an arginine-containing flaxseed protein hydrolysate (Udenigwe et al., 2012). In addition, the observed longer SBP-lowering effects suggest an efficient absorption of the peptides from the digestive tract coupled with longer peptide persistence in the blood. Separation of the CTSH and CBSH led to almost 50% reduction in SBP-lowering efficacy of the peptide fractions (Fig. 5.5 & 5.6). The observed differences in SBP-lowering effects between the hydrolysates and their peptide fractions may be due to a loss of synergistic effects that contributed to the strong antihypertensive properties of CTSH and CBSH, which is similar to the result reported for hemp seed protein hydrolysates and peptide fractions (Girgih et al., 2011). The SBP results are inconsistent with the *in vitro* data which showed higher ACE inhibition by the fractionated peptides (Fig. 5.2A). But the results tend to be consistent with data in Fig. 5.2B, which showed reduced renin inhibition when the CTSH and CBSH were fractionated. Therefore, it is reasonable to propose that the synergistic antihypertensive activity observed for CTSH and CBSH may be due mainly to *in vivo* regulation of renin activity and less of ACE inhibition. We can also assume that the strong ACE-inhibitory peptides in the fractions as observed during *in vitro* tests underwent *in vivo* structural changes (usually enzymatic cleavage) after oral administration to yield less effective ACE inhibitors, which will be consistent with 'substrate' classification (Fujita, Yokoyama, & Yoshikawa, 2000). The observed low SBP-lowering effect of the unhydrolyzed chicken protein after oral administration when compared to the hydrolysates and peptide fractions may be due to the fact that the parent proteins need long time periods to be broken down by the enzymes of the digestive system prior to absorption (Udenigwe et al., 2012), hence the ingested skin yielded lower amounts of antihypertensive peptides. These results demonstrate the potential of CSPH to

be used as ingredients in the formulation of functional foods and nutraceuticals for the control and management of hypertension.

5.4 Conclusions

Results of this study have clearly demonstrated that enzymatic hydrolysates of chicken skin protein possess strong *in vitro* inhibitory activities against ACE and renin. The *in vitro* data suggest that short chain peptides have stronger ACE inhibition potency than long chain peptides. In contrast membrane fractionation seems to reduce peptide synergistic effect and hence reduced *in vitro* renin inhibition. However, unfractionated hydrolysate was more effective in reducing SBP of SHR when compared to the peptide fractions. Thus additional processing is not required subsequent to enzymatic hydrolysis of chicken skin for producing potent antihypertensive hydrolysate; this could contribute to reduced product cost upon commercialization. The results confirm that the antihypertensive effects of chicken skin are not dependent on the animal part from where the skin was harvested. Thus, our work could enhance value-added utilization of chicken skin, which is currently mainly disposed as a waste product by the poultry industry. Long-term studies are required to determine lasting effects of the hydrolysates on SBP and if any adverse effects are associated with oral consumption.

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5.5 References

Ajibola, C. F., Eleyinmi, A. F., & Aluko, R. E. (2011). Kinetics of the Inhibition of Renin and Angiotensin I Converting Enzyme by Polar and Non-polar Polyphenolic Extracts of

Vernonia Amygdalina and Gongronema Latifolium Leaves. *Plant Foods for Human Nutrition, 66*(4), 320-327.

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., Malomo, S. A., Girgih, A. T., & Aluko, R. E. (2014). Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats. *Food Research International, 55*, 7.

Aluko, R. E. (2008). Antihypertensive properties of plant-derived inhibitors of angiotensin I-converting enzyme activity: a review. *Recent Prog Med Plants, 22*, 21.

Aukema, H. M., Gauthier, J., Roy, M., Jia, Y., Li, H., & Aluko, R. E. (2011). Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. *Molecular Nutrition & Food Research, 55*(7), 1044-1051.

Barbana, C., & Boye, J. I. (2011). Angiotensin I-converting enzyme inhibitory properties of lentil protein hydrolysates: Determination of the kinetics of inhibition. *Food Chemistry, 127*, 94-101.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid Analysis of Amino-Acids Using Pre-Column Derivatization. *Journal of Chromatography, 336*(1), 93-104.

Daien, V., Duny, Y., Ribstein, J., du Cailar, G., Mimran, A., Villain, M., Daures, J. P., & Fesler, P. (2012). Treatment of Hypertension With Renin-Angiotensin System Inhibitors and Renal Dysfunction: A Systematic Review and Meta-Analysis. *American Journal of Hypertension, 25*(1), 126-132.

Erdmann, K., Cheung, B. W. Y., & Schroder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry, 19*(10), 643-654.

FAO/WHO. (1991). Protein quality evaluation in human diets. Report of Joint FAO/WHO Expert Consultation, FAO Food and Nutrition paper 51. FAO, Rome, Italy.

Fitzgerald, M. A. (2011). Hypertension treatment update: Focus on direct renin inhibition. *Journal of the American Academy of Nurse Practitioners, 23*(5), 239-248.

Fujita, H., Yokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science, 65*(4), 564-569.

Gehrke, C. W., Wall, L. L., Absheer, J. S., Kaiser, F. E., & Zumwalt, R. W. (1985). Sample Preparation for Chromatography of Amino-Acids - Acid-Hydrolysis of Proteins. *Journal of the Association of Official Analytical Chemists, 68*(5), 811-821.

Girgih, A. T., Udenigwe, C. C., Li, H., Adebiyi, A. P., & Aluko, R. E. (2011). Kinetics of Enzyme Inhibition and Antihypertensive Effects of Hemp Seed (Cannabis sativa L.) Protein Hydrolysates. *Journal of the American Oil Chemists Society, 88*(11), 1767-1774.

He, R., Alashi, A., Malomo, S. A., Girgih, A. T., Chao, D. F., Ju, X. R., & Aluko, R. E. (2013). Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chemistry, 141*(1), 153-159.

He, R., Girgih, A. T., Malomo, S. A., Ju, X. R., & Aluko, R. E. (2013). Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods, 5*, 219-227.

He, R., Malomo, S. A., Girgih, A. T., Ju, X. R., & Auko, R. E. (2013). Glycinyl-Histidinyl-Serine (GHS), a Novel Rapeseed Protein-Derived Peptide Has Blood Pressure-Lowering Effect in Spontaneously Hypertensive Rats. *Journal of Agricultural and Food Chemistry, 61*(35), 8396-8402.

Hou, W. C., Chen, H., & Lin, Y. H. (2003). Antioxidant peptides with angiotensin converting enzyme inhibitory activities and applications for angiotensin converting enzyme purification. *Journal of Agricultural and Food Chemistry, 51*(6), 1706-1709.

Jablecka, A., Checinski, P., Krauss, H., Micker, M., & Ast, J. (2004). The influence of two different doses of L-arginine oral supplementation on nitric oxide (NO) concentration and total antioxidant status (TAS) in atherosclerotic patients. *Medical Science Monitor, 10*(1), Cr29-Cr32.

Jamdar, S. N., Rajalakshmi, V., & Sharma, A. (2012). Antioxidant and Ace Inhibitory Properties of Poultry Viscera Protein Hydrolysate and Its Peptide Fractions. *Journal of Food Biochemistry, 36*(4), 494-501.

Je, J. Y., Park, P. J., Kwon, J. Y., & Kim, S. K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (Theragra chalcogramma) frame protein hydrolysate. *Journal of Agricultural and Food Chemistry, 52*(26), 7842-7845.

Landry, J., & Delhaye, S. (1992). Simplified Procedure for the Determination of Tryptophan of Foods and Feedstuffs from Barytic Hydrolysis. *Journal of Agricultural and Food Chemistry, 40*(5), 776-779.

Li, H. A., & Aluko, R. F. (2010). Identification and Inhibitory Properties of Multifunctional Peptides from Pea Protein Hydrolysate. *Journal of Agricultural and Food Chemistry, 58*(21), 11471-11476.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). Modification of Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Analytical Biochemistry, 87*(1), 206-210.

Martinez-Maqueda, D., Miralles, B., Recio, I., & Hernandez-Ledesma, B. (2012). Antihypertensive peptides from food proteins: a review. *Food & Function, 3*(4), 350-361.

Mundi, S., & Aluko, R. E. (2014). Inhibitory properties of kidney bean protein hydrolysate and its membrane fractions against renin, angiotensin converting enzyme, and free radicals. *Austin Journal of Nutrition and Food Science, 2*(1).

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Research International, 53*(1), 260-267.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2014). In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry, 150*, 366-373.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive Peptides from Muscle Sources: Meat and Fish. *Nutrients, 3*(9), 765-791.

Saiga, A., Iwai, K., Hayakawa, T., Takahata, Y., Kitamura, S., Nishimura, T., & Morimatsu, F. (2008). Angiotensin I-Converting Enzyme-inhibitory Peptides Obtained from Chicken Collagen Hydrolysate. *Journal of Agricultural and Food Chemistry, 56*(20), 9586-9591.

Samanarayaka, A. C. P., Kitts, D. D., & Li-Chan, E. C. Y. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a pacific hake (Merluccius productus) fish protein hydrolysates subjected to simulated gastrointestinal digestion and caco-2 cell permeation. *Journal of Agricultural and Food Chemistry, 58*, 1535 - 1542.

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of Functional Foods, 3*, 229-254.

Segall, L., Covic, A., & Goldsmith, D. J. A. (2007). Direct renin inhibitors: the dawn of a new era, or just a variation on a theme? *Nephrology Dialysis Transplantation, 22*(9), 2435-2439.

Udenigwe, C. C., Adebiyi, A. P., Doyen, A., Li, H., Bazinet, L., & Aluko, R. E. (2012). Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. *Food Chemistry, 132*(1), 468-475.

Udenigwe, C. C., & Aluko, R. E. (2010). Antioxidant and Angiotensin Converting Enzyme-Inhibitory Properties of a Flaxseed Protein-Derived High Fischer Ratio Peptide Mixture. *Journal of Agricultural and Food Chemistry, 58*(8), 4762-4768.

Udenigwe, C. C., Lin, Y. S., Hou, W. C., & Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*(2), 199-207.

Wang, J., Hu, J., Cui, J., Bai, X., Dua, Y., Miyaguchi, Y., & Lin, B. (2008). Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysate in spontaneously hypertensive rats. *Food Chemistry, 111*, 7.

TRANSITION STATEMENT THREE

This study is a further demonstration that enzymatic hydrolysates of chicken skin proteins possess strong *in vitro* inhibitory activities against ACE and renin as well as *in vivo* blood pressure lowering ability in SHRs suggesting that it can be used as an antihypertensive agent to treat and manage hypertension. The study also showed that unfractionated chicken skin protein hydrolysate was more effective in reducing SBP of SHR when compared to the peptide fractions, therefore requiring no further purification processes during commercialization. However, the ability of chicken skin protein hydrolysates to regulate blood pressure in SHRs on a long term basis is not known. The fourth manuscript evaluated the *in vivo* antihypertensive and antioxidant properties of enzymatic chicken skin protein hydrolysates in SHRs with established hypertension during a 6-week chronic feeding trial. The study demonstrated that dietary interventions using chicken skin protein hydrolysates in a chronic feeding trial for 6 weeks significantly lowered SBP in SHRs but have no hypotensive effects on normotensive WKY rats. This correlated with plasma ACE but not renin activity, suggesting the *in vivo* mechanism for blood pressure regulation by CSPHs to be probably due to down-regulation or inhibition of ACE activity. The diet intervention was well tolerated by the SHRs. There were corresponding reductions in the plasma antioxidant enzymes activities by the CSPHs intervention diets at termination compared to baseline. This suggests that the hydrolysates may have been unable to repair age-related irreversible oxidative stress induced damages at that chronic hypertensive state. However, the plasma TAC for the rats was shown to be high. The results confirm the potential of CSPHs to be used as ingredient in formulating functional foods and nutraceuticals in the prevention and treatment of hypertension.

CHAPTER 6

MANUSCRIPT FOUR

ENZYMATIC CHICKEN SKIN PROTEIN HYDROLYSATES REDUCED SYSTOLIC BLOOD PRESSURE VIA INHIBITION OF THE RENIN ANGIOTENSIN SYSTEM AND UPREGULATION OF ENDOGENEOUS ANTIOXIDANT ENZYMES

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6.0 Abstract

This study was aimed at investigating the long-term *in vivo* antihypertensive and antioxidant properties of chicken skin protein hydrolysates (CSPHs) in spontaneously hypertensive rats (SHRs) model. Chicken skin proteins from the thigh and breast muscles were hydrolyzed with either alcalase (3%) or a combination of pepsin/pancreatin (1%), lyophilized and combined in equal proportions to obtain CSPH. SHRs (n=29) rats were randomized into four groups to receive control, CSPH (0.5 and 1%, w/w of diet) and chicken skin meal (CSM 1%, w/w of diet) diets for 6 weeks. The normotensive Wistar Kyoto rats, WKY (n=18) rats were randomized into three groups to receive control, CSPH (1%, w/w of diet) and chicken skin meal (CSM 1%, w/w of diet) diets for 6 weeks. Urine and blood samples were collected at baseline followed by body weight (BW) and systolic blood pressure (SBP) measurements. Body weight, SBP and feed consumption of the rats were also measured weekly. At the end of the study, urine was collected, the rats terminated and blood and organ samples were collected, weighed and stored at -80^{o}C for analyses. The results showed that CSPHs significantly ($p<0.05$) lowered SBP in SHRs (-36, -31 and -26 mmHg for CSPH 1%, CSPH 0.5% and CSM 1% respectively) but had no hypotensive effect on WKY rats. SBP reduction was positively related to plasma ACE but not renin activity. Cumulative BW gain and feed efficiency ratio (FER) of rats were not significantly ($p>0.05$) affected. Though CSPHs significantly ($p<0.05$) reduced plasma antioxidant enzymes activities, the CSPH (0.5 and 1%) samples had less reduction in plasma superoxide dismutase (SOD) activity compared to the other samples. Plasma total antioxidant capacity (TAC) of the rats was shown to be high. Our results confirm the potential of CSPHs to be used as ingredient in formulating functional foods and nutraceuticals in the prevention and management of hypertension.

Keywords: Hydrolysates, Blood pressure, Plasma, hypertension, antioxidant enzymes

6.1 Introduction

There has been an increase of about 30% in the supply of poultry by-products such as organs and skins due to the ever increasing consumption of value-added chicken products (Cliche, Amiot, Avezard, & Gariepy, 2003). Associated with this reported increase in meat and poultry consumption is the generation of waste or under-utilized by-products including offal, bones, blood, skins, horns and hooves in an ever-increasing amount (Di Bernardini et al., 2011). It has therefore, become very important to find ways in which these residual by-products, which are also high in lipids and proteins, can be used or handled (Feddern et al., 2010). An area of research that is generating interest is the development of chicken skin protein hydrolysates and bioactive peptide fractions with functional and health promoting values that could be used especially to reduce symptoms of oxidative stress, hypertension and dyslipidemia which are all risk factors for coronary heart disease (Onuh, Girgih, Aluko, & Aliani, 2013, 2014; Samanarayaka, Kitts, & Li-Chan, 2010).

According to Ahhmed and Muguruma (2010), maintaining normal blood pressure is a physiological balance maintained by the nervous system and the kidneys. However, when these control mechanisms fail, due to a variety of factors, hypertension (high blood pressure) develops. Hypertension, a chronic condition defined by elevated arterial blood pressure at or above 140 mmHg for the systolic blood pressure (SBP) or 90 mmHg for the diastolic blood pressure (DBP), is a leading risk factor for the development of cardiovascular diseases (CVD) and death (Ahhmed & Muguruma, 2010; Erdmann, Cheung, & Schroder, 2008; Girgih, Alashi, He, Malomo, & Aluko, 2013; Onuh et al., 2013). CVD is arguably the single leading cause of death in the United States and other developed nations of the world. It has been projected by the World Health Organization (WHO) that heart disease and stroke will overtake infectious diseases as the

leading cause of death in the world by the year 2020 (Erdmann et al., 2008). Consequently, emphasis is being increasingly placed on improving the diet as well as lifestyle in order to minimize the risk of CVD related conditions, especially hypertension.

Hypertension often results from the loss of elasticity of the walls of the larger arteries which become rigid, creating less space for blood flow and accompanied by increased fluid pressure (Ahhmed & Muguruma, 2010). The renin-angiotensin system (RAS) has been reported to be a major regulator of cardiovascular and renal functions (Erdmann et al., 2008; Girgih et al., 2013; Schmieder, Hilgers, Schlaich, & Schmidt, 2007). Renin cleaves angiotensinogen to the inactive decapeptide (angiotensin-I), which is subsequently hydrolyzed by angiotensin-I-converting enzyme (ACE) to the potent vasoconstrictor, angiotensin-II (Girgih et al., 2013; Martinez-Maqueda, Miralles, Recio, & Hernandez-Ledesma, 2012; Raghavan & Kristinsson, 2009). ACE additionally cleaves bradykinin (a known potent vasodilator) to inactive fragments, which contributes to blood pressure elevation (Raghavan & Kristinsson, 2009). Normal operations RAS can exert strong antiatherosclerotic effects due to the resultant antihypertensive, anti-inflammatory, antiproliferative and antioxidative properties (Schmieder et al., 2007). However, excessive activities of RAS enzymes can result in deleterious effects such as increased blood pressure, and hypertension that leads to CVD complications and eventual death if not treated (Girgih et al., 2013). Therefore, the function of antihypertensive peptides is to lower blood pressure by attenuating the high levels of RAS enzymes, which relaxes the arteries and enhance vascular flexibility (Ahhmed & Muguruma, 2010; Erdmann et al., 2008; Schmieder et al., 2007).

Additionally, the generation of excessive free radical levels during cellular metabolism and respiration has become a major health concern. Oxidative stress, which involves increased production of reactive oxygen species (ROS) in combination with outstripping of endogeneous

antioxidant defense mechanisms, has been implicated in the initiation or progression of many vascular diseases (Erdmann et al., 2008; Vaziri, 2008). Uncontrolled production of free radicals (superoxide, hydroxyl, singlet oxygen etc) resulting from oxidative stress damages cellular molecules by oxidizing lipids, proteins, DNA and enzymes leading to terminal degenerative diseases such as CVD, diabetes, cancer, Alzheimer's disease, etc (Di Bernardini et al., 2011; Erdmann et al., 2008; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). They have also been found to modify low density lipoprotein (LDL) leading to increased atherogenicity of the oxidized LDL (Erdmann et al., 2008). Antioxidants, which have been defined as "substances that significantly decrease the adverse effects of reactive species on normal physiological functions" can play important roles in human health and nutrition as they are known to protect the body against ROS molecules (Di Bernardini et al., 2011; Ryan, Ross et al., 2011; Wu, Pan, Chang, & Shiau, 2005). A number of enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase help to exert some level of control over oxidative processes in the human body. However, the effect of endogenous antioxidants is often weakened when excess free radicals are formed beyond their cellular antioxidant capacity, which leads to cellular damage to lipids, proteins, DNA, and enzymes (Di Bernardini et al., 2011; Vaziri, 2008; Young & Woodside, 2001). Synthetic antioxidants such butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) can be used to control these oxidative processes. However, the use of natural antioxidants, especially bioactive peptides in foods is generating interest not only as natural alternatives to synthetic compounds but for their beneficial effects in terms of health implications and functionality in food systems (Centenaro, Mellado, & Prentice-Hernandez, 2011; Di Bernardini et al., 2011; Erdmann et al., 2008; Ryan et al., 2011).

Food-derived bioactive peptides with antihypertensive and/or antioxidant properties have been obtained from numerous food sources: fish and meat (Di Bernardini et al., 2011; Ryan et al., 2011), hempseed (Girgih et al., 2013), quinoa seed (Aluko & Monu, 2003), and rapeseed (He et al., 2013). Previous research works have demonstrated that chicken skin protein hydrolysates possess *in vitro* antihypertensive and antioxidant properties (Onuh et al., 2013, 2014). Preliminary short term (24 h) *in vivo* studies with spontaneously hypertensive rats (SHRs) also showed that oral administration of chicken skin protein hydrolysates (100 mg/kg body) lowered SBP (-31 mmHg after 6 h), which was positively correlated to the *in vitro* results (Onuh, Girgih, Malomo, Aluko & Aliani, 2015). However, there is scanty information on the ability of chicken skin protein hydrolysates to regulate blood pressure in SHRs on a long term basis. Therefore, the objective of this study was to determine the *in vivo* antihypertensive and antioxidant properties of enzymatic chicken skin protein hydrolysates in SHRs with established hypertension during a 6-week feeding trial.

6.2. Materials and methods

6.2.1. Materials

Chicken skins from the thigh and breast muscles used for this study were supplied by Granny's Poultry (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas), alcalase (from fermentation of *Bacillus licheniformis*, 3.4.21.62), ACE (from rabbit lung, EC 3.4.15.1), trinitrobenzene sulfonic acid (TNBS), N-(3-[2-furyl] acryloyl)-phenylalanylglycylglycine (FAPGG) and sodium dodecyl sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant Renin Inhibitor, Plasma total antioxidant capacity (TAC), superoxide dismutase activity (SOD), glutathione peroxide activity (GPx) and catalase activity (CAT) screening assay kit were purchased from Cayman Chemicals

(Ann Arbor, MI, USA). All other analytical grade reagents and ultrafiltration membranes (1, 3, 5 and 10 kDa molecular weight cut-offs) were purchased from Fisher Scientific (Oakville, ON, Canada).

