COMPOSITION AND FUNCTIONAL BIOACTIVE PROPERTIES OF BAMBARA GROUNDNUT PROTEIN AND HYDROLYSATES

This work is submitted in complete fulfilment for the degree of Doctor of Philosophy (Food Science and Technology) in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences at the Durban University of Technology, Durban, South Africa

Abimbola Kemisola Arise

(B.Tech. (Hons), M.Sc.: Food Science and Technology)

2016

ABSTRACT

Bambara groundnut (*Vigna substerranea*) is an indigenous legume of African origin which is currently experiencing a low level utilisation. It is tolerant to drought and can grow under poor soil conditions in which other lucrative crops such as groundnut cannot grow. Bambara is a good source of protein comparable to that of cowpea and slightly lower than soya bean. In order to assess the potential use of bambara protein as a functional ingredient in food systems and as an important ingredient for the formulation of therapeutic product, the knowledge of its protein composition, structure and functionality becomes important.

The main goal of this thesis was to determine the composition and bioactive properties of bambara protein and its hydrolysates. Specifically, a comparative study was carried out on the protein content, yield and functional properties of protein concentrates prepared from three different bambara landraces using different extraction methods (Salt solubilisation and Acid precipitation). There was no significant difference in protein content, yield and functional properties of the landraces. However, the method of extraction had an influence on their physicochemical and functional properties. Acid precipitation produced bambara protein concentrates with high protein content and yield (79% and 52% respectively) when compared to salt solubilisation (protein content - 57% and yield - 25%). Protein concentrates prepared through salt solubilisation method exhibited better functional properties in terms of water absorption capacity, oil absorption capacity, foaming capacity, foaming stability and emulsion activities when compared to concentrates obtained through acid precipitation.

Furthermore, the composition of bambara proteins produced through isoelectric precipitation was determined. SDS PAGE revealed four major bands; a broad band at 55 kDa which was analysed to be vicilin, two medium bands at 62 kDa and 80 kDa and a high molecular weight (HMW) protein at 141 kDa. Further investigation of bambara protein revealed vicilin (55 kDa) with two sub units as the major protein in bambara and this was also confirmed by the proteomic map. The proteomic map revealed acidic amino acids as the major protein of bambara which is characteristic of vicilin, the map also showed that there were differences in the number of spots across the landraces with 77 spots matching each other. Circular dichroism spectroscopy exhibited reductions in α-helix, and β-pleated sheet conformations as pH varies. In addition, the tertiary structures as observed from the near-UV CD spectra were also influenced by shifts in pH conditions. Differential scanning calorimetry thermograms showed two endothermic peaks at around 67 and 81^{o}C respectively. These can be attributed to thermal denaturation of vicilin and the HMW protein. Subsequent studies used isolates from red bambara since the composition of the landraces were similar.

Bambara protein isolate was subjected to enzymatic hydrolysis using three proteases (alcalase, pepsin and trypsin) to produce various bambara protein hydrolysates (BPHs). BPHs were investigated for antioxidant and antihypertensive activities. The *in vitro* structural and functional characteristics of bambara protein and its enzymatic protein hydrolysate revealed that bambara groundnut possessed antioxidant properties against a variety of physiologically relevant free radicals. High surface hydrophobicity and the molecular size of the peptide seem to be important for scavenging of hydroxyl radicals, ferric reducing power and metal chelation. BPHs and peptide fractions were able to scavenge DPPH radicals with greater affinity for smaller size. Less than 1 and 1-3 kDa pepsin fraction was able to scavenge DPPH radical more than glutathione, BPHs and its fractions scavenge ABTS•+ three folds than the isolate. Scavenging of superoxide radicals was generally weak except for 5-10 kDa peptide fractions. All BPHs inhibited linolenic acid oxidation with greater affinity for the lower molecular size peptide.

BPHs showed potential antihypertensive properties because of the *in vitro* inhibition of activities of angiotensin converting enzyme (ACE) and renin inhibition. The molecular size had significant effect on the ACE inhibitory properties with low molecular weight peptide (<1 kDa) fractions exhibiting significantly higher ($p<0.05$) inhibitory activities. However, enzyme type had synergistic effects on renin inhibition with alcalase hydrolysate showing highest inhibition at 59% when compared to other hydrolysates and their membrane fractions. The fractions with <1 and 1-3 kDa peptides showed a higher potential as antihypertensive and antioxidant peptides. Based on this study, incorporation of bambara protein isolate as an ingredient may be useful for the manufacture of high quality food products. Likewise, the bambara protein hydrolysates, especially the <1 kDa and 1-3 kDa fraction represent a potential source of bioactive peptides in formulating functional foods and nutraceuticals.

DECLARATION

I hereby declare that the work reported in this thesis "**Composition and functional bioactive properties of bambara groundnut protein and hydrolysates**" is my original research work. All sources cited herein are indicated and acknowledged by means of a comprehensive list of references. I hereby certify that the work contained in this thesis has not previously been submitted either in its entirety or in parts for a degree in this or any other university. Its only prior publications are in forms of journal articles and conference papers published during the period of the research. This thesis presents a compilation of manuscripts that were prepared, compiled or published during the course of the research work

……

A.K. Arise

I hereby approve the final submission of the following thesis.

……..…...

Dr Eric O. Amonsou

……..