6.2.2. Preparation of chicken skin protein hydrolysates (CSPH)

Chicken skin protein hydrolysates were prepared as described in our previous work (Onuh et al., 2013). Briefly fresh thigh or breast chicken skins (~250 g) were packed in freeze drying plates, frozen at –20 ^{o}C for 24 h and transferred to –80 ^{o}C for 6 h prior to freeze drying. The freeze dried samples were thereafter manually shredded and defatted repeatedly by mixing ~1 g with 10 mL of food grade acetone. The mixture was stirred in the fume hood for 3 h and decanted manually followed by two additional consecutive extractions of the residue. The defatted skin samples were then air dried overnight in the fume hood chamber at room temperature and subsequently milled with a Warring blender to produce a fine powder that was stored at – 20^{o}C. Dried chicken skin powder from the thigh or breast muscles were mixed with water to give 5% (w/v, protein basis) slurries. Two different enzyme treatments (alcalase or pepsin + pancreatin) were separately used for sample hydrolysis. For the alcalase hydrolysis, the slurry was heated to 55 ^{o}C, adjusted to pH 8.0 using 2 M NaOH and hydrolysis initiated by addition of enzyme (3% w/w, skin protein basis); each mixture was stirred continuously for 4 h. For the pepsin+pancreatin (PP) hydrolysis, the slurry was heated to 37 ^{o}C, adjusted to pH 2.0 using 2 M HCl and the reaction initiated with the addition of pepsin enzyme (1% w/w, skin protein basis); the mixture was then stirred continuously for 2 h. After peptic hydrolysis, the reaction mixture was adjusted to pH 7.5 with 2 M NaOH, pancreatin was added (1% w/w, skin protein basis) and incubated at 37 ^{o}C for 4 h with continuous stirring. At the end of the incubation period, both the alcalase and PP reactions were terminated by heating the slurry to 95

^{o}C for 15 min to ensure complete denaturation of residual enzymes. The mixtures were thereafter centrifuged (7,000 x g at 4 ^{o}C) for 1 h and the resulting supernatant lyophilized and stored at –20 ^{o}C until needed for further analysis and diet formulation. Protein content of the lyophilized CSPHs was determined by the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978). The above digestion protocols were performed in triplicates and the lyophilized samples combined, analyzed for protein content and used for all the assays. Chicken thigh skin hydrolysates hydrolyzed with alcalase and chicken breast skin hydrolysates hydrolyzed with pepsin-pancreatin were combined in equal proportions based on their protein content and used in formulation of the animal experimental diets. The combined use was based on our previous short-term (24 h) oral gavage data, which showed that the two hydrolysates had similar blood pressure-reducing effects in SHR (Onuh et al., 2015).

6.2.3 Animal study protocols using SHRs model

Animal feeding trials were performed using SHRs and the normotensive WKY rats (Charles River laboratories, Montreal, QC, Canada). Twenty nine adult male SHRs and 18 adult male normotensive WKY (34 weeks old) were individually housed in steel cages in a room maintained at 23 $\pm$ 2^{o}C and relative humidity of 50% under a 12 h day and night cycle. The rats were fed regular laboratory diet (chow) and tap water ad libitum. After 4 weeks acclimatization, the SHRs were randomly divided into 4 groups (8, 7, 7, and 7) while the WKY rats were randomly divided into 3 groups of 6 each based on similar average body weight and systolic blood pressure (SBP). Baseline blood was collected through the jugular vein while SBP was measured using the tail-cuff plethysmography method, both in slightly anaesthetised rats according to a previously described method (Aukema, Gauthier, Roy, Jia, Li & Aluko, 2011). The rats were then placed on their respective experimental diets as shown in Table 6.1 and fed ad

libitum for 6 weeks. During the feeding trial period, body weight, SBP and feed consumption of the rats were measured weekly. Prior to the start and at the end of the 6 weeks period of feeding, the rats were transferred to metabolic cages for 48 h to collect urine. After the 6 weeks feeding regimen, the rats were anaesthetized with isoflourane overdose and blood collected after termination of the rats by cardiac puncture. Plasma was obtained from the blood samples by centrifugation at 5000 g for 10 min and stored at –80 ^{o}C for analyses. The above animal work protocols were approved by the University of Manitoba Animal Care Protocol Management and Review Committee in accordance with guidelines from the Canadian Council for Animal Care.

6.2.4 Determination of plasma ACE activity

The plasma ACE activity was determined according to a previously reported spectrophotometric method (Girgih et al., 2013). A 1 ml aliquot of 0.5 mM FAPGG (dissolved in 50 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 µl plasma or ACE (final enzyme concentrations were 0.0313, 0.0625, 0.125, 0.25, 0.5 U/ml), and 200 µl of 50 mM Tris-HCl buffer. Rate of decrease in absorbance was monitored at 345 nm and recorded for 2 min at 23 ^{o}C. The result was expressed as ΔA/min and plotted against ACE enzyme concentration to obtain a standard curve. Plasma ACE activity (U/ml) was obtained by linear regression using the standard curve.

6.2.5 Determination of plasma renin concentration

The plasma renin activity was measured according to a previously reported fluorometric method (Yuan, Wu, & Aluko, 2006). Prior to the assay, renin was diluted with 50 mM Tris–HCl (pH 8.0), containing 100 mM NaCl (assay buffer) to give different concentrations (4.15, 8.3, 16.5, 33, 66, 132, and 250 µg protein/mL). Before the reaction, 20 µL of renin substrate and 160 µL assay buffer were added to the wells. The reaction was initiated by adding 10 µL plasma or

each diluted renin solution to the wells of a 96-well microplate, which was shaken for 10 s to ensure proper mixing and then incubated at 37 ^{o}C for 15 min in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). The fluorescence intensity (FI) was then recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm, and the results were expressed as ΔFI/min. A standard curve was obtained by using linear regression from a plot of ΔFI/min versus renin concentrations. The ΔFI/min obtained for each plasma sample was used to calculate plasma renin concentration (μg/mL) from the regression equation.

6.2.6 Plasma total antioxidant capacity (TAC) and antioxidant enzymes assays

Plasma TAC, superoxide dismutase activity (SOD) and catalase (CAT) activity were each analyzed using the respective assay kit (Cayman Chemicals Inc., Ann Arbor, MI, USA), according to the manufacturer's instructions. For TAC, the samples were analyzed spectrophotometrically using a microplate reader. TAC values were calculated using measured absorbances at 750 nm and results expressed as mM Trolox concentration equivalents/mL of plasma. The SOD activity was determined using the xanthine/xanthine oxidase system to generate superoxide anions, which reduces a chromagen to produce a water-soluble formazan dye. The activity of SOD was determined as the inhibition of chromagen reduction. Briefly, the plasma (or buffer for the blank), xanthine solution, chromagen solution, 10x SOD assay buffer, and water were mixed and added into the clear 96-well microplate. Then, 10 μL pre-diluted xanthine oxidase solution was added into each well and the mixture incubated for 1 h at 37 ^{o}C. The absorbance was read at 460 nm on a microplate reader and SOD activity was calculated based on the standard curve as a function of the chromagen inhibition (U/ml). The CAT assay was performed following the manufacturer's instructions and reading the absorbance at 540 nm.

CAT activity was calculated using a calibration curve and the results expressed as nmol/min/ml of plasma.

6.2.7 Urinary protein excretion

Urinary protein content was measured using the modified Lowry method on 100 μL urine samples as previously described (Markwell, Haas, Bieber, & Tolbert, 1978).

6.2.8. Statistical analyses

All results obtained were expressed as means of triplicate values for statistical analysis. The data were subjected to one-way analysis of variance (ANOVA) while Tukey's multiple comparison tests was used to determine mean treatment differences when significant ($p < 0.05$). An IBM SPSS Statistical package (version 20) was used for all statistical analyses.

6.3. Results and discussion

6.3.1 Experimental diets, body/organ weight and food intake

The experimental diets were formulated to be isocaloric with each delivering 20 – 21 KJ of energy/g diet (Table 6.1). At the beginning of the study, the rats were randomized to the treatment diets in a way that there were no significant differences in weights between the treatment groups. At the end of the study period, the body weights of the rats ranged from 423.68 – 458.26 g. The SHR control rats had the highest average body weight (though not significantly different from SHR 0.5% CSPH, SHR 1% CSM, WKY 1 % CSPH and WKY 1% CSM) while the WKY control rats had a low average body weight (Table 6.2). The SHR on CSPH 1% diet and WKY control rats had significantly low ($p<0.05$) body weight though all the WKY had low average daily feed intake. This was unexpected as the WKY groups had significantly lower ($p<0.05$) average daily feed intake (15.56 – 16.88 g/rat/day) when compared to the SHR groups (18.85 – 20.58 g/rat/day). The high feed intake by the SHR group is probably be due to reduced

Table 6.1: Percent (%) composition of experimental diets showing inclusion levels of chicken skin protein hydrolysates (CSPH) and chicken skin meal (CSM)

Ingredients	Control	0.5% CSPH	1% CSPH	1% CSM
Corn starch	40	40	40	40
Casein	20	19.5	19	19
CSPH[a]	0	0.5	1	0
CSM[a]	0	0	0	1
Maltodextrin	13.2	13.2	13.2	13.2
Sucrose	10	10	10	10
Soy oil + TBHQ	7	7	7	7
Fiber	5	5	5	5
Mineral mix	3.5	3.5	3.5	3.5
Vitamin mix	1	1	1	1
L-cys	0.3	0.3	0.3	0.3
Total	100	100	100	100

[a]Protein weight basis

Table 6.2: Body and tissue weights, average daily feed intake and feed efficiency ratio of male spontaneously hypertensive rats (SHR) and Wistar Kyoto Rats (WKY) fed CSPH, CSM and Control diets for 6 weeks.

Weights (g)	SHR				WKY		
	Control	0.5% CSPH	1.0% CSPH	1.0% CSM	Control	1.0% CSPH	1.0% CSM
Body weight (g)	458.26 ±13.60^{a}	446.47 ± 22.85^{ab}	431.17 ± 24.01^{bc}	441.71 ± 24.69^{abc}	423.68 ± 7.19^{c}	451.27 ± 8.16^{a}	450.38 ± 13.54^{a}
Av. Daily feed intake	20.58 ± 1.82^{a}	19.84 ± 1.34^{ab}	18.85 ± 1.42^{b}	19.90 ± 1.57^{ab}	15.56 ± 0.71^{c}	15.74 ± 0.99^{c}	16.88 ± 0.97^{c}
Cumulative body wt gain (g)	20.29± 4.61^{ab}	26.49 ± 6.27^{a}	19.57 ± 4.73^{ab}	23. 49 ± 3.35^{a}	15.43 ± 9.39^{b}	20.32 ± 9.30^{ab}	19.45 ± 8.41^{ab}
FER	0.74 ± 0.22^{a}	0.96 ± 0.20^{a}	0.77 ± 0.20^{a}	0.89 ± 0.19^{a}	0.69 ± 0.43^{a}	0.94 ± 0.80^{a}	0.77 ± 0.39^{a}

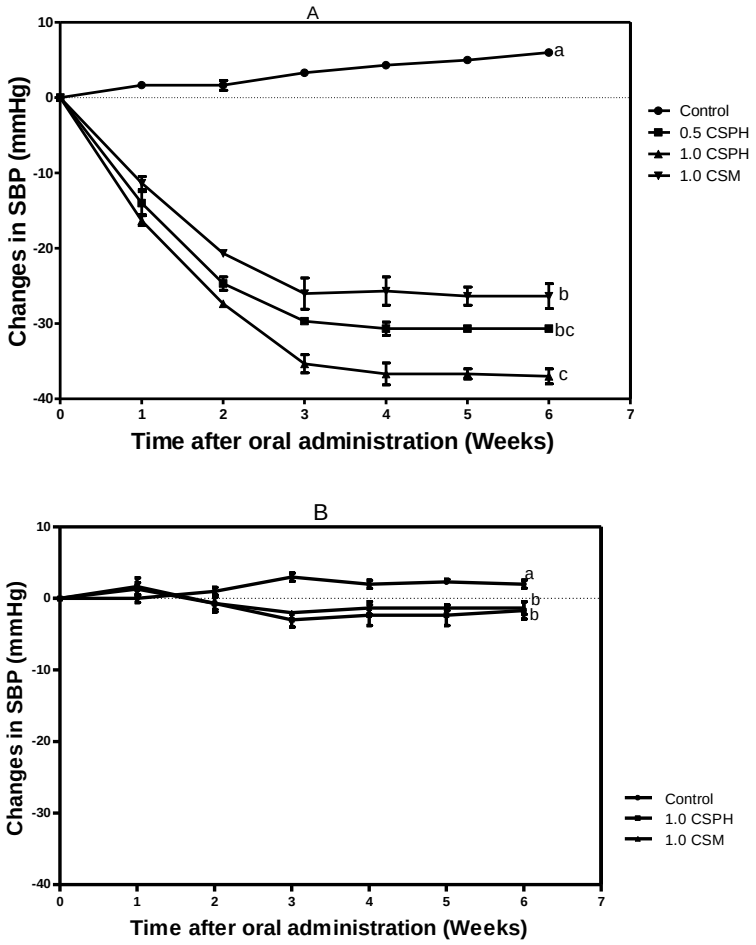
*FER – Feed efficiency ratio

satiety occasioned by their hypertensive state. However, the low average daily feed intake of the WKY groups did not significantly affect ($p>0.05$) the cumulative body weight gain (only WKY control group had significantly lower body weight gain). This also did not affect FER of these rats (0.69 – 0.96) though it was slightly lower in WKY rats fed control diet. This suggests that the rats had very high feed conversion efficiency despite the fact that they consumed significantly lower ($p<0.05$) amount of the diets when compared to the SHR groups. This is similar to the results obtained by Nabha et al (2005) in which tempol treatment had no effects on weight gain and overall fluid intake in both the SHR and WKY groups at both 6 and 11 weeks of age. However, contrary to the observation in this study, they reported lower body weight for the SHR rats compared to the WKY rats. Simao et al (2007) also reported that body weight increased with age in both SHR and WKY rat strains, though the increase was significantly higher in WKY than in SHR when compared against age-matched SHR.

6.3.2 Blood pressure lowering activity of enzymatic CSPH

The blood pressure-lowering activity of enzymatic CSPHs and CSM containing diets when compared to control (casein only) diet was confirmed in a 6 weeks feeding trial using SHRs and the normotensive WKY rats (Fig. 6.1) by monitoring changes in SBP of the rats weekly. The various samples significantly lowered SBP of SHRs when compared to the control (casein only) diet with the CSPH 1% being significantly ($p<0.05$) more effective than the CSM 1% but not CSPH 0.5%. The SBP-lowering effect of the diets was steep for the first 3 weeks but reached a plateau afterwards indicating no SBP lowering effect any longer with a maximum lowering of -36, -31 and -26 mmHg for CSPH 1%, CSPH 0.5% and CSM 1% respectively. The rats on the control (casein only) diet had SBP that gradually increased by 2 mmHg after the first week to 6

Fig. 6.1. Systolic blood pressure (SBP) changes in adult rats fed either control diets or diet containing 0.5 % CSPH, 1.0% CSPH and 1.0% CSM for week 5 measurement (A: SHR and B: WKY). At each time point differences in letters indicate that mean values are significantly ($p<0.05$) different (data for each graph were analyzed separately).



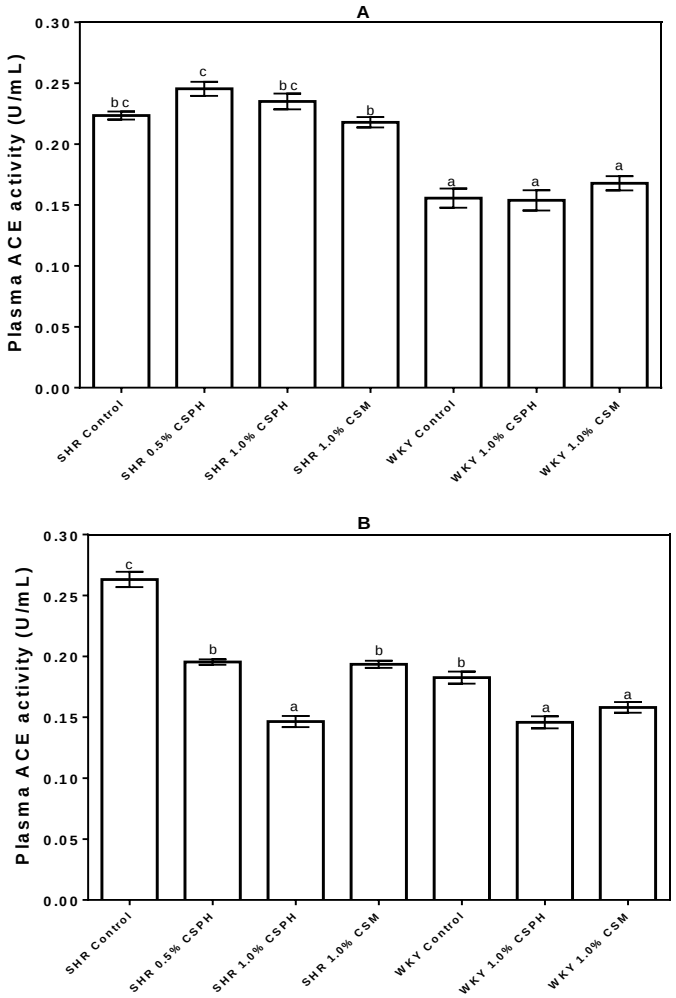
mmHg at week 6. For the WKY, the SBP-lowering effects of the CSPHs samples was significant when compared to the control (casein only) diet though the level of reduction is not high enough, indicating it does not have hypotensive effect in normotensive individuals (Girgih et al., 2013).

Various genetic models of hypertensive rats have been used to study hypertension because they offer simple and useful approach for studying the disease. However, the SHRs model has been reported to be the best and most widely used model as some of the pathophysiological processes closely resembles that of the human essential hypertension (Lu et al., 2008). In our previous study, we observed that CSPHs lowered SBP in SHRs after oral administration and concluded that they may be used on a short-term basis to offer fast and efficient remedy in hypertensive individuals (Onuh et al., 2015). The CSPHs in this present study exhibited better SBP lowering ability than the CSM indicating an efficient absorption of the peptides from the digestive tracts as well as longer peptide that persist in the blood. The results obtained in this study are also in consonance with those obtained for hemp meal protein hydrolysates whereby there was attenuation of SBP and development of hypertension in SHRs (Girgih et al., 2013). However, they are contrary to the results obtained for chicken leg bone protein hydrolysates in which the SBP of all the SHRs increased with age despite the treatments, though at a lower rate for the hydrolysates and captopril when compared to the control (Cheng, Wan, Liu, Lin, & Sakata, 2008). The non-reduction in SBP observed was attributed to a lower dosage used in the study (50 mg/kg body weight/day) when compared to the dose used in the present study (100 mg/kg body weight).

6.3.3 Plasma ACE activity of experimental rats

The plasma ACE activity of SHRs and WKY rats fed CSPH, CSM and control (casein only) diet is shown in Fig. 6.2. At baseline (Fig. 6.2A), the plasma ACE activity in the SHRs were significantly ($p<0.05$) higher with levels ranging from 0.218 – 0.245 U/mL than the WKY rats with levels ranging from 0.154 – 0.168 U/mL. This difference between the plasma ACE activities of the rats is understandable since the SHRs are highly hypertensive at this age (42 weeks old) when compared to the normotensive WKY rats. However, at termination, the diet treatments were observed to significantly ($p<0.05$) alter the plasma ACE activity of both the SHRs and WKY rats with values ranging from 0.147 – 0.263 U/mL for the SHRs and 0.146 – 0.183U/mL for the WKY rats. In both cases, the CSPH 1% treatment had the most significant ($p<0.05$) plasma ACE activity reduction effect with values of 0.147 and 0.146 U/mL for the SHRs and WKY rats respectively followed by the CSPH 0.5% and CSM 1%. However, the plasma ACE activity for the SHR control and WKY control groups increased after the 6 weeks study period suggesting that the casein-only diet had no effect on the plasma ACE activity of the rats. Results of the plasma ACE activity was positively related with the SBP lowering effects of CSPHs suggesting that the diet intervention groups with the maximum SBP reduction also showed the maximum reduction/inhibition of plasma ACE activity for both the SHRs and WKY rats. This is consistent with the results obtained from earlier studies that suggested plasma ACE activity could be a very good biomarker of hypertension in SHRs (Girgih et al., 2013; Kim, Park, & Choue, 2010).

Fig. 6.2. Plasma ACE activity of experimental rats fed diets fed casein-only diet or casein diet that contained chicken skin protein hydrolysates (CSPH) or chicken skin meal (CSM) at (A) – baseline and (B) – termination. Bars with different letters have mean values that are significantly different ($p < 0.05$). Values are means (n=6 rats) ± SD (data for each graph were analyzed separately).



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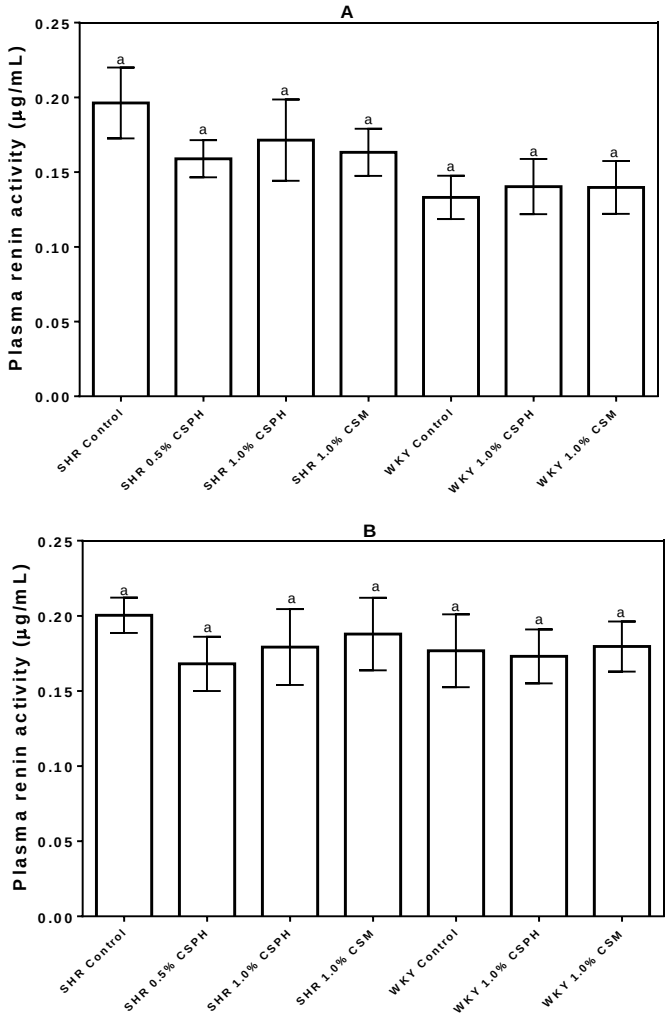
6.3.4 Plasma renin activity

Plasma renin activity of the SHRs and WKY rats fed the experimental diets is shown in Fig. 6.3. The plasma renin activity of WKY rats at baseline (Fig. 6.3A) is lower (0.133 – 0.140 μg/mL) than the plasma renin activity of SHRs (0.159 – 0.196 μg/mL) though not significant ($p<0.05$). This is expected since the WKY rats at this point had correspondingly lower SBP compared to the SHRs. However, at termination, the plasma renin activity for all the rat groups unexpectedly increased with age, though not significantly ($p<0.05$) despite the diet intervention. It has previously been reported that unlike ACE, inhibition of renin is more difficult to achieve, probably due to the difficulty in the down regulation of renin activities both *in vitro* and *in vivo* (Udenigwe et al., 2009). Moreover, the age of the rats may even be more critical, making it difficult to achieve the desired inhibition of plasma renin activities. Renin is known to catalyze the rate-limiting step in the RAAS by converting angiotensinogen to angiotensin I (ACE substrate). The inability of the CSPHs to reduce or inhibit plasma renin activity even though ACE activity was reduced may be indicative of other possible mechanisms beyond RAAS that are responsible for the observed SBP-lowering effects in SHRs.

6.3.5 Plasma SOD activity

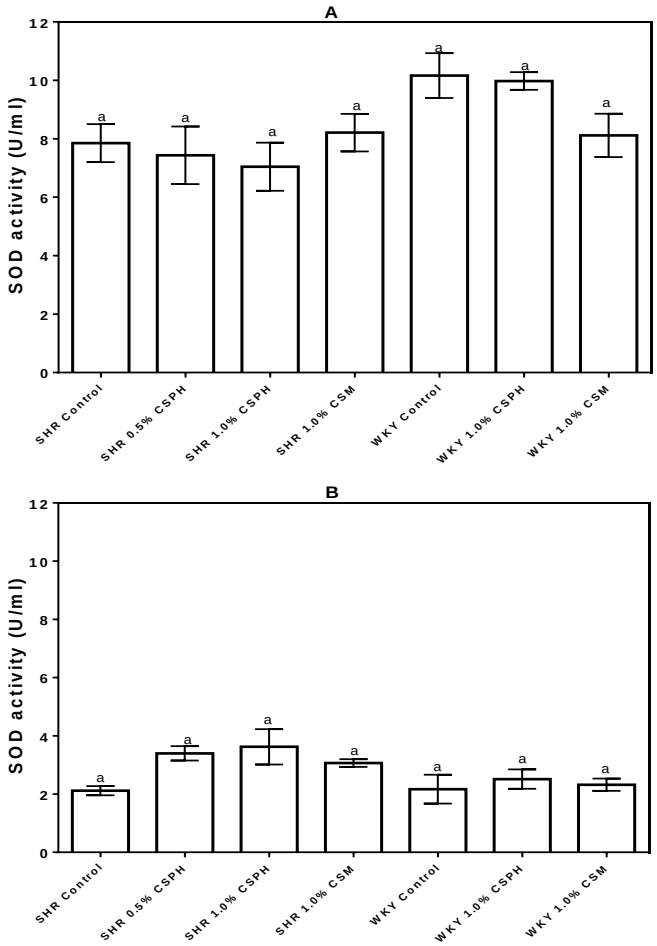
SOD catalyzes the dismutation of superoxide radical to hydrogen peroxide, which is subsequently removed by either catalase or glutathione peroxidase (Girgih et al., 2014; Vaziri, 2008; Young & Woodside, 2001). Fig. 6.4 shows the plasma SOD activities of SHRs and normotensive WKY rats fed CSPHs, CSM and control (casein only) diets. At baseline, there were no significant ($p<0.05$) differences in the plasma SOD activities of the rats, though that of the normotensive WKY rats were higher (8.12 – 10.16 U/mL) than those of the SHRs (7.04 –

Fig. 6.3. Plasma renin activity of experimental rats fed diets fed casein-only diet or casein diet that contained chicken skin protein hydrolysates (CSPH) or chicken skin meal (CSM) at (A) – baseline and (B) – termination. Bars with different letters have mean values that are significantly different ($p < 0.05$). Values are means (n=6 rats) $\pm$ SD (data for each graph were analyzed separately).



8.21 U/mL). This is probably due to the fact that the SHRs (with chronic hypertension due to age) also exhibit high oxidative stress leading to reduced antioxidant enzyme (SOD) defense mechanism (Girgih et al., 2014; Simao et al., 2011). However, at termination, the plasma SOD activity of all the rat groups decreased significantly ($p<0.05$), suggesting that the intervention diets did not ameliorate the oxidative stress in this animal model at this age when chronic hypertension has developed despite the observed SBP reduction. Though, it may be difficult to understand the underlying cause for this observation, however, age of the rats may be a critical factor. Hypertension and oxidative stress are known to develop with age, and this is especially higher in the SHRs resulting in irreversible oxidative stress induced damage (Girgih et al., 2014; Vaziri, 2008; Simao et al., 2011; Nishiyama, Yao, Nagai, Miyata, Yoshizumi, Kagami, et al., 2004 et al., 2004). Another possible explanation for this observation may be due to low feed consumption ratios as these rats, especially the WKY rats had the least average daily feed consumption. The low plasma SOD activity observed for all the rats groups at termination may also imply a high superoxide radical generation in the tissues of the experimental rats. Though superoxide radical is known to be short lived, it is nonetheless a highly reactive and cytotoxic molecule (Girgih et al., 2014; Vaziri, 2008). Girgih et al (2014) reported that though there was a decrease in plasma SOD levels in adult (20-week old) SHRs when compared to growing SHRs, however, addition of hemp meal hydrolysates (HMH) in the diets significantly ($p < 0.05$) increased in plasma SOD in both rat groups with corresponding decreases in total peroxide levels. They concluded that the results indicated that the HMH peptides acted as effective antioxidants against lipid peroxidation by enhancing the antioxidant enzyme levels.

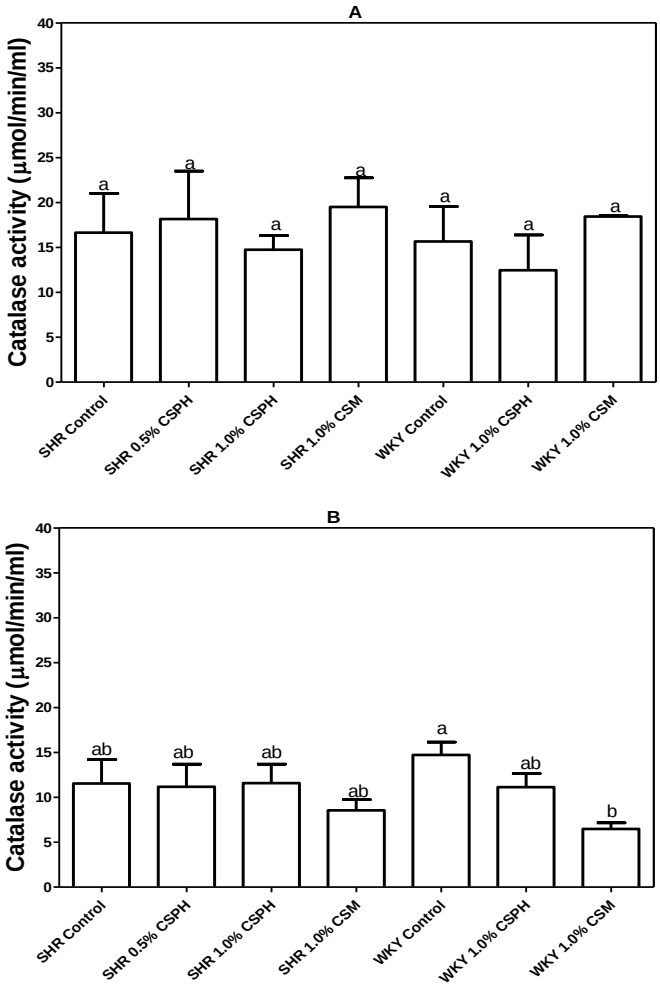
Fig. 6.4. Superoxide dismutase (SOD) activity of plasma of spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto rats (WKY) fed casein-only diet or casein diet that contained chicken skin protein hydrolysates (CSPH) or chicken skin meal (CSM) at (A) – baseline and (B) – termination. Bars with different letters have mean values that are significantly different ($p < 0.05$). Values are means (n=6 rats) $\pm$ SD (data for each graph were analyzed separately).



6.3.6 Plasma catalase activity

The plasma catalase activity of the SHRs and normotensive WKY rats fed CSPHs, CSM and control (casein only) diets did not differ significantly ($p<0.05$) though the values at baseline (Fig. 6.5A) are low (12.46 – 19.50 μmol/min/mL) suggesting a weakened plasma antioxidants protection and therefore, higher induced oxidative stress. The ability of the intervention diets to reverse or improve the plasma catalase activity and hence, better antioxidant protection failed to achieve the desired result as the plasma catalase activity at termination (Fig.6.5B) decreased for all the diet groups with the SHR and WKY CSM 1% groups having the highest ($p<0.05$) decrease (-10.94 and -11.97 μmol/min/mL). The lack of protection by the CSPHs intervention diets on catalase is contrary to the results obtained for reduction of oxidative stress (enhanced SOD and catalase activities) of loach (*Misgurnus anguillicaudatus*) (You, Zhao, Liu, & Regenstein, 2011), sardinelle (*Sardinella aurita*) (Ben Khaled, Ghlissi, Chtourou, Hakim, Ktari, Fatma, et al., 2012) and hemp (HMH) (Girgih et al., 2014) protein hydrolysates. According to Powers and Lennon (1999), catalase enzyme is known to catalyze the conversion of H_2O_2 to water and O_2, thereby suppressing the generation of circulating oxidative stress inducing molecules. The effectiveness of this antioxidant defense mechanism however, reduces with age, fatigue and diseased conditions (especially hypertension) (Girgih et al., 2014), which may possibly explain the observations in this study. Enhanced catalase activities have been reported to also contribute to a decrease in peroxide levels in the blood and consequently, resulting in decrease in the risk or severity of cardiovascular disease events, especially hypertension (Girgih et al., 2014).

Fig. 6.5. Catalase (CAT) activity of plasma of spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto rats (WKY) with casein-only diet or casein diet that contained chicken skin protein hydrolysates (CSPH) or chicken skin meal (CSM) at (A) – baseline and (B) – termination. Bars with different letters have mean values that are significantly different ($p < 0.05$). Values are means (n=6 rats) $\pm$ SD (data for each graph were analyzed separately).



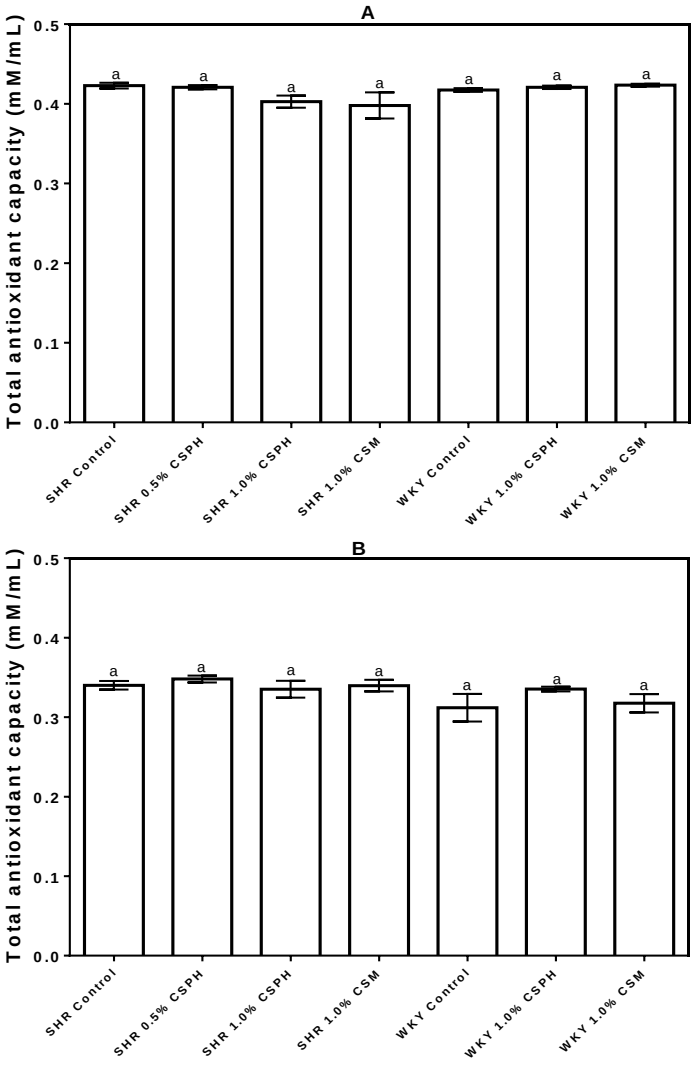
6.3.7 Plasma total antioxidant capacity (TAC)

According to Young (2001), TAC is a biomarker of oxidative stress in biological fluids that can accurately predict the oxidative state when assessed along with individual antioxidant parameters. Though the individual antioxidant parameters in this study were shown to be weakened at both baseline and termination of the study, when combined with the TAC (Fig. 6.6), it can give a true representation of the oxidative state of the SHRs and WKY rats used in this study as well as the effectiveness of the intervention diets. The plasma TAC of SHRs and normotensive WKY rats fed CSPHs, CSM and control (casein only) diets did not differ significantly ($p<0.05$) though the values at baseline (Fig. 6.6A) are correspondingly high with values ranging from 0.399 – 0.423 mM/mL. The SHR Control had the highest TAC values while the SHR CSM 1% had the lowest TAC values. This result is quite surprising given the age of the rats at baseline (42 weeks) as oxidative stress is known to increase with age and subsequently leading to reduced TAC. The plasma TAC levels of the rats groups decreased at termination (Fig. 6.6B) just like the individual antioxidant enzymes, though the decrease is not significant ($p<0.05$). Overall, the plasma TAC for the rats at termination was shown to be high. Girgih et al (2014) observed low TAC values (0.025 – 0.035 mM/mL) for adult rats compared to 0.145 – 0.199 mM/mL for growing rats suggesting that oxidative stress becomes rapidly established with age in SHRs.

6.3.8 Urinary protein excretion

The urinary excretion of protein was similar for all the rat groups at baseline with the exception of the SHR CSM 1% group which is only higher than the SHR control and WKY 1% CSM group (Fig. 6.7) with values ranging from 59.26 – 117. 18 mg/mL. However at termination, the protein excretion in urine was increased significantly ($p<0.05$) by more than 2 fold for the

Fig. 6.6 Total antioxidant capacity (TAC) of plasma of spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto rats (WKY) with casein-only diet or casein diet that contained chicken skin protein hydrolysates (CSPH) or chicken skin meal (CSM) at (A) – baseline and (B) – termination. Bars with different letters have mean values that are significantly different ($p < 0.05$). Values are means (n=6 rats) ± SD.

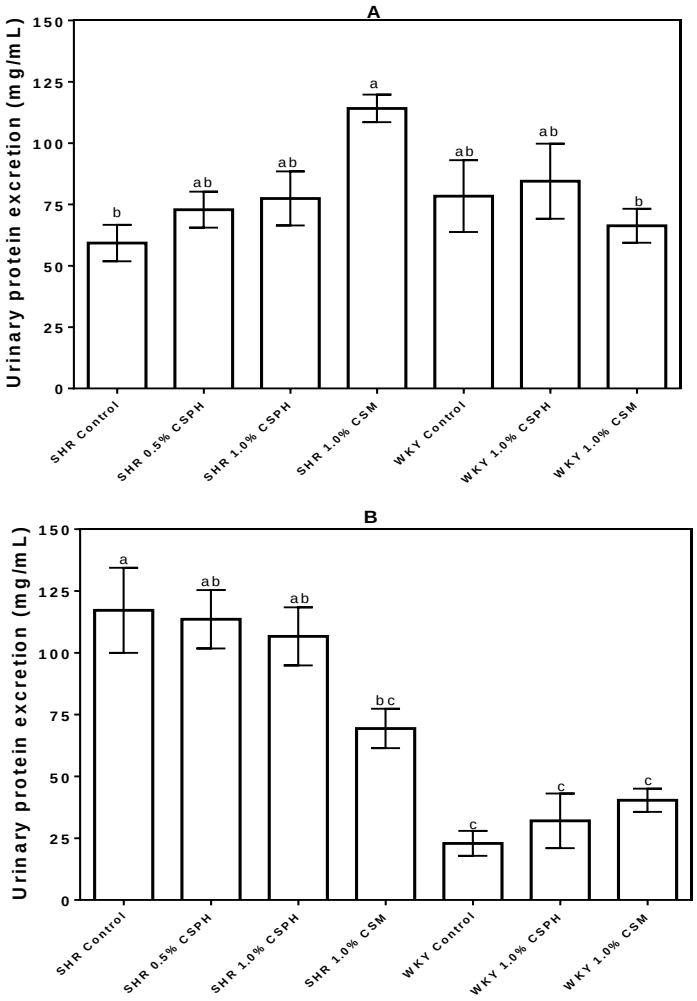


SHR control, SHR 0.5% CSPH and SHR CSPH 1% groups (106 – 117 mg/mL) while it was significantly ($p<0.05$) decreased for the SHR CSM 1% (69.40 mg/mL) and all the WKY rats (22.92 – 40.34 mg/mL). The high excreted proteins in the urine of the SHRs may be due to their hypertensive state resulting in proteinuria, suggesting some degree of oxidative stress and kidney damage (Nishiyama, et al., 2004). This may also be due to the reduced plasma antioxidant enzymes activities as was previously observed, as well as irreversible age-related oxidative stress induced damage. Nishiyama et al (2004) reported that treatment of male Sprague-Dawley rats with 1% NaCl did not alter urinary protein excretion but aldosterone/1% NaCl treated significantly increased urinary excretion of protein (101 mg/day) at 6 weeks. On the other hand, increase in urinary protein excretion was prevented by treatment with eplerenone or tempol to aldosterone/1% NaCl-treated rats (10 and 9 mg/day respectively) indicating that aldosterone/salt-induced renal injury is associated with oxidative stress as well as the ability of antioxidants (tempol) to reverse and prevent renal injury. Similarly, Simao et al (2007) reported that while there were no differences in urinary protein excretion with age in WKY rats (14 and 13.9 mg/day for 3 and 12 months respectively), urinary protein excretion however significantly increased in SHR rats with age (26 and 34 mg/day for 3 and 12 months respectively). This is an indication that oxidative stress increased with age though at a much higher level in the SHR rats than in the WKY rats.

6.4. Conclusions

We have been able to demonstrate from this study that dietary interventions using CSPHs in a feeding trial for 6 weeks significantly lowered SBP in SHRs but have no hypotensive effects on normotensive WKY rats. This observed reduction in SBP also correlated with plasma ACE but not renin activity, suggesting the *in vivo* mechanism for blood pressure regulation by CSPHs

Fig. 6.7. Urinary protein excretion of spontaneously hypertensive rats (SHRs) and normotensive Wistar Kyoto rats (WKY) with casein-only diet or casein diet that contained chicken skin protein hydrolysates (CSPH) or chicken skin meal (CSM) at (A) – baseline and (B) – termination. Bars with different letters have mean values that are significantly different ($p < 0.05$). Values are means (n=6 rats) ± SD



to be down-regulation or inhibition of ACE activity. Though the average daily feed consumption of the rats was affected by the dietary intervention, however, the cumulative body weight gain and FER were not significantly affected implying that the diet was well tolerated by the rats. There was a corresponding reduction in the plasma antioxidant enzymes activities by the CSPHs intervention diets at termination when compared to baseline contrary to our expectation, suggesting that the hydrolysates may have been unable to repair age-related irreversible oxidative stress induced damage at that chronic hypertensive state. However, the plasma SOD activity was higher for the CSPH-fed rats, which suggests some benefits of the diet intervention. Our results confirm the potential of CSPHs to be used as ingredient in formulating functional foods and nutraceuticals in the prevention and treatment of hypertension. Further work is needed to identify other possible biomarkers for hypertension and oxidative stress and potential mechanisms for *in vivo* antioxidant and BP-lowering effects of CSPH.

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6.5 References

Ahhmed, A. M., & Muguruma, M. (2010). A review of meat protein hydrolysates and hypertension. *Meat Science, 86*(1), 110-118.

Aluko, R. E., & Monu, E. (2003). Functional and bioactive properties of quinoa seed protein hydrolysates. *Journal of Food Science, 68*(4), 1254-1258.

Aukema, H. M., Gauthier, J., Roy, M., Jia, Y., Li, H., & Aluko, R. E. (2011). Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy

in experimental kidney disease. *Molecular Nutrition & Food Research, 55*(7), 1044-1051.

Ben Khaled, H., Ghlissi, Z., Chtourou, Y., Hakim, A., Ktari, N., Fatma, M. A., Barkia, A., Sahnoun, Z., & Nasri, M. (2012). Effect of protein hydrolysates from sardinelle (Sardinella aurita) on the oxidative status and blood lipid profile of cholesterol-fed rats. *Food Research International, 45*(1), 60-68.

Centenaro, G. S., Mellado, M. S., & Prentice-Hernandez, C. (2011). Antioxidant activity of protein hydrolysates of fish and chicken bones. *Adv. J. Food Sci. Technol, 3*(4), 9.

Cheng, F. Y., Wan, T. C., Liu, Y. T., Lin, L. C., & Sakata, R. (2008). A study of *in vivo* antihypertensive properties of enzymatic hydrolysate from chicken leg bone protein. *Animal Science Journal, 79*, 6.

Cliche, S., Amiot, J., Avezard, C., & Gariepy, C. (2003). Extraction and characterization of collagen with or without telopeptides from chicken skin. *Poultry Science, 82*(3), 503-509.

Di Bernardini, R., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., & Hayes, M. (2011). Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. *Food Chemistry, 124*(4), 1296-1307.

Erdmann, K., Cheung, B. W. Y., & Schroder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry, 19*(10), 643-654.