Dr Oluwatosin A. Ijabadeniyi

DEDICATION

This thesis is dedicated to my ever loving husband Dr Rotimi Olusanya Arise for understanding and sharing my dreams, believing in my ability to achieve them and for the support and sacrifice made to enable me to achieve them.

ACKNOWLEDGEMENT

I would like to open this page of sincere gratitude by appreciating God. The most gracious, most merciful, my ever loving daddy who gave me the strength and good health coupled with favour to complete this programme. Indeed, Psalm 102:13 works in my life.

I am sincerely thankful to my supervisor, Dr Eric Amonsou, whose supervision, advice, criticism, encouragement, support and guidance from the initial to final level enabled me to develop an understanding of the subject. I always thank God for giving me such a hardworking and intelligent man like him. Thanks sir. I would also like to thank my co-supervisor Dr Tosin Ijabadeniyi. Sincere thanks and appreciation is extended to Prof. Rotimi Aluko for giving me the opportunity to carry out part of this research work in his laboratory. You are wonderful sir.

I acknowledge the financial support of Africa Union and National Research Foundation (NRF). I also acknowledge the support of my colleagues in Durban and Canada; Samson, Debola, Tola, Betty, Amina, Agnes, Faith, Van, Sunday Malomo, Adeola, Ifeanyi, and Ayo. You are all wonderful. My thanks also goes to my pastors and my church members at Winners Chapel, Durban. You made my stay in Durban a home away from home, thanks.

Special thanks to the staff of Department of Biotechnology and Food Technology, thank you all.

I also want to thank Prof. Karim for her support in releasing me to go for this programme. You are highly appreciated.

To my Corlo Court sisters and brothers, you are simply the best. Bro Yemi Awolusi, thanks for your assistance at all times.

I am indebted to my parents Otunba and Chief Mrs Jayeoba, for teaching me good values; thank you. To my sisters for their love, I say thank you.

I would like to express my deepest appreciation to my ever loving husband Dr Rotimi Arise for his unwavering support, care, encouragement, patience, taking over the role of a mother and father simultaneously and his continuous sacrifice. You are indeed my angel. Thanks my one and only. To my wonderful God giving children, Temidayo, Temilola and Temidire, thanks for your patience when I was away from home and for your continuous prayer every night for mummy. Your prayers really work. My sister in law, Ebun Arise thanks for supporting and mothering my children in my absence; you are indeed appreciated.

Thanks to everyone whose names could not be mentioned but may have contributed in one way or another to this work.

FOREWORD

This report is written using the manuscript format. It is composed of four manuscripts (studies) which are presented after the general introduction and literature review chapters. Manuscripts 1 and 3 have been published in the International Journal of Food Science and Technology and Journal of Food and the Journal of Function Royal Society of Chemists respectively. Manuscripts 2 and 4 are under review in a reputable academic journals at the time of filling this report. The last chapter of this thesis therefore, provides a general summary and conclusion of the study, limitations involved and future directions of the study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABS	Absorbance
ACE	Angiotensin converting enzyme
ANOVA	Analysis of Variance
APS	Ammonium persulfate
BP	Blood pressure
BPI	Bambara protein isolate
BPH	Bambara protein hydrolysate
CD	Circular dichroism
DPPH	1,1- diphenyl-2-picrylhydrazyl
2D	Two dimension
Da	Dalton
DSC	Differential Scanning Calorimetry
FAO	Food and Agricultural Organisation of the United Nations
FI	fluorescence intensity
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
IEF	Isoelectric Focusing
IPG	Immobilized pH Gradient
ME	Mercaptoethanol
Mw	Molecular Weight
OHC	Oil holding capacity
PAGE	Polyacrylamide Gel Electrophoresis
PS	Protein solubility
SDS	Sodium dodecyl sulfate
So	Surface hydrophobicity
TCA	Tricholoroacetic acid
Td	Peak Temperature of Transition
TEMED	Tetramethylethylene-Diamine
WHC	Water holding capacity
WHO	World health organisation

PREFACE

PUBLICATIONS

This work has resulted in the following publications:

(a) Journal articles

1) **Abimbola K. Arise**, Eric O. Amonsou and Oluwatosin A. Ijabadeniyi (2015). Influence of extraction methods on functional properties of protein concentrates prepared from South African bambara groundnut landraces. International Journal of Food Science and Technology, 50;1095-1101.
2) **Abimbola K. Arise**, Ifeanyi D. Nwachukwu , Rotimi E. Aluko and Eric .O. Amonsou. Composition, structural and functional properties of protein isolates from bambara groundnut (*Vigna subterranea*) landraces . Under review in Food Hydrocolloids (10th April,2016)
3) **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Oluwatosin A. Ijabadeniyi, Rotimi E. Aluko and Eric O. Amonsou. (2016). Antioxidant activities of bambara groundnut (*Vigna subterranea*) protein hydrolysates and their membrane ultrafiltration fraction. DOI:10.1039/c6f000057
4) **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Sunday A. Malomo, Rotimi E. Aluko and Eric O. Amonsou. Inhibitory properties of bambara protein hydrolysate and its membrane fractions against angiotensin converting enzymes, renin and free radicals Submitted to LWT-journal of Food Science and Technology (21st May,2016)

(b) Conference papers

1) **Arise A.K**., Ijabadeniyi O.A and Amonsou E.O . Composition, Solubility profile and thermal properties of protein isolate from bambara groundnut (*vigna subterranea*) landraces. South African Association of Food Scientist and Technologist (SAAFOST), Durban, September 2015

2) **Arise A.K**. and Amonsou E.O. Physicochemical properties of protein isolates from bambara groundnut (Vigna subterranean) landraces. 29th EFFoST Conference on Food Science Research and Innovation: Delivery sustainable solutions to the global economy and society. 10th -12th November, 2015, Greece.