Feddern, V., Kupski, L., Cipolatti, E. P., Giacobbo, G., Mendes, G. L., Badiale-Furlong, E., & de Souza-Soares, L. A. (2010). Physico-chemical composition, fractionated glycerides and fatty acid profile of chicken skin fat. *European Journal of Lipid Science and Technology, 112*(11), 1277-1284.

Girgih, A. T., Alashi, A., He, R., Malomo, S. A., & Aluko, R. E. (2013). Preventive and treatment effects of a hemp seed (Cannabis sativa L.) meal protein hydrolysate against high blood pressure in spontaneously hypertensive rats. *European Journal of Nutrition*.

Girgih, A. T., Alashi, A. M., He, R., Malomo, S. A., Raj, P., Netticadan, T and Aluko, R. E. (2014). Hemp Seed Meal Protein Hydrolysate Reduces Oxidative Stress Factors in Spontaneously Hypertensive Rats. *Nutrients* 2014, *6*, 5652 – 5666.

He, R., Alashi, A., Malomo, S. A., Girgih, A. T., Chao, D. F., Ju, X. R., & Aluko, R. E. (2013). Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chemistry, 141*(1), 153-159.

Kim, S. M., Park, S., & Choue, R. (2010). Effects of Fermented Milk Peptides Supplement on Blood Pressure and Vascular Function in Spontaneously Hypertensive Rats. *Food Science and Biotechnology, 19*(5), 1409-1413.

Lu, Y., A, J., Wang, G., Hao, H., Huang, Q., Yan, B., Zha, W., Gu, S., Ren, H., Zhang, Y., Fan, X., Zhang, M., & Hao, K. (2008). Gas chromatography/time-of-flight mass spectrometry based metabonomic approach to differentiation hypertension- and age-related metabolic variation in spontaneously hypertensive rats. *Rapid Communications in Mass Spectrometry, 22*, 7.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). Modification of Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Analytical Biochemistry, 87*(1), 206-210.

Martinez-Maqueda, D., Miralles, B., Recio, I., & Hernandez-Ledesma, B. (2012). Antihypertensive peptides from food proteins: a review. *Food & Function, 3*(4), 350-361.

Nabha, L., Garbern, J. C., Buller, C. L., & Charpie, J. R. (2005). Vascular oxidative stress precedes high blood pressure in spontaneously hypertensive rats. *Clin. Exp. Hypertens., 27*, 71– 82.

Nishiyama, A., Yao, L., Nagai, Y., Miyata, K., Yoshizumi, M., Kagami, S., Kondo, S., Kiyomoto, H., Shokoji, T., Kimura, S., Kohno, M., & Abe, Y. (2004). Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. *Hypertension, 43*(4), 841-848.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Research International, 53*(1), 260-267.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2014). In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry, 150*, 366-373.

Onuh, J. O., Girgih, A. T., Malomo, S. A., Aluko, R. E., & Aliani, M. (2015). Kinetics of *in vitro* renin and angiotensin converting enzyme inhibition by enzymatic chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. *Journal of Functional Foods,* 14, 133 – 143.

Powers, S. K., & Lennon, S. L. (1999). Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. *Proceedings of the Nutrition Society, 58*(4), 1025-1033.

Raghavan, S., & Kristinsson, H. G. (2009). ACE-inhibitory activity of tilapia protein hydrolysates. *Food Chemistry, 117*(4), 582-588.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive Peptides from Muscle Sources: Meat and Fish. *Nutrients, 3*(9), 765-791.

Samanarayaka, A., G. P., Kitts, D. D., & Li-Chan, E. C. Y. (2010). Antioxidative and angiotensin-I-converting enzyme inhibitory potential of a pacific hake (Merluccius productus) fish protein hydrolysate subjected to simulated gastrointestinal digestion and caco-2 cell permeation. *J. Agric. Food Chem, 58*, 8.

Schmieder, R. E., Hilgers, K. F., Schlaich, M. P., & Schmidt, B. M. W. (2007). Renin-angiotensin system and cardiovascular risk. *Lancet, 369*(9568), 1208-1219.

Simao, S., Gomes, P., Pinto, V., Silva, E., Amaral, J. S., Igreja, B., Afonso, J., Serrao, M. P., Pinho, M. J., & Soares-da-Silva, P. (2011). Age-related changes in renal expression of oxidant and antioxidant enzymes and oxidative stress markers in male SHR and WKY rats. *Experimental Gerontology, 46*(6), 468-474.

Udenigwe, C. C., Lin, Y. S., Hou, W. C., & Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*(2), 199-207.

Vaziri, N. D. (2008). Causal Link Between Oxidative Stress, Inflammation, and Hypertension. *Iranian Journal of Kidney diseases, 2*(1), 10.

Wu, H. C., Pan, B. S., Chang, C. L., & Shiau, C. Y. (2005). Low-molecular-weight peptides as related to antioxidant properties of chicken essence. *Journal of Food and Drug Analysis, 13*(2), 176-183.

You, L. J., Zhao, M. M., Liu, R. H., & Regenstein, J. M. (2011). Antioxidant and Antiproliferative Activities of Loach (Misgurnus anguillicaudatus) Peptides Prepared by Papain Digestion. *Journal of Agricultural and Food Chemistry, 59*(14), 7948-7953.

Young, I. S. (2001). Measurement of total antioxidant capacity. *Journal of Clinical Pathology, 54*(5), 339-339.

Young, I. S., & Woodside, J. V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology, 54*(3), 176-186.

Yuan, L., Wu, J., & Aluko, R. E. (2006). Kinetics of renin inhibition by sodium houttuyfonate analogs. *Biosci Biotechnol Biochem, 70*, 6.

TRANSITION STATEMENT FOUR

Several mechanisms and pathways are known to regulate the development and progression of hypertension. In the fourth study, the chicken skin protein hydrolysates diet intervention in SHRs was effective in lowering blood pressure. However, *in vivo* analysis of the plasma antihypertensive and antioxidant activities did not provide enough information with regards to the underlying factors responsible for the blood pressure-lowering effect beyond a significant reduction in plasma ACE activity. Metabolomics study was therefore, undertaken in the fifth study to provide more insights into other mechanisms and metabolic pathways beyond RAS that may be responsible for these changes. To achieve this goal, urine and plasma metabolomics from the fourth study were investigated using the non-targeted approach in order to generate metabolites profiles for each of the intervention groups. These metabolite profiles were subsequently analyzed to determine if any of the metabolites observed to be significantly changing (up-regulated or down-regulated) may be associated with activated pathways in the hypertension (SBP) regulatory system. Results of the metabolomics study revealed several distinct urinary and plasma metabolite changes that may be considered biomarkers responsible for attenuation of systolic BP and hypertension. The evidence also supports the vasodilative action of nitric oxide (NO) production via the arginine metabolic pathway as well the antioxidant role of the hydrolysates and peptides by inhibiting and/or scavenging free radical formation. Finally, this study could therefore promote the use of CSPH as an ingredient in the development of nutraceuticals and functional foods for the management of hypertension and enhance value-added utilization of chicken skin and protect the environment.

CHAPTER 7

MANUSCRIPT FIVE

LONG-TERM ORAL ADMINISTRATION OF CHICKEN SKIN PROTEIN HYDROLYSATE AFFECTS URINARY AND PLASMA METABOLITE PROFILES IN SPONTANEOUSLY HYPERTENSIVE RATS

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7.0 Abstract

Metabolite profiles of the urine and plasma of spontaneously hypertensive rats (SHRs) and Wistar Kyoto rats (WKY) fed chicken skin protein hydrolysates (CSPH) were investigated to establish potential biomarkers for hypertension and oxidative stress regulation. Chicken skin proteins from the thigh and breast muscles were hydrolyzed with alcalase (3%) or a combination of pepsin/pancreatin (1%). The digests were lyophilized and combined in an equal proportion to obtain CSPH that was used in feed formulation. Spontaneously hypertensive rats (SHRs) were randomized into four groups (7-8 rats/group) that received casein-only (control), CSPH (0.5 or 1%, w/w) and chicken skin meal (CSM, 1% w/w) diets for eight weeks. Normotensive Wistar Kyoto rats (6 rats/group) were fed similar diets, with the exception of 0.5% CSPH. Urine and plasma samples were collected at baseline and termination of the study period followed by liquid chromatography/time of flight mass spectrometry (LC-QTOF-MS) analysis. Partial least square (PLS) of the detected metabolites in urine and plasma of the rats showed a clear separation between the rats at baseline and termination. Several metabolites with significant changes (≥ 2-fold changes, $P<0.05$) in urine and plasma of CSPH-fed SHRs may be considered biomarkers for hypertension. These metabolites are associated with L-arginine metabolism/nitric oxide (NO) production, oxidative stress, amino acid, phospholipid and fat metabolism. Some of the significant metabolite changes identified were Symmetric Dimethylarginine (SDMA), N2-acetyl-L-ornithine, buthionine sulfoximine, uric acid, Vitamin E succinate (α-tocopherol succinate), L-isoleucine, creatinine, and phospholipids. Overall, the evidence supports the vasodilative actions of NO production via the L-arginine pathway and inhibition of oxidation through scavenging of free radicals by the CSPH peptides in the attenuation of systolic blood pressure and hypertension.

Keywords: Metabolomics, L-arginine, hypertension, vasodilation, metabolites, biomarkers

7.1 Introduction

Hypertension has been defined as a clinical condition, with a manifestation of elevated arterial blood pressure (≥ 140 mmHg systolic or 90 mmHg diastolic) and other associated metabolic disorders (Jiang, Nie, Li, & Xie, 2012). It is the most prevalent chronic disease worldwide and has been reported to be a major risk factor for cardiovascular diseases and death (Aa et al., 2010). Although the cause of primary (essential) hypertension in about 85 - 90% of cases remains unknown, lifestyle may actually support its development. Hypertension is a multifactorial disorder that may likely be due to a complex combination of genetic (heredity), environmental and other factors such as diet, salt intake, age, race and gender as well as smoking, lack of exercise, stress and excessive alcohol intake, which have all been implicated as influencing the onset of hypertension (Ahhmed & Muguruma, 2010; Akira et al., 2008; Akira, Masu, Imachi, Mitome, & Hashimoto, 2012; Jiang et al., 2012). Therefore, lifestyle modifications and diet therapy are the most important ways of effectively controlling hypertension, especially consumption of foods high in proteins that have long been known to possess bioactive peptides (Kim et al., 2013; Vercruysse, Van Camp, & Smagghe, 2005; Vermeirssen, Camp, & Verstraete, 2004).

Research results have indicated that bioactive peptides from different food protein hydrolysates decreased blood pressure and enhanced blood flow by interfering with the renin angiotensin system (RAS) activities (Girgih, Alashi, He, Malomo, & Aluko, 2014). Bioactive peptides also decreased blood lipids levels, as well as increased blood antioxidant enzymes levels and total blood antioxidant capacity in rats (Simao et al., 2011). We recently reported the systolic blood pressure lowering effect of chicken skin protein hydrolysates in spontaneously hypertensive rats SHRs, a model of human essential hypertension (Onuh, Girgih, Malomo, Aluko, & Aliani, 2015). These findings also corroborate earlier *in vitro* results on the potential

antihypertensive and antioxidative properties of chicken skin protein hydrolysates (Onuh, Girgih, Aluko, & Aliani, 2013, 2014). Though various genetic models of hypertensive rats have been used to study hypertension because they offer simple and useful approach for studying the disease, the SHRs model however, is the most widely used because some of the pathophysiological processes closely resemble those observed in human essential hypertension (Lu et al., 2008). Therefore, their use alongside their normotensive equivalent, the Wistar Kyoto rats (WKY) could furnish researchers with the knowledge of blood pressure regulation in essential hypertension, biomarkers and appropriate diagnosis of the condition (Akira et al., 2012).

Metabolomics is primarily concerned with the study of low molecular weight compounds in bio-fluids and other matrices (Bruce et al., 2009; Kristensen, Engelsen, & Dragsted, 2012). It is a systematic approach to the study of *in vivo* metabolic profiles that will provide information on drug toxicity, disease processes and gene function at several stages in the discovery and development processes (Law et al., 2008; Lu et al., 2008). Metabolomics science has also been defined as "the measurement of the metabolome or the full set of low molecular weight endogeneous compounds (or metabolites) present within cells, tissues, organisms or bio-fluids that can reflect genetic modifications, exposure to pathogens, toxic agents, pharmaceuticals and nutritional and environmental changes" (Aa et al., 2010; Theodoridis, Gika, & Wilson, 2008). These metabolites play key roles within *in vivo* physiological and metabolic pathways, and as such, metabolomics approach is very vital in exploring the various mechanisms underlying disease conditions, especially hypertension and their complications (Aa et al., 2010; Lu et al., 2008).

As suggested previously (Aa et al., 2010), the relationship between hypertension and metabolic disorders elicits a lot of attention. Although several studies have confirmed the metabolic factors involved in hypertension, most of the metabolomics studies were restricted to the investigation of hypertension and age-related metabolic pattern (Kim et al., 2013; Roberts et al., 2013). A detailed study of the metabolic changes and regulatory effects of antihypertensive agents, especially bioactive peptides, on hypertension have not been studied. Such a detailed study is envisaged to generate data that will broaden our understanding of the metabolic changes involved in hypertension. Therefore, the objective of this study was to determine the urine and plasma metabolomics profile of SHRs and WKY rats fed CSPHs supplemented diet in other to establish possible biomarkers for hypertension and oxidative stress as well as determine possible mechanisms for the observed blood pressure regulating effects.

7.2 Materials and methods

7.2.1. Materials

Chicken skins from the thigh and breast muscles used for this study were supplied by Granny's Poultry (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas), alcalase (from fermentation of *Bacillus licheniformis*, 3.4.21.62), ACE (from rabbit lung, EC 3.4.15.1), and sodium dodecyl sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other analytical grade reagents were purchased from Fisher Scientific (Oakville, ON, Canada).

7.2.2. Preparation of chicken skin protein hydrolysates (CSPH)

Chicken skin protein hydrolysates were prepared as described in our previous work (Onuh et al., 2013). Briefly fresh thigh or breast chicken skins (~250 g) were packed in freeze drying plates, frozen at –20 ^{o}C for 24 h and transferred to –80 ^{o}C for 6 h prior to freeze drying.

The freeze dried samples were thereafter manually shredded and defatted repeatedly by mixing ~1 g with 10 mL of food grade acetone. The mixture was stirred in the fume hood for 3 h and decanted manually followed by two additional consecutive extractions of the residue. The defatted skin samples were then air dried overnight in the fume hood chamber at room temperature and subsequently milled with a Waring blender to produce a fine powder that was stored at – 20^{o}C. Dried chicken skin powder from the thigh or breast muscles were mixed with water to give 5% (w/v, protein basis) slurries. Two different enzyme treatments (alcalase or pepsin + pancreatin) were separately used for sample hydrolysis. For the alcalase hydrolysis, the slurry was heated to 55 ^{o}C, adjusted to pH 8.0 using 2 M NaOH and hydrolysis initiated by addition of enzyme (3% w/w, skin protein basis); each mixture was stirred continuously for 4 h. For the pepsin+pancreatin (PP) hydrolysis, the slurry was heated to 37 ^{o}C, adjusted to pH 2.0 using 2 M HCl and the reaction initiated with the addition of pepsin enzyme (1% w/w, skin protein basis); the mixture was then stirred continuously for 2 h. After peptic hydrolysis, the reaction mixture was adjusted to pH 7.5 with 2 M NaOH, pancreatin was added (1% w/w, skin protein basis) and incubated at 37 ^{o}C for 4 h with continuous stirring. At the end of the incubation period, both the alcalase and PP reactions were terminated by heating the slurry to 95 ^{o}C for 15 min to ensure complete denaturation of residual enzymes. The mixtures were thereafter centrifuged (7,000 x g at 4 ^{o}C) for 1 h and the resulting supernatant lyophilized and stored at –20 ^{o}C until needed for further analysis and diet formulation. Protein content of the lyophilized CSPHs was determined by the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978). The above digestion protocols were performed in triplicates and the lyophilized samples combined, analyzed for protein content and used for all the assays. Chicken thigh skin hydrolysates hydrolyzed with alcalase and chicken breast skin hydrolysates hydrolyzed with

pepsin-pancreatin were combined in equal proportions based on their protein content and used in formulation of the animal experimental diets. The combined use was based on our previous short-term (24 h) oral gavage data, which showed that the two hydrolysates had similar blood pressure-reducing effects in SHR (Onuh et al., 2015).

7.2.3 Animal study protocols using SHRs model

Animal feeding trials were performed using SHRs and the normotensive WKY rats (Charles River laboratories, Montreal, QC, Canada). Twenty nine adult male SHRs and 18 adult male normotensive WKY (34 weeks old) were individually housed in steel cages in a room maintained at 23 $\pm$ 2^{o}C and relative humidity of 50% under a 12 h day and night cycle. The rats were fed regular laboratory diet (chow) and tap water ad libitum. After 4 weeks acclimatization, the SHRs were randomly divided into 4 groups (8, 7, 7, and 7) while the WKY rats were randomly divided into 3 groups of 6 each based on similar average body weight and systolic blood pressure (SBP). Baseline blood was collected through the jugular vein while SBP was measured using the tail-cuff plethysmography method, both in slightly anaesthetized rats according to a previously described method (Aukema, Gauthier, Roy, Jia, Li & Aluko, 2011). The rats were then placed on their respective experimental diets as shown in Table 1 and fed ad libitum for 6 weeks. During the feeding trial period, body weight, SBP and feed consumption of the rats were measured weekly. Prior to the start and at the end of the 6 weeks period of feeding, the rats were transferred to metabolic cages for 48 h to collect urine. After the 6 weeks feeding regimen, the rats were anaesthetized with isoflourane overdose and blood collected by cardiac puncture to terminate the rats. Plasma was obtained from the blood samples by centrifugation at 5000 g for 10 min and stored at –80 ^{o}C for analyses. The above animal work protocols were

approved by the University of Manitoba Animal Care Protocol Management and Review Committee in accordance with guidelines from the Canadian Council for Animal Care.

7.2.4 Sample preparation

7.2.4.1 Extraction of urine metabolites for metabolomics studies

Before analysis, the frozen urine samples were thawed at room temperature and vortexed (10 sec). A 250 µL aliquot of each urine sample was placed in a clean 2 mL Eppendorf tubes and 10 µL of norvaline solution (0.03 mg/mL) added. The samples were centrifuged at 10,000 *g* for 10 min at room temperature. The supernatant was then removed, placed in clean 2 mL tube and 500 µL of acetonitrile added. The sample was vortexed (20 secs) and quenched at –20^{o}C for 30 min followed by centrifugation at 10,000 *g* for 20 min at room temperature. The supernatant was removed and dried completely under nitrogen evaporator and kept at -20°C prior to reconstitution for LC-QTOF-MS analysis. The dried samples were reconstituted in 200 µL of water:acetonitrile (4:1), vortexed (20 secs) and transferred to glass inserts in brown LC vials. Also, a blank consisting of 4:1 water:acetonitrile mixture was used throughout this study.

7.2.4.2 Extraction of plasma metabolites for metabolomics analysis

Frozen plasma samples were thawed on ice (20 min) and 100 µL aliquots mixed with acetonitrile (200 µL) in clean 2 mL eppendorf tubes. The mixture was vortexed vigorously (30 sec) and centrifuged for 10 min (10,000 x g at 4°C). The supernatant (approximately 250 µL) was transferred into a new 2 mL Eppendorf tube and completely dried for 1 h under a gentle stream of N_2 followed by storage at -20 ^{o}C until needed for LC-QTOF-MS analysis. Prior to LC-QTOF-MS injection, the dried samples were reconstituted with 100 µL of acetonitrile:deionized water 4:1 and transferred into glass inserts in brown LC vials.

7.2.5 LC-QTOF-MS analysis

LC-QTOF-MS metabolomics analysis was performed on a 1290 Infinity Agilent Rapid Resolution HPLC system containing a binary pump and degasser, well-plate auto-sampler with a thermostat, and thermostatic column compartment coupled to a 6538 UHD Accurate Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA) equipped with dual electro-spray ionization (ESI) source. An Agilent ZORBAX SB-Aq column 2.1mm×100mm, 1.8 µm was used for chromatographic separations of urine samples with the column temperature maintained at 60°C. The mobile phases A (water) and B (acetonitrile) contained 0.1% formic acid. The run time was set at 10 min with a gradient of 0-6 min 2% B; 6-8 min 60% B; 8-8.50 60% B; 8.50-8.60 min 2% B and 8.60-10 min 2% B with a post run time of 2 min prior to new injections. Sample carryover during every successive injection was minimized by washing the injection needle with the mobile phase in two separate vials (5 washings per vial) prior to each injection. The temperature of the auto sampler was maintained at 4°C throughout the runs. For individual urine sample analysis, 2 µL of urine extracts were injected and the flow rate was maintained at 0.7 mL/ min. For plasma metabolomics, a 3x50 mm, 2.7 µm Agilent Poroshell column (Agilent Technologies) and similar mobile phases A and B were used for separating the metabolites at 60°C column temperature. For individual plasma sample analysis, 2 µL of extracts were injected with the HPLC flow rate maintained at 0.7 mL/min using a gradient of 0, 0.5, 16, 17 and 22 min with 30, 30 100, 100 and 30% of solvent B, respectively. A post-run time of 2 min prior to new sample injection was used and the temperature of the auto sampler was maintained at 4°C.

The MS data acquisitions were done in the positive ionization mode. The parameters used included capillary voltage (4,000 V), the fragmentor (175 V), the skimmer (50 V) and the OCT 1 RFVpp (750 V). Nitrogen gas (N_2) used for drying was set to 11 L/min at 300°C while the nebulizer at 50 psig and MS spectra was acquired over 50-1,000 m/z range. The collision energy

was set at 25 V in targeted MS/MS mode and references with masses of 121.0508 and 922.0097 were used for all runs. Targeted MSMS analysis of the pure standards and urine/plasma extracts were subsequently used for identification of the potential biomarkers. In-built collision energy in MassHunter Software was applied using appropriate equation with a slope of 5 and offset value of 2.5. A full range mass scan from 50-1200 *m/z* with an extended dynamic range of 2 GHz standardized at 3200 was applied. For data acquisition, the rate was maintained at 3 spectra/s using a time frame of 333.3 ms/spectra and a transient/spectrum ratio of 1932. The Molecular Feature Extraction (MFE) was applied to all replicates and features with abundance > 4000 counts were extracted. Average values for RT and m/z values of each feature were then calculated.

7.2.6 Data Processing and Statistical Analysis

The metabolomics workflow employed for the LC-QTOF- MS data processing comprised several algorithms used by Agilent MassHunter Qualitative (MHQ, B.05) and Mass Profiler Professional (MPP, 12.6). The raw chromatographic data files were first acquired and stored as "*.d" files using an Agilent MassHunter Acquisition software (B.05) for processing in MHQ. The MFE which is a naïf extraction process was the first algorithm applied to the total ion chromatograms (TIC) files. The settings of the MFE parameters allows extraction of detected features with absolute abundances > than 4,000 counts giving information regarding $[M + H]^+$, isotopes and their corresponding Na^+ adducts. These extracted ions were then treated as single features used to generate potential formula. The collected information (retention time, RT, exact masses and ion abundances) was then converted into compound exchange format ("*.cef") and exported to MPP for further comparative and statistical analyses. The individual "*.cef" files were binned and combined to generate new "*.cef" files through alignment and normalization

and ‘Find by ion’ algorithm was subsequently used in MHQ for further data mining procedures. This targeted feature algorithm helped to minimize the false positive and negative features found by MFE procedure. A second set of individual “*.cef” files were also generated from the original individual “*.d” files and exported into MPP for statistical and differential analysis. The features detected in at least one condition were accepted using a frequency filtration employed to ensure that potential feature extraction artifacts were not eliminated.

Other MPP filtration procedures used included number of detected ions (set to ‘2’) and charge states (set to ‘all charge states permitted’). The RT compound alignment parameters were set to 0.15 min with a mass tolerance of 2.0 mDa. All data were normalized using a percentile shift algorithm set to 75 and they were baselined to median of all samples. Norvaline was employed as internal reference standard to correct for potential differences in diuresis between animals that may contribute to variations in concentration differences. Moderated T-Test ($P<0.01$), volcano plots (> 2 fold changes and $P<0.01$) and Partial least square (PLS) statistical analyses were performed using MPP software (version 12.6).

7.2.7 Identification of urine metabolites/biomarkers

Metabolites or potential biomarkers contributing to the discrimination with at least two-fold significant changes (≥ 2-fold changes, $P<0.05$) in any of the different groups of rats were first annotated based on their exact mass data corresponding to *m/z* peaks by searching them against Metlin (> 64,000 metabolites) and SimLipids (> 39,000 lipids) database. Their identities were further confirmed using their mass spectra and retention times compared to pure commercial standards when available. Mass fragmentation experiments were also conducted on the standards, urine and plasma samples. The compounds for which pure standards were not available were identified and confirmed either by comparison of their ion fragmentation (MS/MS

spectra) from previously published studies or percent confidence score based on exact mass. A mass error of less than 5 ppm was set to formulate the elemental composition.

7.3. Results and discussions

Our previous results showed that SHRs fed 1% (w/w) CSPH had significant SBP-lowering effect (-36 mmHg) better than the control (casein only diet, 6 mmHg), 0.5% (w/w) CSPH (-31 mmHg), 1% (w/w) CSM (-26 mmHg) diet and the normotensive WKY rats. However, analysis of the plasma antihypertensive and antioxidant activities did not reveal the necessary information with regards to the underlying cause responsible to the SBP lowering effect beyond a significant reduction (inhibition) in plasma ACE activity for the CSPH treatment groups. We attributed the unexpected reduced antioxidant enzymes activities to be due probably to the age of the rats (42 weeks) and possibly the study duration (6 weeks). Several mechanisms and pathways are known to regulate the development and progression of hypertension. However, since our initial *in vivo* results did not reveal any of such mechanisms/pathways beyond ACE inhibition (RAS pathway), we postulated that the results of the metabolomics approach used in this trial may give more insights into other mechanisms/metabolic pathways that may be responsible for the observed SBP-lowering effect of the CSPH intervention. To achieve this goal, we investigated the urine and plasma using a non-targeted approach in order to generate metabolite profiles for each of the intervention groups. These metabolite profiles were subsequently analyzed to determine if any of the metabolites that changed significantly (up-regulated or down-regulated) may be associated with activated pathways in the hypertension regulatory system.

LC-QTOF-MS was used to detect small molecule metabolites (entities) in rat's urine and plasma in the positive mode. Though some differences in TIC were observed for the different

rats groups, more subtle changes for the metabolites/entities were found by pattern recognition approach using principal component analysis (PCA) and partial least square – discriminant analysis (PLS-DA) because they are better suited to statistically differentiate clusters produced by different groups. The PLS analyses of multivariate data of urine and plasma metabolites/entities of all rat groups in this study showed clear separations. A total of 388 entities out of 6209 detected in the urine samples were significantly different after a one way ANOVA ($P<.05$) while for the plasma samples, a total of 185 out of 4189. Recursive analysis was then performed on these entities that were changing significantly in both the urine and plasma samples. A T-test ($P< 0.05$) between the SHRs at baseline and termination, SHRs and normotensive WKY rats, SHR control rats and the various treatments urine and plasma samples (at baseline and termination) also revealed significant changes in the concentrations of the entities.

Since our previous study suggested that the 1% CSPH diet group had the most significant functionality with respect to lowering of SBP, a fold change and a moderated T- statistical test were performed between the 1% CSPH group urine and plasma samples at baseline and termination in order to identify the entities that are significantly different ($p < 0.05$) with a ≥ 2 fold change. The PLS analyses of multivariate data of metabolites/entities that were detected in urine and plasma of this rats group at baseline and termination also showed a clear separation (Figure 7.1 and 7.3) respectively. The volcano plot (Figure 7.2) suggests that 120 entities/metabolites were significantly changing in urine of SHRs fed CSPH 1% diet intervention while Figure 7.4 showed 185 entities/metabolites were also changed in the plasma of SHRs fed same intervention diet. Each of these metabolites is represented by a box in the volcano plot. Among the metabolites/entities that were significantly changed, 25 and 28 entities/metabolites in

Figure 7.1. The partial least square (PLS) analyses of multivariate data of metabolites/entities that were detected in urine of all SHR rats fed 1% (w/w) CSPH diet intervention at baseline and termination (6 weeks)

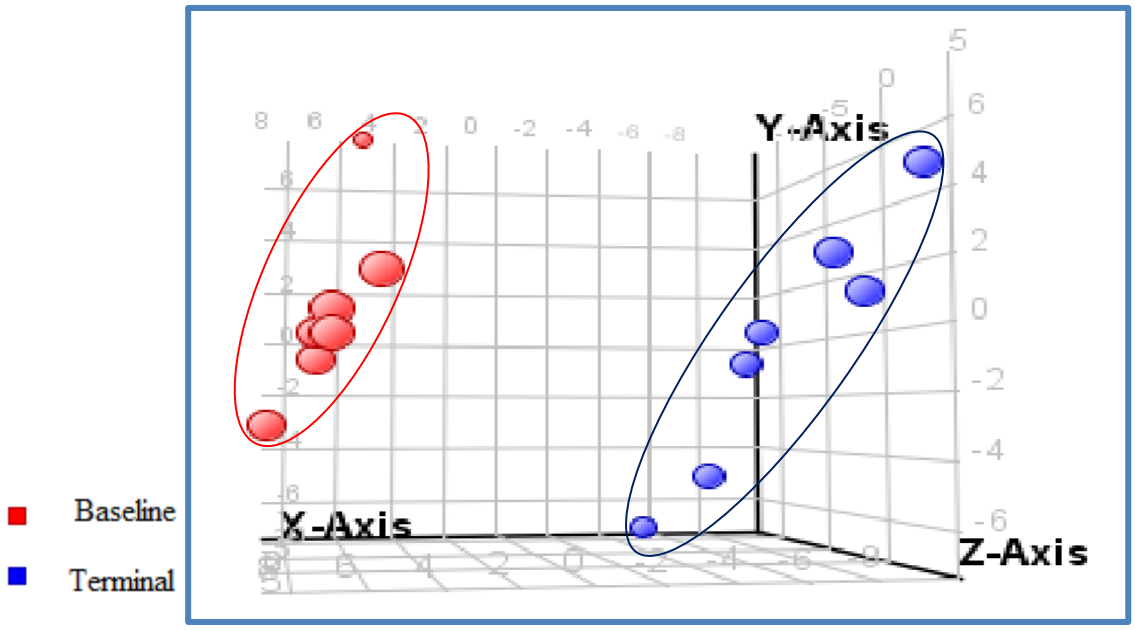
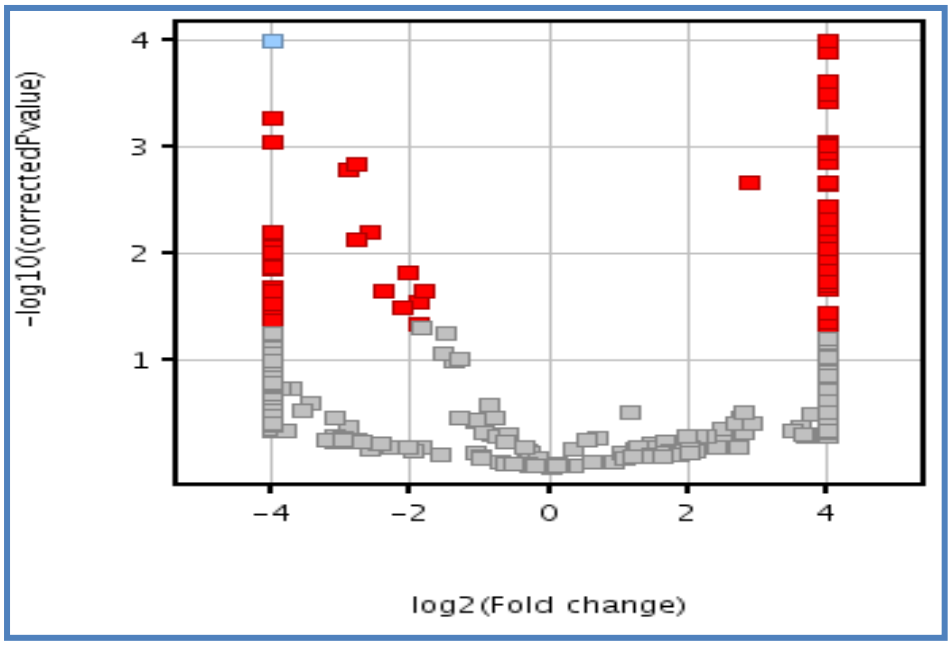


Figure 7.2. Volcano plot of the 120 entities/metabolites that changed significantly ($p<0.05$) in urine of SHR rats fed CSPH 1% diet intervention at termination (6 weeks) compared to baseline (0 week)



the urine and plasma respectively, were annotated based on their exact mass and MS information using "ID browser" and MassHunter Qualitative software available in Metlin database, which contained approximately 64,000 metabolites. The identities of the metabolites/entities were also confirmed by comparing the fragmentation patterns of their ions using either pure commercial standards or the MS/MS spectra available in the libraries. The related metabolic pathways were further searched in KEGG pathways using the single experiment analysis within the pathway analysis of the MPP software.

N_2-Acetyl-L-ornithine (*m/z* 175.1077) with elution time of 2.96 mins is given here to illustrate the process used in the identification of the biomarkers of significant importance in the pathophysiology of hypertension. $C_7H_{14}N_2O_3$ was generated in the ESI (+ mode) as the most probable molecular formula for this metabolite from which the MS/MS spectrum information was then used to obtain the compound molecular structure. The elemental composition, fragmentation pattern and MS/MS of the metabolite were compared to the Metlin database in order to confirm the presence of N2-acetyl-L-ornithine. The other compounds that might be responsible for observed blood pressure-lowering effects in SHRs fed CSPHs were confirmed in similar manner (Table 7.1).

Though hypertension is reported to be a complex multifactorial disorder with several influencing metabolic pathways, arginine metabolism is well-known. Arginine is a very important amino acid because it is a known precursor of nitric oxide (NO), a vasodilator involved in blood pressure regulation (Godzien, Garcia-Martinez, Martinez-Alcazar, Ruperez, & Barbas, 2013; Hsu, Huang, Lau, Lin, & Tain, 2012). NO is produced in the blood vessels by endothelial nitric oxide synthase (eNOS) which catalyzes the conversion of L-arginine to L-citrulline with potent vasoactive actions (Elms et al., 2013). NO deficiency or reduced levels

Figure 7.3. The PLS analyses of multivariate data of metabolites/entities that were detected in plasma of SHRs rats fed CSPH 1% diet intervention at baseline and termination (6 weeks)

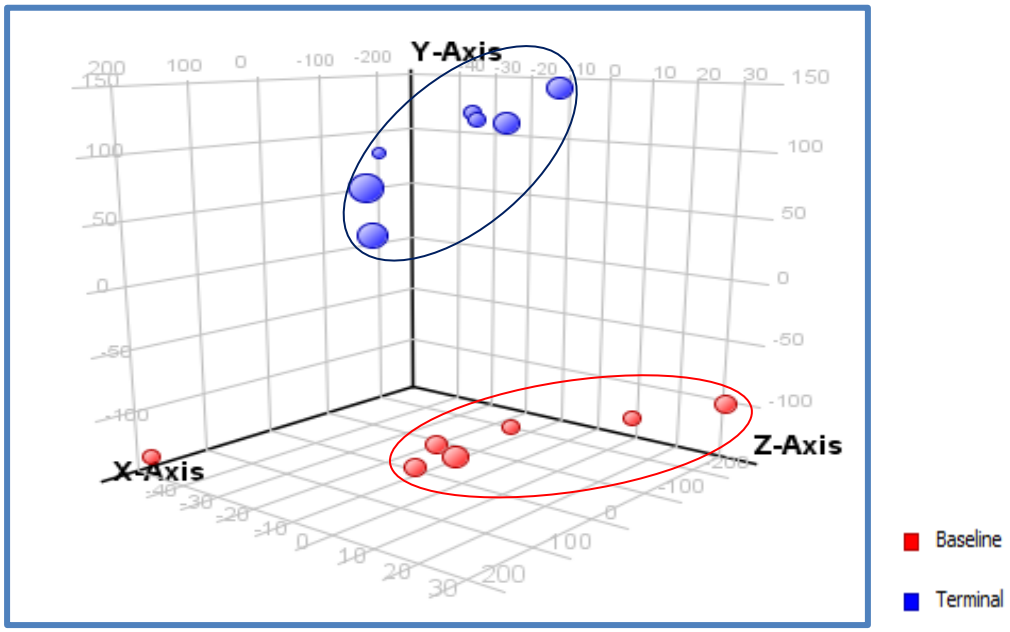
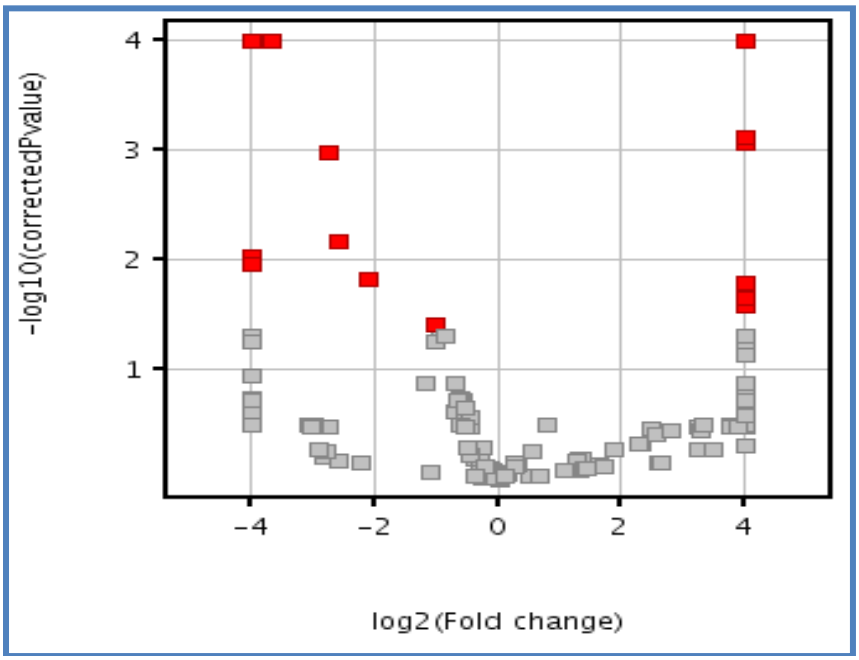


Figure 7.4. Volcano plot of the 28 entities/metabolites that changed significantly ($p<0.05$) in plasma of SHR rats fed CSPH 1% diet intervention at termination (6 weeks) compared to baseline (0 weeks)



therefore, is closely associated with arteriosclerosis, hypertension, diabetic and hypertensive organ damage (Elms et al., 2013; Hsu et al., 2012). Though NO deficiency is multifactorial in nature, increased levels of asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) have been implicated as biomarkers of NO availability, various cardiovascular diseases, hypertension, endothelial dysfunction and renal function (Hsu et al., 2012; Pullamsetti et al., 2005). While ADMA inhibits eNOS activities by direct competition for L-arginine in NO production, SDMA on the other hand indirectly inhibits NO availability by competing with L-arginine for transporter uptake (Hsu et al., 2012; Kielstein, Fliser, & Veldink, 2009; Pullamsetti et al., 2005). Reduction in plasma ADMA by melatonin treatment caused reductions in blood pressure, in addition to the preservation of renal L-arginine availability as well as improved plasma L- arginine-ADMA ratio (AAR) in SHRs, which suggests that L-arginine and ADMA may be considered as potential biomarkers for disease prediction as well as responses to therapeutic treatments (Hsu et al., 2012; Tain, Huang, Lin, Lau, & Lin, 2010). In addition to AAR, the plasma ADMA-SDMA ratio (ASR) was reported to be a useful biomarker for hypertension. Considering the importance of ADMA and SDMA in the metabolic pathway for arginine synthesis and NO production, SDMA, which was significantly down-regulated in the SHRs at the end of the experiment was annotated using exact mass and MS information. However, a list of the entities/metabolites under that notation gave 2 additional isobaric possibilities (ADMA and Arg-OEt) with similar molecular formula, mass and score. An analysis of the MS/MS profile suggests that it is unlikely to be ADMA but possibly SDMA. The down-regulation in the level of SDMA in SHRs fed 1% CSPH intervention diet suggests that the peptides suppressed the development of the metabolites thereby preserving L-arginine and

Table 7.1. Selected metabolites with significant changes (≥ 2-fold changes, P<0.05) in urine and plasma of spontaneously hypertensive rats (SHR) fed chicken skin protein hydrolysate 1% (CSPH 1%) at baseline and termination.

Metabolites	**Biofluid**	**m/z (ESI +)**	**RT (Min)**	**Formula**	**Identification mode**	**Associated pathways**	**Direction of change**	**†Abundance**	
								Baseline *vs*. Terminal	
Symmetric Dimethylarginine SDMA	Urine	203.1490	2.970	$C_8H_{18}N_4O_2$	MS	Arginine metabolism [22]	Up-regulated	3.6±2.9	ND
N_2-acetyl-L-ornithine	Urine	175.1077	2.946	$C_7H_{14}N_3O_2$	MS/MS	Arginine metabolism [27]	Down-regulated	19.6±12.0	9.8±2.4
N_1-Acetylspermidine	Urine	188.1760	1.545	$C_9H_{21}N_3O$	MS/MS	Polyamines metabolism [28]	Up-regulated	3.2±1.2	21.4±4.3
Buthionine sulfoximine	Urine	223.1107 *245.0925	2.230	$C_8H_{18}N_2O_3S$	MS	Oxidative stress [30]	Down-regulated	4.8±2.3	ND
Uric acid	Urine	169.0352	2.228	$C_5H_4N_4O_3$	MS	Activation of RAS/ROS	Down-regulated	39.7±3.6	26.9±20.4

						generation [32]			
Vitamin E succinate	Plasma	531.4101	14.895	$C_{33}H_{54}O_5$	MS	Free radical	Down-regulated	6.3±0.4	ND
(α-tocopherol succinate)		*553.3908				scavenging [34]			
Pentahomomethionine	Urine	220.1365	4.266	$C_{10}H_{21}NO_2S$	MS/MS	Glucosinolate	Up-regulated	3.4±1.3	108.6±31.4
						metabolism [28]			
L-isoleucine	Urine	132.1013	1.774	$C_6H_{13}NO_2$	MS/MS	Amino acid	Up-regulated	5.4±2.8	14.7±5.5
						metabolism [36]			
Creatinine	Urine	114.0663	1.687	$C_4H_7N_3O$	MS/MS	Arginine and	Up-regulated	81.9±27.1	309.9±50.4
						proline			
						metabolism [21]			
Tranexamic acid	Plasma	158.1174	0.355	$C_8H_{15}NO_2$	MS	-	Down-regulated	10.5±1.6	ND
13-Docosenamide	Plasma	338.4244	12.683	$C_{22}H_{43}NO$	MS	-	Up-regulated	ND	37.1±24.4
PS(O-20:0/13:0)	Plasma	736.5439	16.337	$C_{39}H_{78}NO_9P$	MS	Phospholipids	Up-regulated	ND	4.8±1.1
						metabolism [1]			
PS(O-18:0/13:0)	Plasma	708.5129	16.343	$C_{37}H_{74}NO_9P$	MS	Phospholipids	Up-regulated	ND	21.8±5.1
						metabolism [1]			

[†]***Abundance: The area obtained for the peaks corresponding to selected metabolites;*** [¥]***ND: not detected or abundance of the mass was below the cut-off threshold level.*** [‡]***Standard Deviation, *[M+H +Na]***$^+$**.**

invariably, making NO more available for its vasodilative roles. This may possibly result from reduced synergy with ADMA thereby preventing eNOS inhibition and consequently, enhanced pulmonary vasodilation and reduced blood pressure (Pullamsetti et al., 2005).