3) **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Sunday A. Malomo, Rotimi E. Aluko and Eric O. Amonsou. Inhibitory properties of bambara protein hydrolysate and its membrane fractions against angiotensin converting enzymes, renin and free radicals. Food

Safety and Security 2016 Autumn Scientific Conference (FSaS), Johannesburg. South African, 16-18th May, 2016

4) **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Oluwatosin A. Ijabadeniyi, Rotimi E. Aluko and Eric O. Amonsou. (2016). Antioxidant activities of bambara groundnut (*Vigna subterranea*) protein hydrolysates and their membrane ultrafiltration fraction. IUFoST-world congress of Food Science and Technology, Dublin, Ireland 21st-25th August, 2016

CHAPTER ONE

1. INTRODUCTION

Bambara groundnut (*Vigna subterranea*) is an underutilised legume of African origin (Eltayeb *et al.* 2011; Mazahib *et al.* 2013). It is the third most important legume after groundnut (*Arachis hypogeal*) and cowpea (*Vigna unguiculata*) in Africa (Adebowale *et al.* 2011; Hillocks *et al.* 2012). However, its economic importance has not been fully exploited. In Southern Africa, bambara is cultivated mainly in Limpopo, Mpumalanga and KwaZulu–Natal provinces of South Africa (Mabhaudhi and Modi 2013). The protein content of bambara groundnut (20.5-24.0%) is similar to that of cowpea and slightly lower than that of soya bean (Hillocks *et al.* 2012; Marques *et al.* 2015; Thammarat *et al.* 2015). Furthermore, bambara is tolerant to drought and poor soil, adaptable to hot tempreatures and low rainfall with good resistance to pests and diseases (Thammarat *et al.* 2015). It is suitable for intercropping with other crops and does not take up large areas of land like other lucrative crops such as groundnut. Despite its drought tolerant ability and nutritional potential, bambara is underutilised (Yao *et al.* 2015). Traditionally, the fresh bambara seeds may be consumed raw, grilled or dried while immature, the fresh pods are boiled with salt and pepper and eaten as a snack (Adegbola and Bamishaiye 2011; Murevanhema and Jideani 2013). Findings from recent studies suggest the possibility of using bambara in various food products such as in biscuit and cake production (Okafor *et al.* 2015), vegetable milk and yoghurt (Falade *et al.* 2014; Murevanhema and Jideani 2014) .

The underutilisation of bambara may therefore be attributed to limited research and lack of market value. For instance, the limited scientific knowledge on the characteristics of its storage protein has resulted in its poor usage in food and health products processing. Bambara storage protein, just as soya protein may be useful in foods to improve functionality (Cui *et al.* 2013; Nishinari *et al.* 2014). In addition, intrinsic characteristics of proteins such as hydrophobicity have been found to significantly influence their functionality including solubility, foaming and

emulsification. These functional properties may determine the suitability of a protein as an ingredient in various food applications (Shevkani *et al.* 2014; Shevkani *et al.* 2015). Protein concentrates and isolates from legumes such as cowpea and soya bean have been used for various food applications (Shevkani *et al.* 2015). Hence, investigative study on bambara protein concentrates and isolates may be important to unlock the potential of this crop and enhance its application for novel product development. Therefore, the knowledge of composition and structure as well as the functionality of bambara protein, is very essential in order to facilitate its utilisation in food systems.

Furthermore, there is a growing interest in food proteins and peptides beyond their basic nutritional effects (Udenigwe and Aluko 2012; Hernández-Álvarez *et al.* 2013). For instance, many biological peptides which are inactive within parent proteins of various foods, have been exploited during enzymatic digestion or food processing. These biological peptides have been found to exhibit health benefits such as immune defence, antihypertensive, antioxidant, antibacterial and enhancement of intestinal activities. Specifically, bioactive peptides derived from food proteins which were digested with different proteases under different hydrolysis conditions have been associated with the management of renin–angiotensin system, that regulates peripheral blood pressure via conversion of angiotensin I to angiotensin II (Medina-Godoy *et al.* 2012; Ajibola *et al.* 2013; Ruiz-ruiz *et al.* 2013). Previous studies reported the inhibition of angiotensin-I-converting enzyme (ACE) for food proteins such as soya bean and bean protein (Nakahara *et al.* 2011; Ruiz-ruiz *et al.* 2013). Bioactive peptides within the sequence of native proteins have also been associated with antioxidant activity and could be used as functional ingredients in food formulations, in order to prevent oxidative stress related diseases and/or to improve the shelf life of food products (Corrêa *et al.* 2011; Carrasco-Castilla *et al.* 2012; Ruiz-ruiz *et al.* 2013). Previous studies suggested protein hydrolysates from pulses such as kidney bean as potential peptides with ACE inhibitory and antioxidant acitivity (Mundi and Aluko 2014). Hence, there is a need to explore the bioactive properties of bambara protein isolates and hydrolysates, which may also serve as a new source of peptide in certain nutraceutical applications. This research work was

therefore aimed at evaluating the chemical composition and functional bioactive properties of bambara groundnut protein and hydrolysates.