N_2-acetyl-L-ornithine confirmed by MS/MS analysis is known to also play a major role in arginine and proline metabolism. According to Xu, Labedan, & Glansdorff (2007), acetylation of L-glutamate at the *N*-α position by amino-acid N-acetyltransferase (EC 2.3.1.1) is the first step in the arginine biosynthetic pathway. This acetylation process of the early arginine precursors differentiates them from intermediates of proline biosynthesis and prevents spontaneous cyclization of the semialdehyde arginine precursor. In this pathway, the acetylated precursors are continuously formed until acetylornithine. However, the subsequent formation of intermediate products (ornithine) is catalyzed by either acetylornithine deacetylase (acetylornithinase, AO) (EC 3.5.1.16) or ornithine acetyltransferase, OAT (EC 2.3.1.35), which invariably involves recycling of the glutamate acetyl group. Ornithine is subsequently converted into arginine via citrulline and arginosuccinate. As had been stated previously, arginine is a precursor of the endothelial vasodilator, NO. Therefore the role of N_2-Acetyl-L-Ornithine in the attenuation of blood pressure is very crucial. The metabolite was significantly ($p<0.05$) down-regulated in the urine samples by the intervention diet at termination when compared to baseline data. Though, the exact mechanism responsible for this reduction in N_2-acetyl-L-ornithine is unknown, however, it may likely be due to enhanced activities of the enzymes responsible for its conversion to ornithine and invariably arginine and citrulline (Xu et al., 2007). This may also have been responsible for reduction in SHRs SBP in this group since its utilization would have implied increased NO production and therefore, greater vasodilation.

Ornithine is also a precursor of polyamines, urea and creatine (it is converted to guanidinoacetate which reacts with glycine to form creatine) and subsequently creatinine (Godzien et al., 2013). This may likely explain the up-regulation in the content of N_1-acetylspermidine and creatinine in the urine samples of the rats. Though polyamines and in particular acetylated polyamines are known to play essential roles in cell growth and differentiations, their association with hypertension is speculative, very remote and still not well understood (Orlinska, Olson, Gebb, & Gillespie, 1989). Evidence from our previous SBP study does not also support this position as this particular rat group had the most SBP reduction. In addition, increased levels of spermidine and spermine had previously been reported to be associated with folate deficiency (Godzien et al., 2013). It is however not possible to precisely attribute the increase observed in the present study to folate deficiency since this metabolite was not determined.

Though creatine and creatinine are known biomarkers of renal failure, it is however difficult to conclude that the up-regulation in creatinine is associated with kidney problem especially given the association between N_2-acetyl-L-ornithine and creatine in the formation of creatinine. The higher creatine and creatinine contents observed in the SHRs at termination could therefore, be probably due to the synthesis of creatine (and consequently creatinine) from guanidinoacetate utilizing S-adenosyl methionine as a major requirement (Godzien et al., 2013). It may also have been possible that the peptides acted as antihypertensive agent to reduce SBP without actually affecting any change in metabolites (Aa et al., 2010) or that the changes were already irreversible due to age of the rat at the commencement of the trial.

Oxidative stress has been demonstrated to play a major role in elevation of SBP in many animal and experimental models of hypertension even though human results have been less

consistent (Vargas et al., 2012). In one of such studies, oxidative stress and hypertension were induced in normal Sprague Dawley rats given oral administration of buthionine sulfoximine (Vaziri, Wang, Oveisi, & Rad, 2000). Buthionine sulfoximine is known to cause the depletion of glutathione by selectively inhibiting an important enzyme (γ-glutamylcysteine) in glutathione synthetic pathway. However, administration of antioxidant therapy was reported to attenuate the induced hypertension in this model, suggesting the role of oxidative stress in hypertension (Vaziri et al., 2000). Buthionine sulfoximine-induced oxidative stress has been implicated in cardiovascular and renal disorders especially elevated BP and increased heart rate (Vargas et al., 2012). Buthionine sulfoximine was significantly ($p<0.05$) reduced in the SHR urine at termination, which suggests that SBP attenuation in this group may probably be due to reduced oxidative stress. It is therefore strongly believed that the CSPH intervention diet may have acted as an antioxidant to reverse the oxidative stress and consequently, the high baseline SBP. We had previously reported CSPH to possess strong antioxidant properties, which could be beneficial in reducing oxidative stress (Onuh et al., 2014).

Uric acid was found to be significantly ($p<0.05$) reduced between the urine samples of the SHRs at baseline and termination. Hyperuricemia (elevated serum uric acid levels) is positively associated with cardiovascular disease risk, especially hypertension and is a biomarker for ischaemia, insulin resistance and vascular function (Ciceroa, Salvib, D'Addatoa, Rosticcia, & Borghia, 2013; Waring, Webb, & Maxwell, 2000). Though the underlying mechanisms for this relationship are still not properly elucidated, reduction in endothelial NO levels and stimulation of the vascular RAS, which leads to angiotensin II production with attendant proliferation of vascular smooth muscle cells and ROS generation has been implicated (Ciceroa et al., 2013). This may result in vasoconstriction, elevated BP, and subsequently, arteriosclerosis and

hypertension if not treated (Ciceroa et al., 2013). Therefore, the reduction in uric acid observed for the SHRs by the CSPH intervention diet probably explains the attenuated SBP that we previously reported (Onuh et al., 2015). We had also previously reported the peptides in CSPH to act as inhibitors of RAS (Onuh et al., 2013) and as antioxidants (Onuh et al., 2014).

The role of free radical generation in the pathogenesis of essential hypertension is well known (Newaz, Nawal, Rohaizan, Muslim, & Gapor, 1999; Young & Woodside, 2001). Free radicals may reduce NOS activity, and thereby decrease NO either by direct reduction of NO synthesis or disruption of endothelial receptor signal transduction. They can also act directly on NO and make it less available. Treatment of SHRs with α-tocopherol (an antioxidant) prevented development of age-related increase in BP by up-regulating NOS activity and NO levels in blood vessels (Newaz et al., 1999). The mechanism for this action was thought to involve free radical scavenging by α-tocopherol, which acts as an antioxidant. The vitamin E succinate (α-tocopherol succinate) levels in the SHR plasma was significantly ($p<0.05$) reduced by the CSPH intervention diet at termination when compared to baseline. The reduction may have resulted from increased utilization to scavenge free radicals in the blood vessels. Though, the exact mechanisms by which the peptides are involved in the antioxidant action of vitamin E is not yet known, however, it may probably be synergistic in nature. We had previously attributed the depletion of antioxidant enzymes at termination to be probably due to age-related irreversible oxidative stress induced changes. The peptides may also inhibit oxidation directly without any synergistic improvement of the vitamin E level (Aa et al., 2010).

Another important metabolite that was significantly ($p<0.05$) down-regulated in the plasma samples of SHRs by the CSPH intervention is tranexamic acid. Tranexamic acid is a commonly used anti-fibrinolytic drug given to patients with menorrhagia, haemophilia and other

bleeding conditions. However, it has been reported to induce arterial thrombosis and myocardial infarction though the exact mechanism has not yet been elucidated (Sirker, Malik, Bellamy, & Laffan, 2008). The reduction of tranexamic acid in the SHRs fed CSPH intervention diet at termination may likely suggest the suppression of this deleterious metabolite by the peptides. This reduction in plasma tranexamic acid was positively related to reduction in SBP, which indicates a likely pathway towards hypertension attenuation.

Also, phospholipids annotated as PS (m/z 736.5439), PS (m/z 708.5129) and PE (19:0/0:0) were found to be significantly ($p<0.05$) up-regulated in the plasma of SHRs fed CSPH intervention diet at termination when compared to the baseline values. This may indicate abnormality in phosphate metabolism as phosphates have been reported to emulsify and dissolve fats in plasma, thereby preventing hypertension caused by vasoconstriction (Jiang et al., 2012). Though the exact cause of the phospholipids elevation is still unknown, it may be attributed to age-related irreversible oxidative stress-induced changes as previously stated. Contrary to the above observations, however, 2 triglycerides, TG(17:/17:1) and TG(17:0/17:2) were significantly ($p<0.05$) down-regulated in the urine of SHRs fed CSPH intervention diet at termination when compared to baseline, which suggests peptide-dependent suppression of fat oxidation.

7.4. Conclusions

CSPH dietary intervention in SHRs model using a non-targeted metabolomics approach revealed several distinct urinary and plasma metabolites changes that may be considered biomarkers responsible for SBP and hypertension attenuation. The metabolite changes support the evidence of a potential mechanism that involve the vasodilative actions of NO production via the arginine metabolic pathway. The evidence also supports the inter-relationship between oxidative stress and hypertension and the antioxidant role for the hydrolysates and peptides by

inhibiting formation and/or by scavenging of free radicals. The study demonstrated possible alternate mechanisms for the attenuation of hypertension and blood pressure control beyond ACE and renin inhibitions. To the best of our knowledge, this is the first metabolomics study to evaluate metabolite profiles changes in CSPH-fed SHR as related to SBP and hypertension attenuation. This study could therefore, promote the CSPH use as an ingredient in the development of nutraceuticals and functional foods for hypertension management. It could also enhance value-added utilization of chicken skin, which is currently mainly disposed as a waste product by the poultry industry while protecting the environment. Since this research may not be exhaustive in nature, further studies, especially using growing (young) SHRs need to be explored to better provide a more detailed understanding of the mechanisms underlying hypertension control by protein hydrolysates in general and CSPH in particular. Data from such studies could serve as a basis for future clinical trials to determine the antihypertensive efficacy of CSPH in humans.

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7.5 References

Aa, J. Y., Wang, G. J., Hao, H. P., Huang, Q., Lu, Y. H., Yan, B., Zha, W. B., Liu, L. S., & Kang, A. (2010). Differential regulations of blood pressure and perturbed metabolism by total ginsenosides and conventional antihypertensive agents in spontaneously hypertensive rats. *Acta Pharmacologica Sinica, 31*(8), 930-937.

Ahhmed, A. M., & Muguruma, M. (2010). A review of meat protein hydrolysates and hypertension. *Meat Science, 86*(1), 110-118.

Akira, K., Masu, S., Imachi, M., Mitome, H., Hashimoto, M., & Hashimoto, T. (2008). H-1 NMR-based metabonomic analysis of urine from young spontaneously hypertensive rats. *Journal of Pharmaceutical and Biomedical Analysis, 46*(3), 550-556.

Akira, K., Masu, S., Imachi, M., Mitome, H., & Hashimoto, T. (2012). A metabonomic study of biochemical changes characteristic of genetically hypertensive rats based on H-1 NMR spectroscopic urinalysis. *Hypertension Research, 35*(4), 404-412.

Aukema, H. M., Gauthier, J., Roy, M., Jia, Y., Li, H., & Aluko, R. E. (2011). Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. *Molecular Nutrition & Food Research, 55*(7), 1044-1051.

Bruce, S. J., Tavazzi, I., Parisod, V., Rezzi, S., Kochhar, S., & Guy, P. A. (2009). Investigation of Human Blood Plasma Sample Preparation for Performing Metabolomics Using Ultrahigh Performance Liquid Chromatography/Mass Spectrometry. *Analytical Chemistry, 81*(9), 3285-3296.

Ciceroa, A. F. G., Salvib, P., D'Addatoa, S., Rosticcia, M., & Borghia, C. (2013). Association between serum uric acid, hypertension, vascular stiffness and subclinical atherosclerosis: data from the Brisighella Heart Study. *Journal of Hypertension, 32*, 8.

Elms, S., Chen, F., Wang, Y. S., Qian, J., Askari, B., Yu, Y. F., Pandey, D., Iddings, J., Caldwell, R. B., & Fulton, D. J. R. (2013). Insights into the arginine paradox: evidence against the importance of subcellular location of arginase and eNOS. *American Journal of Physiology-Heart and Circulatory Physiology, 305*(5), H651-H666.

Girgih, A. T., Alashi, A., He, R., Malomo, S. A., & Aluko, R. E. (2013). Preventive and treatment effects of a hemp seed (Cannabis sativa L.) meal protein hydrolysate against high blood pressure in spontaneously hypertensive rats. *European Journal of Nutrition*.

Girgih, A. T., Alashi, A. M., He, R., Malomo, S. A., Raj, P., Netticadan, T and Aluko, R. E. (2014). Hemp Seed Meal Protein Hydrolysate Reduces Oxidative Stress Factors in Spontaneously Hypertensive Rats. *Nutrients* 2014, *6*, 5652 – 5666.

Godzien, J., Garcia-Martinez, D., Martinez-Alcazar, P., Ruperez, F. J., & Barbas, C. (2013). Effect of a nutraceutical treatment on diabetic rats with targeted and CE-MS non-targeted approaches. *Metabolomics, 9*(1), S188-S202.

Hsu, C. N., Huang, L. T., Lau, Y. T., Lin, C. Y., & Tain, Y. U. (2012). The combined ratios of L-arginine and asymmetric and symmetric dimethylarginine as biomarkers in spontaneously hypertensive rats. *Translational Research, 159*(2), 90-98.

Jiang, H. Q., Nie, L., Li, Y. L., & Xie, J. (2012). Application of ultra-performance liquid chromatography coupled with mass spectrometry to metabonomic study on spontaneously hypertensive rats and intervention effects of Ping Gan prescription. *Journal of Separation Science, 35*(4), 483-489.

Kielstein, J. T., Fliser, D., & Veldink, H. (2009). Asymmetric Dimethylarginine and Symmetric Dimethylarginine: Axis of Evil or Useful Alliance? *Seminars in Dialysis, 22*(4), 5.

Kim, M. J., Yang, H. J., Kim, J. H., Ahn, C. W., Lee, J. H., Kim, K. S., & Y., K. D. (2013). Obesity-Related Metabolomic Analysis of Human Subjects in Black Soybean Peptide Intervention Study by Ultraperformance liquid Chromatography and Quadrupole-Time-of-Flight Mass Spectrometry. *Journal of Obesity*, 11.

Kristensen, M., Engelsen, S. B., & Dragsted, L. O. (2012). LC-MS metabolomics top-down approach reveals new exposure and effect biomarkers of apple and apple-pectin intake. *Metabolomics, 8*(1), 64-73.

Law, W. S., Huang, P. Y., Ong, E. S., Ong, C. N., Li, S. F. Y., Pasikanti, K. K., & Chan, E. C. Y. (2008). Metabonomics investigation of human urine after ingestion of green tea with gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry and H-1 NMR spectroscopy. *Rapid Communications in Mass Spectrometry, 22*(16), 2436-2446.

Lu, Y., A, J., Wang, G., Hao, H., Huang, Q., Yan, B., Zha, W., Gu, S., Ren, H., Zhang, Y., Fan, X., Zhang, M., & Hao, K. (2008). Gas chromatography/time-of-flight mass spectrometry based metabonomic approach to differentiation hypertension- and age-related metabolic variation in spontaneously hypertensive rats. *Rapid Communications in Mass Spectrometry, 22*, 7.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). Modification of Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Analytical Biochemistry, 87*(1), 206-210.

Newaz, M. A., Nawal, N. N. A., Rohaizan, C. H., Muslim, N., & Gapor, A. (1999). alpha-tocopherol increased nitric oxide synthase activity in blood vessels of spontaneously hypertensive rats. *American Journal of Hypertension, 12*(8), 839-844.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Research International, 53*(1), 260-267.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2014). In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry, 150*(0), 366-373.

Onuh, J. O., Girgih, A. T., Malomo, S. A., Aluko, R. E., & Aliani, M. (2015). Kinetics of *in vitro* renin and angiotensin converting enzyme inhibition by enzymatic chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. *Journal of Functional Foods,* 14, 133 – 143.

Orlinska, U., Olson, J. W., Gebb, S. A., & Gillespie, M. N. (1989). Acetylated Polyamines in Lungs from Rats with Monocrotaline-Induced Pneumotoxicity. *Fundamental and Applied Toxicology, 13*(2), 277-284.

Pullamsetti, S., Kiss, L., Ghofrani, H. A., Voswinckel, R., Haredza, P., Klepetko, W., Aigner, C., Fink, L., Muyal, J. P., Weissmann, N., Grimminger, F., Seeger, W., & Schermuly, R. T. (2005). Increased levels and reduced catabolism of asymmetric and symmetric dimethylarginines in pulmonary hypertension. *Faseb Journal, 19*(6), 1175-+.

Roberts, M. D., Cruthirds, C. L., Lockwood, C. M., Pappan, K., Childs, T. E., Company, J. M., Brown, J. D., Ryan G. Toedebusch, R. G., & Booth, F. W. (2013). Comparing serum responses to acute feedings of an extensively hydrolyzed whey protein concentrate versus a native whey protein concentrate in rats: a metabolomics approach. *Appl. Physiol. Nutr. Metab., 39*, 10.

Simao, S., Gomes, P., Pinto, V., Silva, E., Amaral, J. S., Igreja, B., Afonso, J., Serrao, M. P., Pinho, M. J., & Soares-da-Silva, P. (2011). Age-related changes in renal expression of oxidant and antioxidant enzymes and oxidative stress markers in male SHR and WKY rats. *Experimental Gerontology, 46*(6), 468-474.

Sirker, A., Malik, N., Bellamy, M., & Laffan, M. A. (2008). Acute myocardial infarction following tranexamic acid use in a low cardiovascular risk setting. *British Journal of Haematology, 141*(6), 907-908.

Tain, Y. L., Huang, L. T., Lin, I. C., Lau, Y. T., & Lin, C. Y. (2010). Melatonin prevents hypertension and increased asymmetric dimethylarginine in young spontaneous hypertensive rats. *Journal of Pineal Research, 49*(4), 390-398.

Theodoridis, G., Gika, H. G., & Wilson, I. D. (2008). LC-MS-based methodology for global metabolite profiling in metabonomics/metabolomics. *Trac-Trends in Analytical Chemistry, 27*(3), 251-260.

Vargas, F., Rodríguez-Gómez, I., Pérez-Abud, R., Tendero, P. V., Baca, Y., & Wangensteen, R. (2012). Cardiovascular and Renal Manifestations of Glutathione Depletion Induced by Buthionine Sulfoximine. *American Journal of Hypertension, 25*(6), 7.

Vaziri, N. D., Wang, X. Q., Oveisi, F., & Rad, B. (2000). Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. *Hypertension, 36*(4), 688-688.

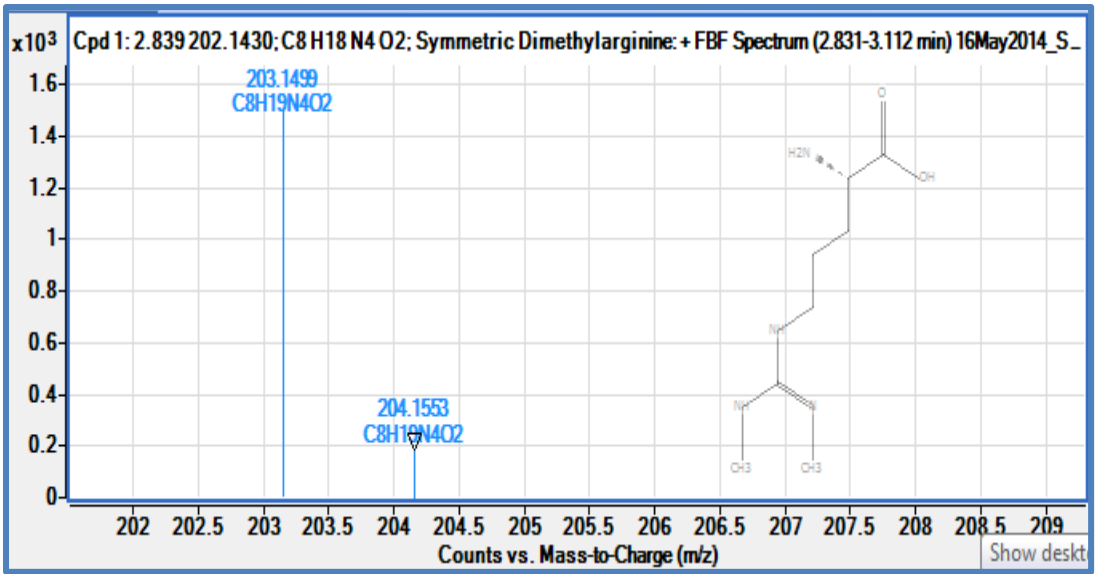
Vercruysse, L., Van Camp, J., & Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. *Journal of Agricultural and Food Chemistry, 53*(21), 8106-8115.

Vermeirssen, V., Camp, J. V., & Verstraete, W. (2004). Bioavailabilty of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition, 92*, 10.

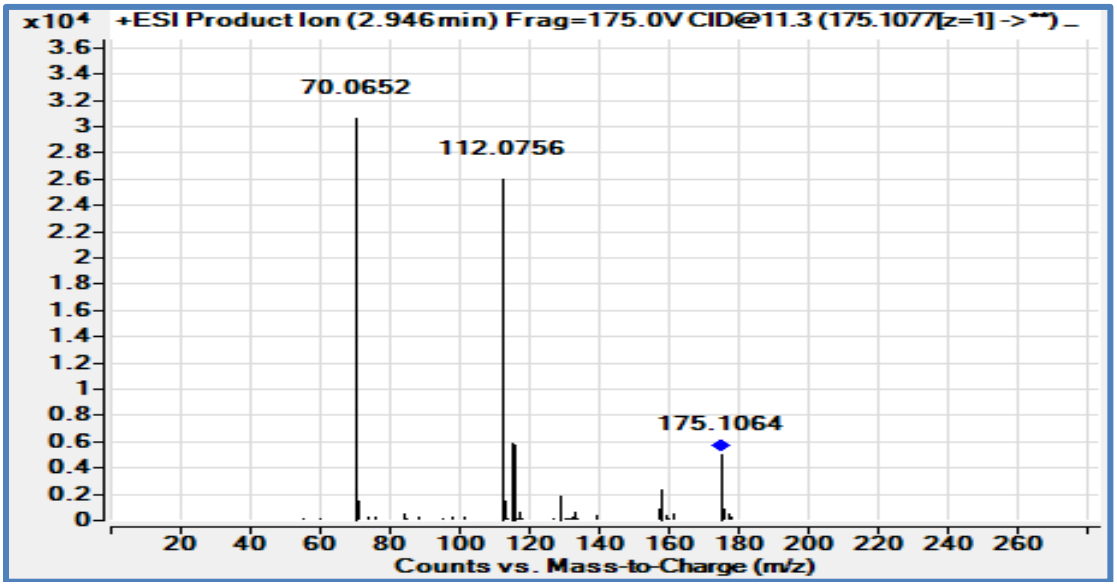
Waring, W. S., Webb, D. J., & Maxwell, S. R. J. (2000). Uric acid as a risk factor for cardiovascular disease. *Qjm-Monthly Journal of the Association of Physicians, 93*(11), 707-713.