1.1. Hypotheses

1). Acid precipitation of bambara protein concentrate will result in a higher yield and protein content than salt solubilisation as previously reported (Boye *et al.* 2010; Adebowale *et al.* 2011). Salt solubilisation method will result in superior functional properties such as higher water absorption capacity and foaming than the acid precipitation method. Differences in water absorption capacity could be due to the exposure of the hydrophilic and ionic group which allows for improved affinity with the surrounding water (Boye *et al.* 2010; Adebowale *et al.* 2011).

2) The major storage protein in bambara will be globulin which will be composed of the vicilin-like (7S) and legumin-like (11S) similar to other legumes such as cowpea (Shevkani *et al.* 2015). However, differences may be observed in terms of the number and molecular weight of constituent polypeptide subunits between bambara and other legumes. Variations in the number of vicilin (7S) subunits have been reported for bean, kidney, mung and black bean (Rui *et al.* 2011; Kudre *et al.* 2013; Shevkani *et al.* 2015). These variations were attributed to post-translational proteolytic processing of the pre-protein and the differential extent of glycosylation (Sathe 2002; Amonsou 2010).

3) Optimized *in vitro* enzymatic hydrolysis of bambara protein isolate will lead to the release of multifunctional peptides that possess antioxidant and antihypertensive activities. In addition, peptide with low molecular weight will have higher ACE inhibitory activities. This may be due to the fact that smaller size peptides have been suggested to exhibit a high rate of intestinal absorption, without any structural changes (Mundi and Aluko 2014). Legume peptides released through enzymatic hydrolysis has been reported to possess antioxidant and antihypertensive activities with smaller size having greater ACE inhibitory activities (Ajibola *et al.* 2013; Ruiz-ruiz *et al.* 2013; Alashi *et al.* 2014; Mundi and Aluko 2014).

1.2. Objectives of the Study

1) To determine the functional properties of protein concentrates obtained from bambara landraces using different extraction methods.
2) To determine the composition, structure and functional properties of protein isolates from bambara landraces.
3) To determine the antioxidant potential and ACE and renin inhibition ability of enzyme hydrolysate and ultrafiltration membrane fractions of bambara proteins

1.3. Contribution to knowledge

This research explores the composition, structure, functional and bioactive properties of protein from bambara groundnut grown in South Africa. The influence of extraction methods on protein functionality of bambara groundnut has been previously reported, but not for bambara grown in South Africa. Since the variation in chemical composition of legumes protein may vary with cultivar, environmental conditions and agricultural practices, we considered that it is important to study the influence of extraction methods on functional properties of South African bambara landraces (Chapter 3). In this study, salt solubilisation method gave a higher foaming, emulsifying and water holding capacities than the acid precipitation methods. Hence, food manufacturers may consequently find bambara protein produced through salt solubilisation method as a useful ingredient in the formulation of a variety of specialty foods, such as ready-to-eat breakfast foods.

Chapter 4 reports on the effects of pH on the circular dichroism (CD) and fluorescence spectroscopy properties of bambara protein isolates since a search of the literature has not shown any report on conformation dynamics of bambara protein isolate as monitored by far-UV CD spectroscopy in the current study. The use of CD to monitor the changes in conformation with change in temperature will help to increase our knowledge on monitoring the effect of heat preparation processes, especially as affected by change in pH. In addition, proteomic map has not been established for bambara protein. This is necessary so as to identify the types of

protein expressed in bambara landraces in terms of their charge and molecular weight.

Also, to our knowledge, different studies performed by different researchers, even with legumes, have not reported the antioxidant and antihypertensive potencies of protein hydrolysate and peptides from bambara protein. Therefore, novel antioxidant and antihypertensive peptides may emerge if further *in vitro* work is performed with bambara protein. Chapter 5 and 6 will, therefore, report the *in vitro* antioxidant and the antihypertensive properties of bambara protein hydrolysate and the membrane ultra-filtered fractions using different proteases. This will contribute novel information on the production of multifunctional protein hydrolysates (in this case antioxidant, renin inhibition and ACE inhibition) that can be used to produce simultaneous effects on different metabolic disorders. Multifunctional bioactive agents are highly desirable because their use is more cost effective and obviates the need for multiple therapeutic agents against chronic metabolic disorders.

CHAPTER TWO

2. LITERATURE REVIEW

This chapter is divided into three parts. The first part is a review on bambara groundnut which mainly discusses the distribution, nutritional values and uses of the bambara groundnut. The second part provides a review on the composition, structure and functional properties of legumes and the third part focuses on bioactive properties of legume proteins