Xu, Y., Labedan, B., & Glansdorff, N. (2007). Surprising arginine biosynthesis: a reappraisal of the enzymology and evolution of the pathway in microorganisms. *Microbiology and Molecular Biology Reviews, 71*(1), 36-47.

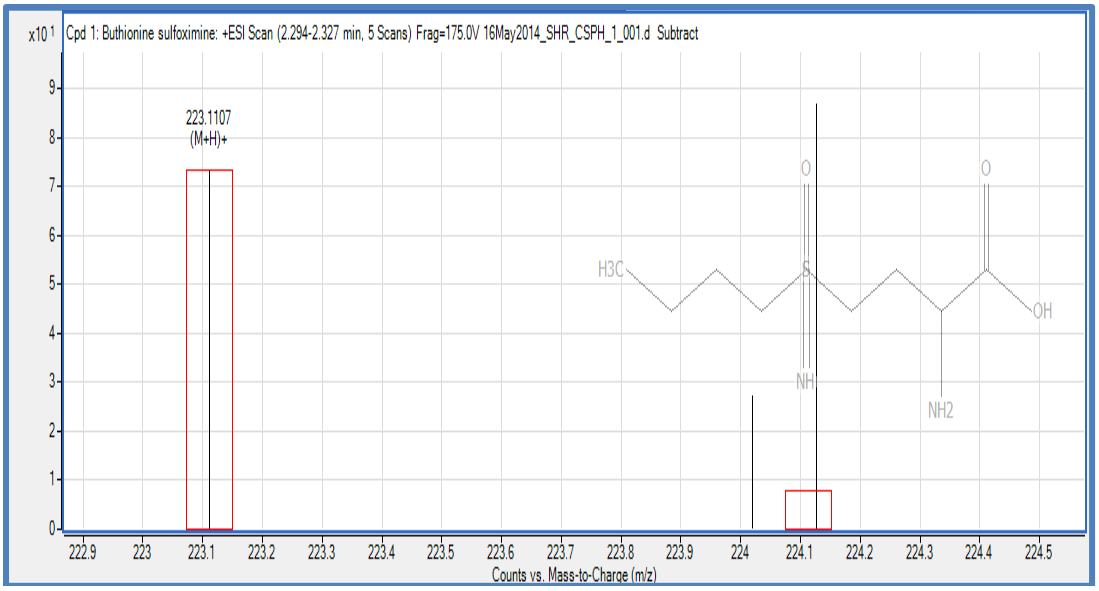
Young, I. S., & Woodside, J. V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology, 54*(3), 176-186.



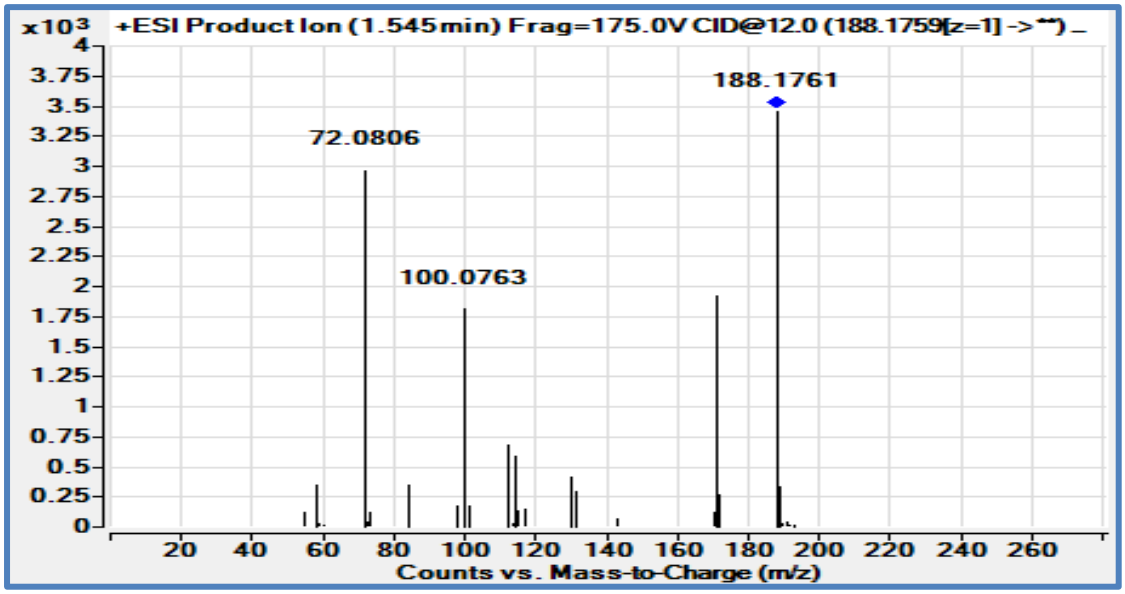
Supplementary material Figure 1. Mass spectrum of Symmetric Dimethylarginine



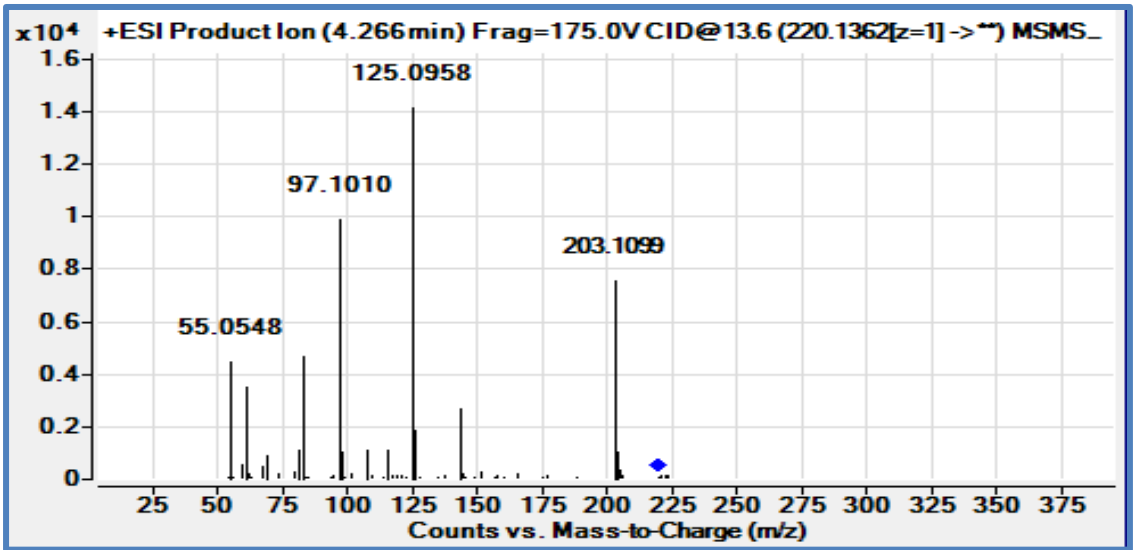
Supplementary material Figure 2. Mass spectrum of N_2-Acetyl-L-Ornithine



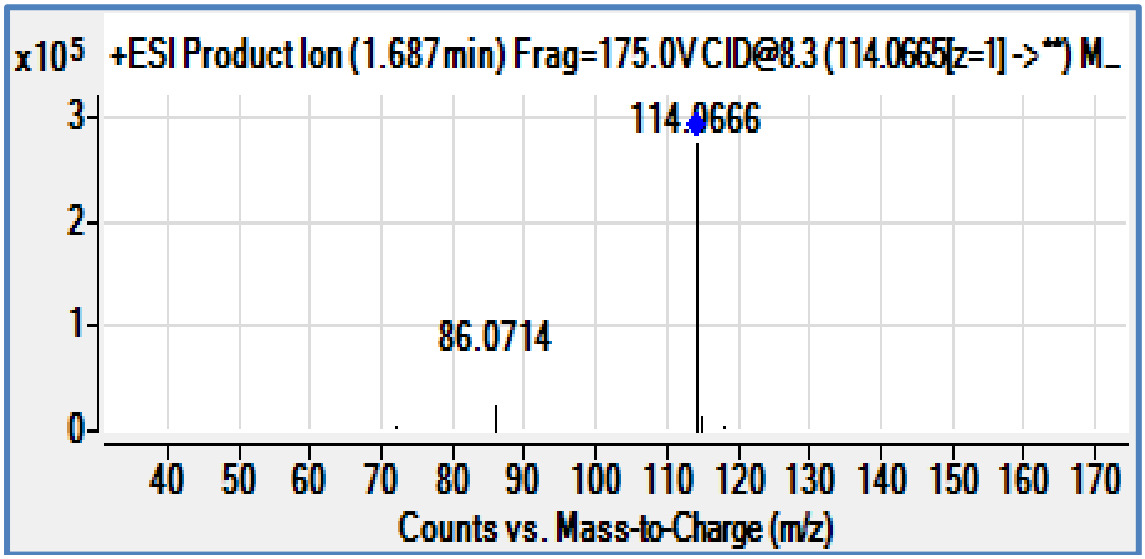
Supplementary material Figure 3. Mass spectrum of Buthionine sulfoxime



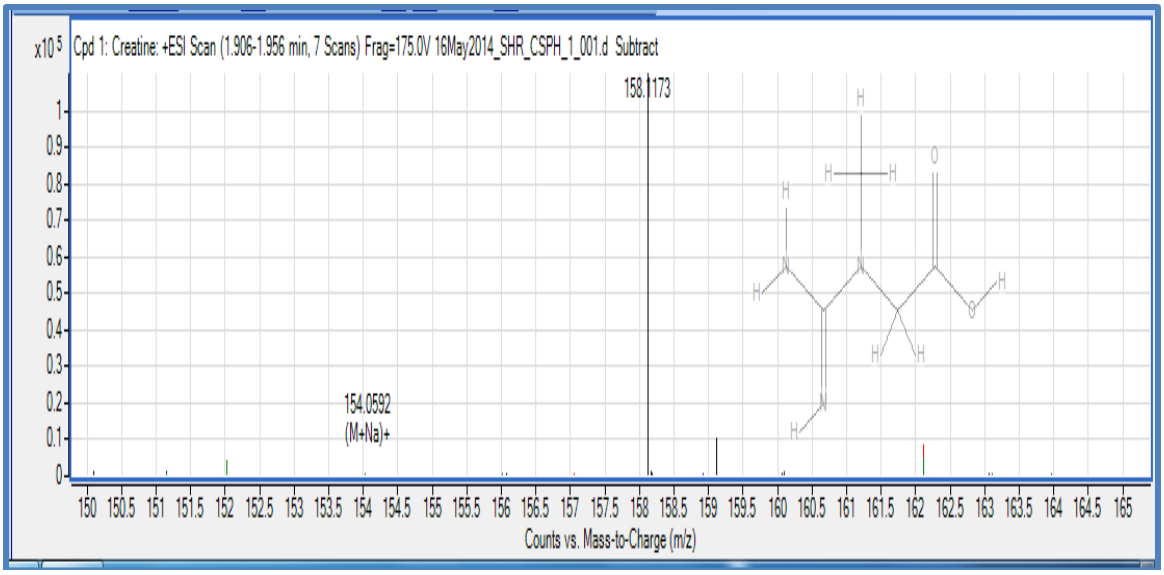
Supplementary material Figure 4. Mass spectra N_1-Acetylspermidine



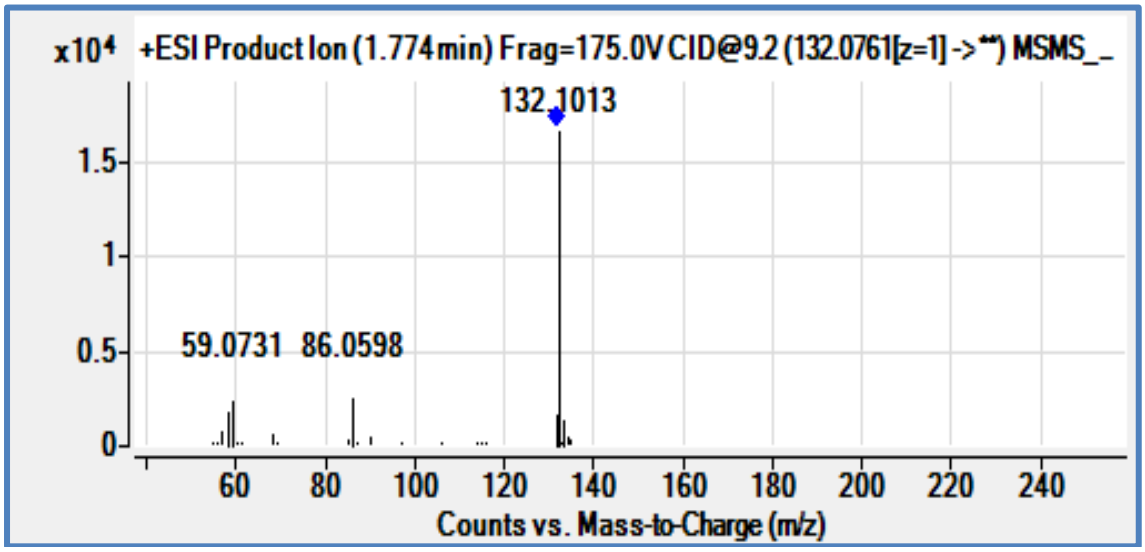
Supplementary material Figure 5. Mass spectra of pentahomomethionine



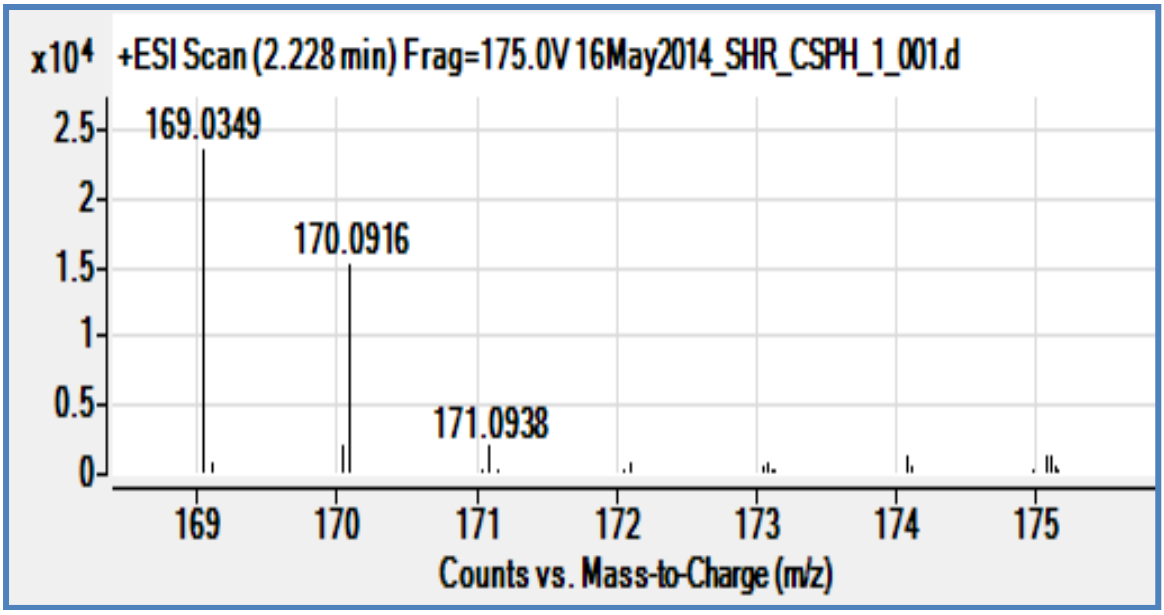
Supplementary material Figure 6. Mass spectra of creatinine



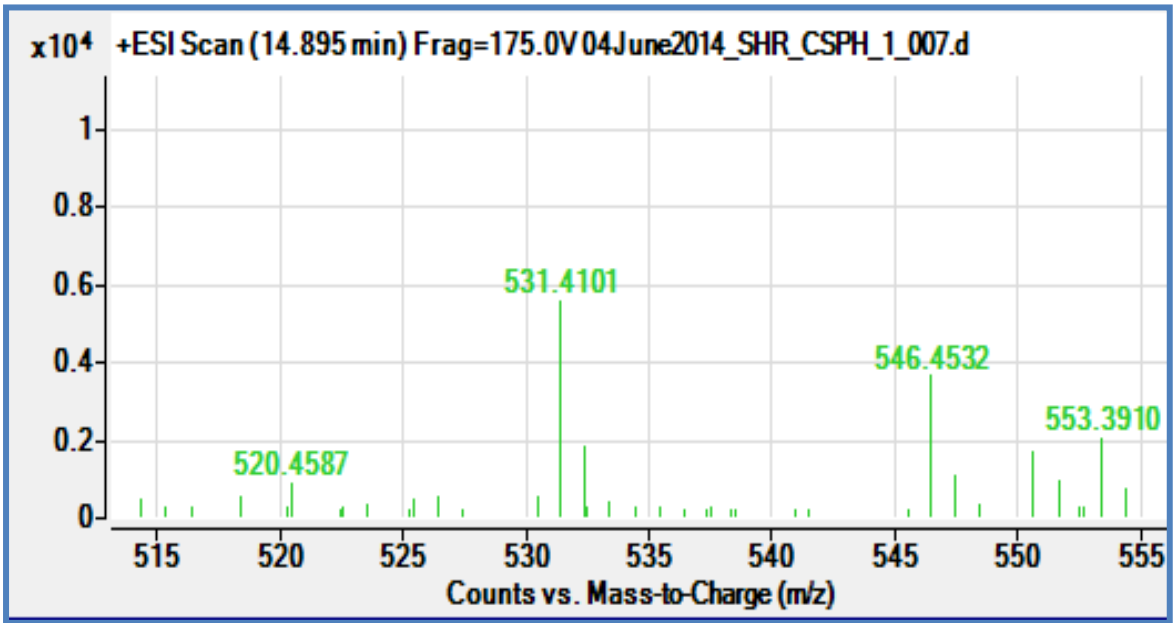
Supplementary material Figure 7. Mass spectra of creatine



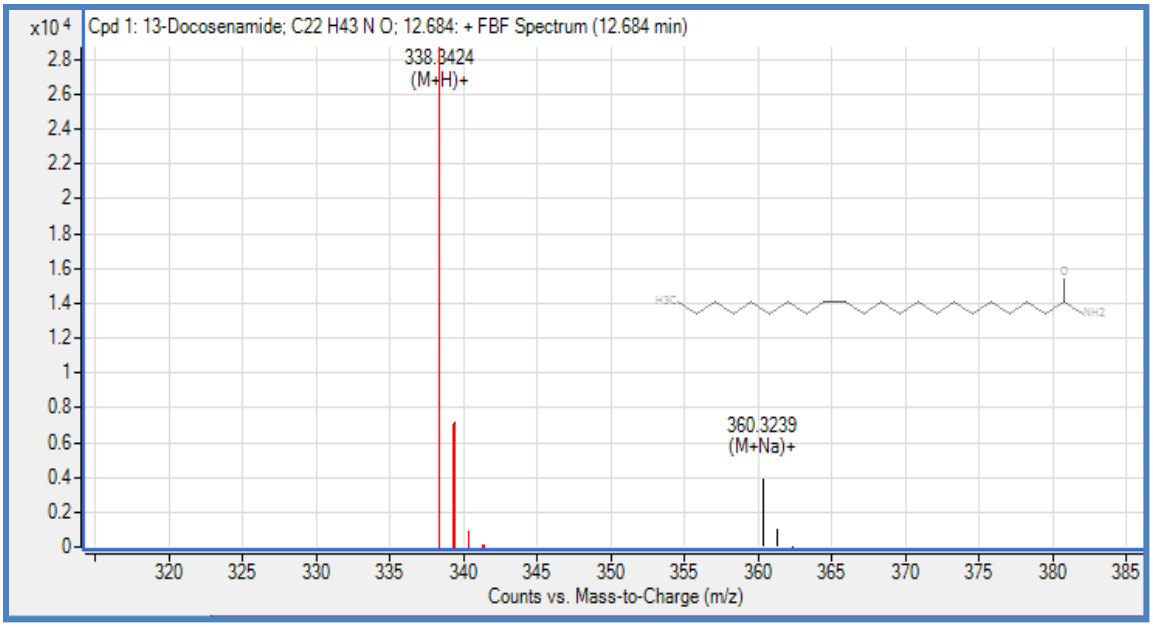
Supplementary material Figure 8. Mass spectra of L-isoleucine



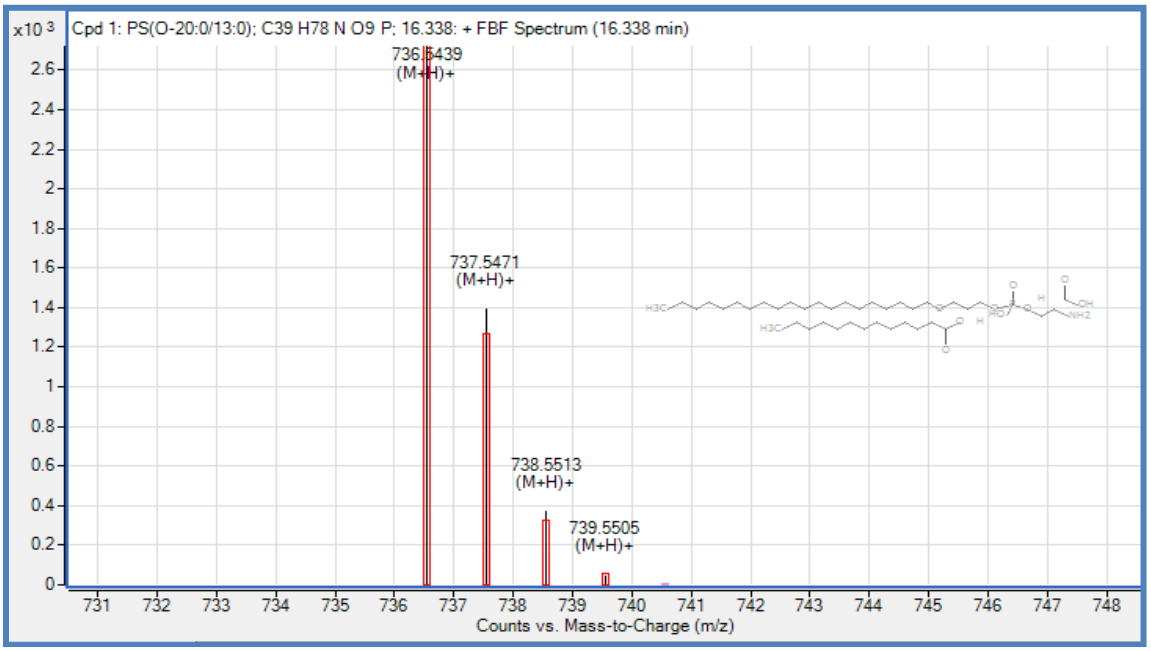
Supplementary material Figure 9. Mass spectra of uric acid



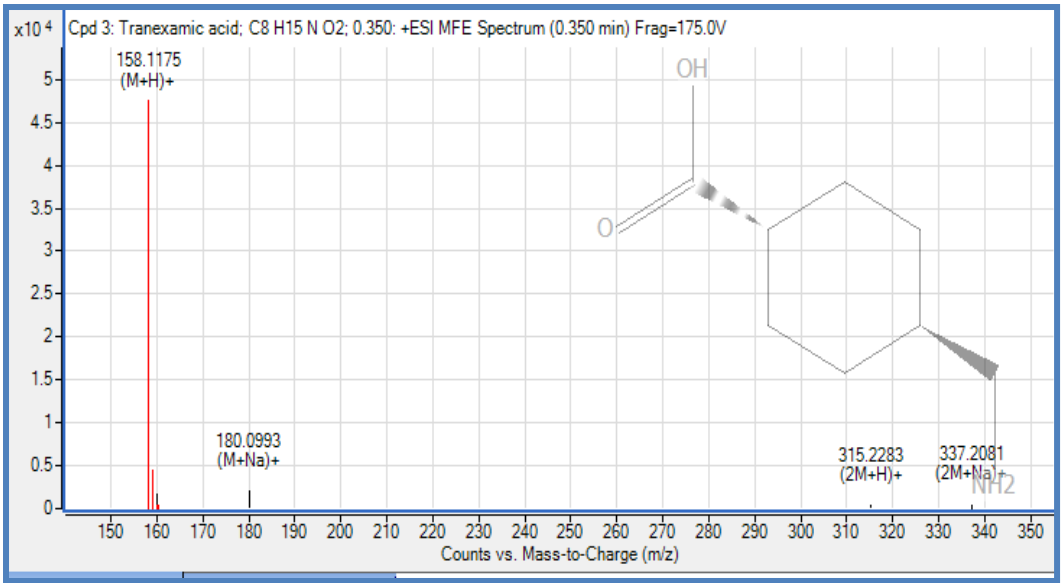
Supplementary material Figure 10. Mass spectra of Vitamin E succinate



Supplementary material Figure 11. Mass spectra of Docosenamide



Supplementary material Figure 12. Mass spectra of PS(O-20:0/13:0)



Supplementary material Figure 13. Mass spectra of Tranexamic acid

CHAPTER EIGHT

GENERAL DISCUSSIONS AND CONCLUSIONS

8.1 Discussion on statistical interactions

Due to the complex nature of the experimental designs and statistical models we used throughout this thesis, an additional general discussion will focus on significant statistical interactions reported in Tables 3.2, 3.3, 4.2 and 4.3 by examining the SPSS-generated interactions plots in the parameters measured.

In the first study on the inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates (Table 3.2 and Table 3.3), an investigation of the interaction plots for the DH revealed that the DH for the CBSH was significantly ($P<0.05$) and consistently higher than the CSTH for all enzyme types as well as enzyme concentrations. However, while the DH of the alcalase hydrolysates for both muscle types increased sharply from 1 – 2%, the rate of increase at 3 and 4% were more gradual especially for the CBSH than the CTSH. On the other hand, there was a steady increase in the DH for the pepsin-pancreatin hydrolysates of both muscle types suggesting that both enzymes behave differently in their hydrolytic activities.

There were significant interactions between the muscle type and enzyme type, muscle type and enzyme concentration and enzyme type and enzyme concentration in the protein content indicating a difference in the nature of the interactions. The protein content for the alcalase hydrolyzed CBSH increased steadily with increasing enzyme concentration while the protein content of the alcalase hydrolyzed CTSH increased from 1 – 2% but decreased afterwards. However, for the pepsin-pancreatin hydrolysis, the protein content of the CBSH was unaffected from 1 – 3% and increased sharply from 3 – 4% while the protein content for the CTSH decreased sharply from 1 – 3% and increased sharply also from 3 – 4% with the CBSH

being significantly higher at 4% than the CTSH. Though the muscle type and membrane fractions showed significant differences in the main effects, there was no significant difference in their interaction effects.

The peptide yield of the hydrolysates showed significant interaction only in muscle type and enzyme concentration especially for the pepsin-pancreatin hydrolyzed chicken muscle skins. The peptide yield for CBSH was higher at 1% (70%) than for CTSH (65%) and both increased to 75% at enzyme concentration of 2% after which the increase in peptide yield for the CTSH became slowed, while that for the CBSH was sharp afterwards. Overall, the CBSH had significantly higher peptides than the CTSH. Also, pepsin-pancreatin hydrolysis was more effective than alcalase in peptide generation. We earlier attributed this to be due probably to predigestion with pepsin which may have caused more exposure of susceptible peptide bonds to the actions of pancreatin. There is also a significant interaction effect between the different membrane sizes and the muscle types in their peptide yield. The peptide yield for both CBSH and CTSH were similar at < 1 kDa, < 3 kDa and < 5 kDa but the CBSH was significantly higher in peptide yield at < 10 kDa than the CTSH.

For ACE inhibitions, only the enzyme type had significant difference in the main effect of the statistical model studied. However, there were significant differences in the interaction effects between muscle type and enzyme type, muscle type and enzyme concentration, and enzyme type and enzyme concentration. For alcalase hydrolysis, ACE inhibition for the CBSH showed a rapid increase from 1 – 2% (70 – 80% inhibition) and decreased sharply to 73% at 3% enzyme concentration after which it increased to 70% at 4% enzyme concentration suggesting that it had no definite pattern. However, the CTSH inhibition of ACE activity increased markedly from 55% at 1% enzyme concentration to 73% at 3% enzyme concentration and

plateaued at that point onward. This suggests that the ACE inhibitory activity for the alcalase hydrolysates increases with increasing enzyme concentration for both muscle skin types. For the pepsin-pancreatin hydrolysis, the ACE inhibition for the CBSH decreased gradually from 71% at 1% enzyme concentration to 70% at 2% enzyme concentration after which there was a sharp decrease until a final inhibitory activity of 52% at 4% enzyme concentration. However, the ACE inhibition for the CTSH increased with increasing enzyme concentration from 75% at 3% enzyme concentration after which there was a sharp decrease to 66% at 4% enzyme concentration. This indicates that the ACE-inhibitory activity for the pepsin-pancreatin hydrolysates decreased with increasing enzyme concentration, which is contrary to the observation with the alcalase hydrolysates. There was also a significant interaction between the membrane fractions and the muscle skin types in their ACE-inhibitory activities with the CTSH having significantly higher ACE inhibition at < 1 kDa than the CBSH while the CBSH had significantly higher ACE inhibition at < 10 kDa than the CTSH. The results suggest that the CTSH has higher contents of lower molecular weight peptides than the CBSH.

For renin inhibition, there were significant differences in the interaction effects between muscle type and enzyme type, muscle type and enzyme concentration, and enzyme type and enzyme concentration. For alcalase hydrolysis, renin inhibition increased sharply from 2% - 22% at 1% and 2% enzyme concentration, respectively and flattened at 3% after which it decreased to 15% at 4% enzyme concentration suggesting that use of enzymes beyond 3% concentration will not be economical. However, for CBSH, there was a gradual increase from 21% - 22% at 1 and 2% enzyme concentrations, respectively and a sharp decrease and subsequent loss of activity at 3 and 4% enzyme concentrations, which suggest that use of enzyme beyond a concentration of 2% is also not economical for renin inhibition. Fractionation of the hydrolysates showed that the

CTSH significantly had better renin inhibition at all peptide sizes compared to the CBSH and this also increases with increasing membrane size.

In the second study on the in vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions (Table 4.2 and Table 4.3), an investigation of the plot of the interactions for the DPPH revealed that the DPPH radical scavenging activity for the CBSH was significantly ($P<0.05$) and consistently higher than the CSTH for all enzyme types (both alcalase and pepsin-pancreatin) as well as enzyme concentrations (except 4% enzyme concentration). The results suggest that use of enzymes beyond a concentration of 3%, is not economical if good DPPH scavenging activity is desired. However, contrary to the above observation, the DPPH scavenging activity for the CTSH increased with increasing enzyme concentration for both alcalase and pepsin-pancreatin hydrolysis, though activity was lower than CBSH. This may be attributed to the lower proteolysis and peptide content in the CTSH compared to the CBSH. However, for the membrane fractions, the DPPH radical scavenging activity was observed to decrease with increasing peptide size suggesting that lower sized peptides are better DPPH scavengers than higher molecular weight sized peptides. Also, there was no significant interaction effect between the muscle skin type and membrane fractions in their DPPH scavenging activities indicating the nature of the differences between them. The CTSH was significantly higher than the CBSH at all membrane sizes.

For metal chelation, an investigation of the plot of the interactions revealed that the CBSH had lower metal chelation than the CTSH, increasing with increasing enzyme concentration up to 2% enzyme concentration after which it flattens out whereas the CTSH flattens out at 3%. This suggests that for optimum metal chelation, CBSH and CTSH should be hydrolyzed at 2 and 3% enzyme concentrations respectively. The plot of the interactions also

revealed that while the alcalase hydrolysis decreases with enzyme concentration, the pepsin-pancreatin hydrolysis increases with increasing enzyme concentration indicating that alcalase gives better metal chelation at lower concentration than pepsin-pancreatin. An investigation of the plot of the interactions for the membrane fractions also revealed that the CTSH had significantly higher metal chelation than the CBSH at all membrane sizes suggesting possible differences in the susceptibilities of the muscle skin types to proteolytic degradation.

An investigation of the plot of the interactions for hydroxyl radical scavenging activities revealed that the alcalase hydrolysates had significantly higher hydroxyl radical scavenging activity than the pepsin-pancreatin hydrolysates at all enzyme concentrations used. Both decreased sharply initially from 1 – 2% enzyme concentration but the alcalase hydrolysates increased afterwards while the pepsin-pancreatin hydrolysate decreased gradually until enzyme concentration of 4%. Also an investigation of the interaction plot of the muscle skin type and enzyme concentration revealed that both the CBSH and CTSH decreased initially from 1 – 2% enzyme concentration, the CBSH increased afterwards while the CTSH decreased indicating that the CBSH exhibited significantly better scavenging at higher enzyme concentration the CTSH. There was also a significant interaction between the muscle skin type and peptide membrane size with the CTSH being significantly higher than the CBSH in hydroxyl radical scavenging activity at peptide size < 1 and 3 kDa while the CBSH on the other hand is significantly higher than the CTSH at peptide size < 5 kDa. This indicates that the CTSH are better hydroxyl radical scavengers at lower peptide sizes than the CBSH.

A plot of the interactions of the chicken skin protein hydrolysates for their superoxide oxide scavenging activities (Table 4.3) revealed that the CTSH had better superoxide radical scavenging activity at peptide size < 1 kDa while the CBSH had better scavenging activity at

peptide size < 10 kDa. This showed that CTSH is a better scavenger at lower peptide size than CBSH. However, a plot of the interactions for the oxygen radical absorption capacity (ORAC) revealed that both CTSH and CBSH behave similarly in their ability to quench peroxyl radicals as the plots of their interactions follow same trend, though the CTSH was higher than the CBSH at peptide size < 1, 3 and 5 kDa but similar at 10 kDa.

8.2 General discussions

Good nutrition is vital to improved health as it has the potential to reduce the risk as well as progression of numerous life-style related chronic diseases such as CVD and cancer, which are known to be responsible for millions of deaths yearly (Ahhmed & Muguruma, 2010). Therefore, it is the duty of food, nutrition and health-related professionals to promote healthy eating habits in order to arrest this negative health tide. Hypertension (elevated blood pressure) is the most common CVD type and the incidence of this chronic disease is increasing worldwide at an alarming rate. Though several pharmaceutical drugs (captopril, enalapril, lisinopril, and aliskiren) are presently being used to treat the condition, the inability of these drugs to treat the underlying cause of the disease is however, a source of concern to patients, scientists and medical professionals. More importantly, these drugs are synthetic in nature, costly and could exhibit some side effects owing to prolonged use. The search for safer, cheaper and natural alternatives to these drugs has therefore, become very paramount. Consequently, consumers are increasingly becoming more attracted to foods that offer health benefits beyond basic nutrition.

The development of functional foods and nutraceuticals with demonstrated physiological benefits of reducing the risk of chronic diseases beyond basic nutrition could assist in meeting the current growing demand for food-derived (natural) bioactive compounds with potential health benefits while also saving overall health-care costs (Ahhmed & Muguruma, 2010; Erdmann, Cheung, & Schroder, 2008). This development has also led to the identification of

several bioactive substances (especially peptides) from plant and animal tissues and by-products useful in the management and treatment of chronic diseases. Bioactive proteins and peptides with multi-functional activities can be used as functional ingredients to formulate functional foods and nutraceuticals that can be used to treat, manage and ameliorate many of the life-style associated chronic conditions such as diabetes, osteoporosis, oxidative stress, hypertensions and associated CVD. Bioactive peptides have been demonstrated to lower blood pressure by inhibiting the RAS, relax the arteries and allowing more flexibility as well as reducing oxidative stress induced damages to cellular components through free radical scavenging (Erdmann et al., 2008; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011).

In response to the demand for safer, cheaper and more affordable sources of food-derived bioactive peptides, this study evaluated the potentials of chicken skin, a waste product of the poultry processing industry to be used as a bioactive ingredient in the formulation of functional foods and nutraceuticals. Chicken skin proteins from the thigh and breast muscles were enzymatically hydrolyzed with either alcalase or a combination of pepsin/pancreatin resulting in peptides that could be used to prevent, treat, manage and ameliorate the chronic metabolic disorders associated with oxidative stress and hypertension. Metabolomics profiling was also employed to understand the pathogenesis of hypertension, oxidative stress and associated chronic conditions and the regulation of these processes by the chicken skin peptides. This study provided detailed information on the potential antioxidant and antihypertensive properties of chicken skin protein hydrolysates. To the best of our knowledge, it is the pioneering research effort that could guide other subsequent studies on chicken skin protein utilization in the development of functional foods and nutraceuticals. The study also provided novel information regarding cellular metabolites changes and associated physiological pathways that are involved

in the regulation of hypertension and oxidative stress by chicken skin peptides. This will be very useful to scientists, pharmacists and medical professionals involved in the development of drugs, treatment and management of hypertensive patients.

8.3 General conclusions

The major findings in this research work that will be regarded as novel contributions to scientific knowledge are:

1. Chicken skin protein hydrolysates (CSPHs) produced through alcalase or simulated gastrointestinal digestions were shown to exhibit inhibitory activities against ACE and renin during *in vitro* tests. Fractionation of the hydrolysates with ultrafiltration membranes also produced peptides of different sizes with strong ACE and moderate renin inhibitory activities. This work has shown that poultry skin is a suitable raw material for the production of potentially bioactive peptides that can be used as ingredients to formulate antihypertensive functional foods and nutraceuticals. The high yield exhibited by the hydrolysates is a good indication of the potential viability for future commercialization of the bioactive chicken skin products.
2. CSPHs and its membrane ultrafiltration fractions can scavenge reactive oxygen species during *in vitro* studies. This is because these peptide products exhibited varying abilities to scavenge or quench DPPH, hydroxyl, peroxyl and superoxide radicals as well as chelate metal ions. The effectiveness of CSPHs was shown to be dependent on the source of the chicken skin (breast or thigh), type of protease used during chicken skin protein digestion (alcalase was better) as well as molecular size of the peptides (1 kDa permeate peptides had the best antioxidant activities). The breast skin protein hydrolysate exhibited slightly better free radical scavenging activities whereas upon membrane ultrafiltration, the thigh skin protein hydrolysate fractions showed better antioxidant activities.

Therefore, the CSPHs or 1 kDa permeate may be suitable ingredients for the formulation of functional foods and nutraceuticals that can potentially be used to prevent or manage diseases associated with oxidative stress.

3. CSPHs possess strong *in vitro* inhibitory activities against ACE and renin. The *in vitro* data suggest that short chain peptides have stronger ACE inhibition potency than long chain peptides. In contrast membrane fractionation seems to reduce peptide synergistic effect and hence reduced *in vitro* renin inhibition. However, unfractionated hydrolysate was more effective in reducing SHR systolic blood pressure (SBP) when compared to the peptide fractions. Thus additional processing is not required subsequent to enzymatic hydrolysis of chicken skin for producing potent antihypertensive hydrolysate; this could contribute to reduced product cost upon commercialization. The results confirm that the antihypertensive effects of chicken skin are not dependent on the animal part from where the skin was harvested. Thus, our work could enhance value-added utilization of chicken skin, which is currently disposed as a waste product by the poultry industry.
4. Dietary interventions using CSPHs in a feeding trial for 6 weeks significantly lowered SHRs SBP but have no effect in normotensive WKY rats. The observed SBP reduction was positively related to plasma ACE but not renin activity, suggesting the *in vivo* mechanism for blood pressure regulation by CSPHs to be down-regulation or inhibition of ACE activity. Heart weight of the experimental rats was not significantly reduced by CSPH diets probably due to the short duration of the trial, age and state of the rats. Though the average daily feed consumption of the rats was affected by the dietary intervention, the cumulative body weight gain and feed efficiency ratio were not significantly affected, which implies that the diet was well tolerated by the rats. There

was a corresponding reduction in plasma antioxidant enzyme activities by the CSPHs intervention diets at termination when compared to baseline contrary to our expectation, suggesting that the hydrolysates may have been unable to repair age-related irreversible oxidative stress induced damage at that chronic hypertensive state. However, even though the plasma SOD activity was low, the CSPHs-containing diets had the least reduction, suggesting some benefits of the diet peptide intervention. Overall, the plasma total antioxidant capacity was high. Our results confirm the potential of CSPHs to be used as ingredient in formulating functional foods and nutraceuticals in the prevention and treatment of hypertension.

5. CSPH dietary intervention in SHRs model using a non-targeted metabolomics approach revealed several distinct urinary and plasma metabolite changes that may be considered biomarkers responsible for SBP and hypertension attenuation. The metabolite changes support the evidence of a potential mechanism that involve the vasodilative actions of NO production via the arginine metabolic pathway. The evidence also supports the antioxidant role of the chicken skin peptides through inhibition and/or scavenging of free radicals. To the best of our knowledge, this is the first metabolomics study to evaluate metabolite profile changes of SHRs fed CSPH diet intervention in relation to SBP and hypertension. This study could therefore promote CSPH use as an ingredient in the development of nutraceuticals and functional foods for the hypertension management. This work could also enhance value-added utilization of chicken skin which is currently mainly disposed as a waste product by the poultry industry, which can enhance the economic value of the poultry industry while ensuring a cleaner environment.

8.4 Novelty of the thesis findings

Though several attempts have previously been made at product innovations in order to diversify chicken skin utilization, reduce cost of waste disposal and minimize environmental damage, this is the first work to report on the development of chicken skin based peptides with health promoting values. This work has demonstrated that chicken skin is an active source of bioactive peptides that are capable of preventing and reducing oxidative stress and hypertension. Also, there is a general agreement in the literature that there is an intrinsic relationship between hypertension and metabolic disorders. However, a detailed study of the metabolic changes and regulatory effects of bioactive peptides on hypertension has not been previously studied. Our research has, for the first time, been able to use a non-targeted metabolomics approach to reveal several distinct metabolite changes that may be considered biomarkers responsible for the attenuation of systolic BP, hypertension and oxidative stress. The data generated from this study will broaden our understanding of the metabolic changes involved in hypertension and assist scientists in the development of antihypertensive products that are more potent than currently available compounds. .

8.5 Limitations of this thesis work

While we acknowledge that this thesis work may not have been exhaustive in nature as is the case with most studies of this nature, however, the following research areas were not considered in order to better support our results due to time constraints:

1. A detailed study to isolate and sequence the bioactive peptides responsible for the observed blood pressure-lowering effects after the metabolomics study. The information would have yielded the amino acid sequence in the peptide chains that are responsible for the observed effects.

2. Cell culture (*Ex vivo*) studies with chicken skin protein hydrolysates to simulate *in vivo* gastrointestinal tolerance of peptide oral administration in order to determine optimal dosages and toxic levels.

8.6 Anticipated future direction of this study

In view of the novelty of the results of this study and the intended impact it is likely to have, we anticipate that further studies, especially using growing (young) SHRs need to be explored to better provide a more detailed understanding of the preventive mechanisms underlying hypertension control by protein hydrolysates in general and CSPH in particular. Cytotoxicity tests that will provide necessary information on the safety levels and oral administration dosage will also need to be conducted in order to assure safety of the chicken skin bioactive peptides in humans when used as a functional ingredient in nutraceuticals and functional foods for hypertension control. This will also possibly serve as a basis for a future clinical trial to determine the blood pressure-lowering efficacy of CSPH. Finally, peptide sequencing of the amino acid chains in the chicken skin protein hydrolysates responsible for the observed blood pressure-lowering effects needs to be performed in order to have a proper fundamental scientific understanding of the structure-function relationships of bioactive peptides.

8.7 References

Ahhmed, A. M., & Muguruma, M. (2010). A review of meat protein hydrolysates and hypertension. *Meat Science, 86*(1), 110-118.

Erdmann, K., Cheung, B. W. Y., & Schroder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *Journal of Nutritional Biochemistry, 19*(10), 643-654.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: meat and fish. *Nutrients, 3*(9), 765-791.