2.1. Background

Bambara groundnut (*Vigna subterranea)* is a legume specie of African origin and belongs to the family of fabaceae and sub family of faboidea (Mazahib *et al.* 2013). It is an intermediate, herbaceous annual legume that self-pollinates with well-developed tap-root and with many profuse geotropic short lateral roots which are about 20 cm long. Bambara has lateral stems which develops from the root and on which leaves are borne. The leaves are trifoliate while petiole is long, stiff and grooved with green or purple colour base. The leaves and flower buds arise alternately at each node. Its podding habit is similar to that of groundnut in that the pale yellow flower stalk bends downwards after fertilization, pushing the young developing pod into the soil where it will develop and mature (Hillocks *et al.* 2012; Yao *et al.* 2015). Bambara grows well in an average temperature of 20 ^{o}C-28 ^{o}C and has a growth period of at least 3 to 5 months. The plant is highly adaptable and tolerates harsh conditions better than most crops. It grows well on well-drained soil and can also grow on soil that is low in nutrients and requires pH of about 5.0-6.5. It is not prone to risk of total crop failure especially in low and uncertain rainfall (Brough *et al.* 1993; Adegbola and Bamishaiye 2011; Hillocks *et al.* 2012). In this era of global warming and food security threat in Africa, bambara will be a crop of hope to alleviate malnutrition and poverty as a result of its drought tolerant characteristics especially in regions of the world where water availability is a serious issue (Basu *et al.* 2007) The main producing areas of bambara in South Africa are Limpopo, Mpumalanga and Kwazulu-Natal provinces (Fig.2.1). Seed colours also varies from white to cream, yellow, brown, purple red and black as shown in Fig 2.2

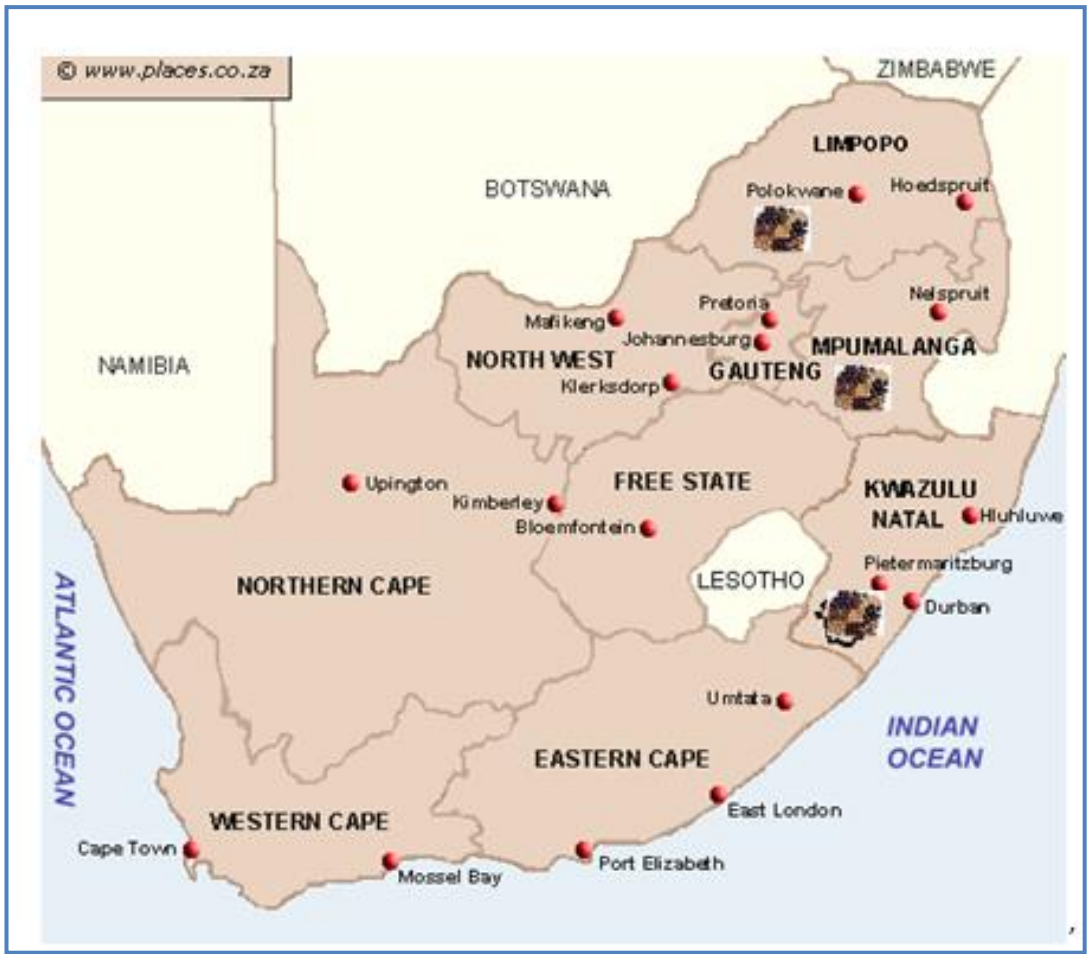


Fig.2.1 : Distribution of bambara in South Africa (Source: Modified from Hilocks et al.,2012)

Fig.2.2: **Varieties of bambara groundnut (*Vigna substerranea*) differentiated based on the colour of the seed coat.**

2.2. Uses of Bambara Groundnut

Bambara groundnuts are consumed in many ways, such as eating it fresh or grilled while immature. Fresh pods are boiled with salt and pepper and eaten as snacks in many West African countries. Bambara groundnuts are also roasted and crushed to make soup with or without condiments (Goli *et al.* 1997; Murevanhema and Jideani 2013). Recent research has established the possibility of using bambara groundnut in various food products such as biscuit and cake production (Okafor *et al.* 2015), vegetable milk and yoghurt (Falade *et al.* 2014; Murevanhema and Jideani 2014). Bambara groundnut paste is used in the preparation of steamed products such as Okpa in Nigeria. Okpa is a cooked, dough-like gel made from bambara paste. It is usually wrapped in banana leaves and boiled. Apart from its nutritional uses, bambara medicinal uses have been reported. The Lio tribe in Kenya use water from boiled bambara seeds to cure diarrhoea (Adegbola and Bamishaiye 2011). Bambara leaves are applied to abscesses and infected wounds and sap from bambara leaves is applied to the eye to treat epilepsy. Bambara roots are sometimes taken as an aphrodisiac and pulverized bambara seeds are mixed with water and used to treat cataracts in Senegal. The Igbos in Nigeria use the plant to treat venereal diseases (Hillocks *et al.* 2012). The black landraces have the reputation of being used for treating impotence in Botswana (Adegbola and Bamishaiye 2011). Chewing and swallowing of raw seeds is being used to curb nausea and vomiting in South African pregnant women (Jideani and Diedericks 2014)

2.3. Nutritional profile of bambara groundnut

The seeds of bambara make a nutritious and complete food due to its sufficient quantities of protein (20.5-24.0%), carbohydrate content (54.5-69.3%), and fat (5.3-7.8%) with the level of essential sulphur containing amino acid higher than that found in most legumes (Brough *et al.* 1993; Ijarotimi and Esho 2009; Mune *et al.* 2011; Murevanhema and Jideani 2013). Bambara is also a good source of fibre, calcium, iron and potassium. It has the potential to provide a balanced diet in areas where animal protein is expensive and the cultivation of other legumes is

economically risky due to unfavourable environmental conditions (Murevanhema and Jideani 2013; Yao *et al.* 2015). The composition of bambara groundnut is presented in Table 2.1. Despite all these attributes, bambara remains underutilised and neglected even though it has the potential to play a crucial role in food security, income generation and food culture of the rural poor.

Table 2.1: Composition of bambara groundnut

Component	Value
Proximate g/100g	
Ash	2.0 - 3.6
Carbohydrate	54.5 - 69.3
Crude fat	1.6 - 6.7
Crude fibre	1.8 - 12.9
Crude protein	17.0 - 27.0
Iron	5.9 - 7.1
Potassium	1240 -1290
Phosphorus	296 - 320
Sodium	3.7 - 4.8
Calcium	7.8 – 13.5

Source: Hillocks *et al.* (2012); Adegbola and Bamishaiye (2011); Murevanhema and Jideani (2013)

2.4. Protein Composition of Legumes

Legumes are an important source of plant proteins. Most proteins in legumes are present as storage proteins. The storage proteins of seeds serve as a major nitrogen source and are utilized during germination of the seed to provide necessary free amino acids and nitrogen to the growing plant during initial stages of germination. Indigenous legumes such as African yam bean (Ajibola *et al.* 2011; Abioye *et al.* 2015), bambara groundnut (Murevanhema and Jideani 2014) and marama bean (Amonsou *et al.* 2012) have been found to be good sources of protein similar to soya bean (Adebowale *et al.* 2011; Amonsou *et al.* 2012). The protein content (20.6-25.0%) of bambara groundnut (Chinedu and Nwinyi 2012; Mazahib *et al.* 2013; Murevanhema and Jideani 2014) is similar to those reported for other pulses such as cowpea (Olalekan and Bosede 2010), kidney bean (Qayyum *et al.* 2012) and mung bean (Dahiya *et al.* 2013). The chemical composition of some legumes is shown in

Table 2.2. The variation in chemical composition of legumes protein may be attributed to differences in genotypes, environmental conditions and agricultural practices as described by Abioye *et al.* (2015)

Table 2.2: Chemical composition of some legumes[1]

[2]Legume type	Species	Protein	Carbohydrate	Fat	Ash	Fibre
Bambara groundnut	*Voadnzeia subterranea*[a]	32.40	51.79	7.35	5.78	2.68
African yam bean	*Sphenogtylis stenocarpa*[a]	37.21	44.40	9.49	5.35	3.55
Bambara groundnut	*Vignal subterranea*[b]	20.60	56.51	6.60	3.25	6.34
Jack bean	*Canavalis ensiformes*[c]	26.20	57.83	1.95	6.51	1.07
Pigeon pea	*Cajanus cajan*[c]	24.46	56.83	4.78	4.58	1.10
Cowpea	*Vigna unguiculata*[c]	24.13	56.60	4.37	4.73	0.97
Soya bean	*Glycine max*[d]	42.80	19.80	22.80	5.20	2.30
Bauhinia seed	*B.galipinii*[d]	38.50	23.30	24.20	4.50	1.4
Chick pea	*Acararietinum*[e]	22.83	57.19	5.43	3.04	3.50
Lentil	*Lensculinaris medicus*[e]	31.12	52.63	0.81	2.62	3.68
Kidney bean	*Phaseolus vulgarius*[e]	20.09	57.67	2.46	3.85	6.78
Mung bean	*Vigna radiataf*	22.70	58.99	1.36	3.35	4.70

[1]Values are reported in % dry basis, [2]Sources ; [a]Chinedu and Nwinyi (2012); [b]Mazahib *et al.* (2013); [c]Olalekan and Bosede (2010); [d]Amonsou *et al.* (2014); [e]Qayyum *et al.* (2012) and [f]Dahiya *et al.* (2013).

2.4.1. Amino acid composition

The main amino acids of legumes storage protein are glutamic and aspartic acid which may include glutamine and asparagine, respectively (Mune *et al.* 2011). These amino acids account for 25-40% of the total amino acids in leguminous seed as shown in Table 2.3 (Adebowale *et al.* 2011; Kudre *et al.* 2013; Latif *et al.* 2013; Liu *et al.* 2013; Pastor-Cavada *et al.* 2014). A higher amount of *arginine* has been reported (Table 2.3) in Faba bean (Pastor-Cavada *et al.* 2014) and peanut (Latif *et al.* 2013) but with similar comparison in bambara groundnut compared with soya (Adebowale *et al.* 2011) and cowpea (Elhardallou *et al.* 2015). Adebowale *et al.* (2011) had reported a higher arginine content of approximately 8.1% for bambara groundnut. There has been recognition for protein sources (including Bambara) that is rich in arginine and glutamine. Arginine helps in preventing heart diseases, while

glutamine helps support the immune system and improve athletic performance (Adebowale *et al.* 2011). The lysine content of bambara (6.3 g/100 g protein) is similar to that of mung bean (Kudre *et al.* 2013) and soybean (Adebowale *et al.* 2011). Based on FAO/WHO (1989) reference, indigenous legumes such as bambara groundnut, mung bean (Kudre *et al.* 2013) and faba bean (Pastor-Cavada *et al.* 2014) contained adequate sources of lysine similar to soya bean (Adebowale *et al.* 2011). Thus, these indigenous legumes offer some potential significant nutritional attributes which makes legumes a good protein supplement to cereals that are known to be deficient in lysine. The methionine content of bambara (Kudre *et al.* 2013) is higher than that of marama bean (Amonsou *et al.* 2012) and faba bean (Pastor-Cavada *et al.* 2014) but similar to black bean (Kudre *et al.* 2013) and soya bean (Adebowale *et al.* 2011). However, higher methionine content (1.8%) was reported by Ijarotimi and Esho (2009) for bambara. This higher methionine and arginine content of bambara, if researched into, may lead to differences in its functionality when compared to other legumes.

Table 2.3: **Amino acid composition of some legume proteins** [1]

[2] Legume type	ASP	GLU	ARG	LYS	HIS	ALA	ILE	LEU	MET	PHE	PRO	VAL	TRP	CYS	SER	THR	TYR	Gly
Bambara groundnut[a]	9.6	15.4	5.9	6.3	3.0	3.5	3.8	7.3	1.3	5.3	2.7	4.3	7.3	ND	3.2	2.8	3.3	3.1
Marama bean [b]	9.4	15.2	8.0	5.7	2.7	3.5	4.3	7.9	1.0	3.7	7.2	4.8	ND	6.1	5.5	3.2	11.4	5.9
Kidney bean [c]	10.9.	15.3	5.3	4.9	3.4	3.8	5.2	8.5	1.6	5.9	3.0	5.2	ND	0.9	4.6	3.7	3.2	3.6
Mung bean [a]	8.5	12.5	6.4	6.2	2.8	3.7	3.9	7.4	1.3	5.8	3.0	4.6	6.4	0.5	3.9	2.8	3.2	3.2
Faba bean [d]	11.9	16.4	11.8	7.1	2.8	4.4	3.9	7.9	0.9	5.8	4.1	4.8	0.7	1.1	4.6	4,8	2.5	4.1
Black bean [a]	9.6	14.1	6.4	6.0	2.9	3.6	4.0	7.4	1.3	5.7	2.9	4.6	7.6	ND	3.6	2.5	3.3	3.2
Soya bean [e]	11.6	19.1	7.7	6.4	2.8	4.3	4.0	7.8	1.4	5.2	ND	5.0	ND	ND	5.6	4.1	3.8	4.7
Peanut [f]	11.2	18.7	11.4	3.4	2.1	4.0	3.1	6.2	1.1	3.6	4.3	3.8	0.9	1.2	4.9	2.7	5.1	4.6
Cowpea [g]	12.2	18.9	6.8	6.9	2.5	4.4	4.6	7.7	1.2	5.7	3.9	5.4	3.8	1.0	5.5	3.8	3.2	4.1
[3] FAO/WHO				5.8	1.9		2.8	6.6	1.7	6.3		3.5				3.4		

[1] Amino acid values are expressed in g/100g protein. Recalculation has been made where necessary. ND: Not determined. [2] Sources :[a] Kudre *et al.* (2013); [b]Amonsou *et al.* (2012); [c]Liu *et al.* (2013); [d]Pastor-Cavada *et al.* (2014); [e]Adebowale *et al.* (2011); [f]Latif *et al.* (2013) and [g]Elhardallou *et al.* (2015).
[3] FAO/WHO recommended pattern for pre-school children age : 2-5 years

Some differences in the proportion of hydrophobic amino acid and relative ratios of acidic and basic amino acids have been observed based on the side chain properties (Table 2.4). Bambara protein has high hydrophobic content (Kudre *et al.* 2013) compared to faba bean (Pastor-Cavada *et al.* 2014), soya bean (Adebowale *et al.* 2011), peanut (Latif *et al.* 2013) and cowpea (Elhardallou *et al.* 2015). Further information on the hydrophobicity of the protein explains the propensities for protein solubility as well as the emulsifying properties. The hydrophobic amino acids clearly play an important role in the thermal stability or conformation of globulins (Adebowale *et al.* 2011). Also, the amino acid composition has greater influence on the biological activity of a peptide (Ajibola *et al.* 2011). In addition, high hydrophobicity has been reported to enhance antioxidative activity (Alashi *et al.* 2014). Adebowale *et al.* (2011) and Kudre *et al.* (2013) working with some varieties of bambara linked the amino acid to their functional properties without any linkage to the bioactive properties. Further research into bioactive properties of bambara protein may reveal its biological activity.

Table 2.4: Amino acid distribution based on side chain characteristics in some legume protein

Legume type	Acidic	Basic	Uncharged polar	Hydrophobic	total
Bambara groundnut	25.0	15.2	12.5	35.5	88.2
Marama bean	24.6	16.4	23.9	32.4	97.3
kidney bean	26.2	13.6	16.0	33.2	89.0
Mung bean	21.0	15.4	13.6	36.1	86.1
Faba bean	28.3	21.7	17.1	32.5	99.6
Black bean	23.8	15.3	12.8	36.8	88.7
Soya bean	30.7	16.9	18.2	27.7	93.5
Peanut	29.9	16.9	18.5	27.0	92.3
Cowpea	31.1	16.2	17.6	34.0	98.9

Values (expressed in g/100 g protein) were calculated from the amino acid data from table 2.4 based on classification done by (Tang *et al.* 2009). Acidic (Glutamic+Aspartic), Basic (arginine+lysine+histidine), Uncharged polar (cysteine+serine+threonine+ tyrosine+glycine), Hydrophobic(Alanine+Isoleucine+Leucine+methionine+phenylalanine+proline+valine+tryptophan).

2.4.2. Protein types

Protein has been classified into four based on their solubility in some solvents (Osborne 1924). These are albumins (water soluble), globulins (salt soluble), prolamins (alcohol soluble) and gluteins (alkali/acid soluble). The major storage protein of most legumes such as kidney bean (Mundi and Aluko 2013), bambara groundnut (Odeigah and Osanyinpeju 1998), faba bean (Kumar *et al.* 2015), pea seed (Rubio *et al.* 2014), cowpea (Tchiagam *et al.* 2013),

rapeseed and soya bean (Sari *et al.* 2013) are the globulins followed by albumin. However, Adebowale *et al.* (2007), reported albumin as the major storage protein in mucuna bean. Based on their sedimentation coefficient (s), globulins are classified as 7S and 11S. These are named vicilin and legumin respectively. Both albumins and globulin have claimed to induce a number of health beneficial effects (antihypertensive, antioxidant, anti-carcinogenic and hypoglycaemic) upon dietary consumption (Rubio *et al.* 2014). Adebowale *et al.* (2011) and Kudre *et al.* (2013) carried out SDS-PAGE on total storage protein of bambara and reported vicilin as the major protein in bambara. However, the author did not carry out any further investigation for confirmation of vicilin as the major protein and its sub unit. Therefore, further characterisation of the storage protein, especially of underutilised legume such as bambara groundnut, could help to gain an understanding into its functionality in the food system.

2.4.3. Sub-unit composition

The protein profile of legume globulins has been elucidated through one dimensional SDS-PAGE. It has been reported that differences in terms of number of molecular weight of polypeptide subunits of legume proteins have an effect particularly on the emulsion and gelation capacity of proteins. These functional properties of protein are very important in food system (Mundi and Aluko 2012). Differences in terms of number and molecular weight of constituent polypeptide sub-units have been reported for legumes such as bambara (Adebowale *et al.* 2011), Mug bean and black bean protein (Kudre *et al.* 2013), cowpea (Peyrano *et al.* 2015), pinto bean and soya bean (Tan *et al.* 2014) and hemp seed (Malomo *et al.* 2014). The globulin fraction of kidney bean protein was composed of two major polypeptides with molecular weight of around 43 and 45 kDa and three minor polypeptides in the range < 30 kDa (Mundi and Aluko 2012). Mung bean protein extract showed major polypeptide bands at 42, 51 and 54 kDa (Kudre *et al.* 2013). Adebowale *et al.* (2011), reported three major bands at 35, 43 and 112 kDa for bambara protein under both reducing and non-reducing conditions. Also, Mune Mune and Sogi (2015), obtained three major bands but at 40, 65 and 72 kDa under both conditions. However, Kudre *et al.* (2013), reported two major bands at 52 and 62 kDa under both reducing and non-reducing conditions. The difference observed in the number of bands may be due to different extraction methods used in the preparation of the protein isolates (Mune Mune and Sogi 2015). Conversely, Benjakul *et al.* (2000), observed a different result in which the author stated an appearance of a new band under reducing condition which suggest the presence of disulphide bonds between the

bands. The differences in these authors' observations may be as a result of the nature of sample used, Benjakul *et al.* (2000) used seed flour while Kudre *et al.* (2013) and Adebowale *et al.* (2011) used protein isolate. Therefore, there is need to further explore the SDS of bambara protein so as to confirm the actual bands that it possesses as this will help in its functionality in the food system (Tang 2015).

Differences in structure and sub-unit composition of the two fractions (7S and 11S) of the legume globulin have been reported. The 11S is an oligomeric whose quaternary structure is composed of six monomeric pairs of acidic- basic sub units (α and β) linked by disulfide bridges. This α-β is synthesized from a single mRNA as a precursor polypeptide that is cleaved to yield the disulfide bonded α-β complex (Mujoo *et al.* 2003). Tan *et al.* (2014), reported that the parent subunit in soya bean belongs to the legumin-like 11S type storage proteins which are characterized by disulphide linked by α-β paired sub unit. This is evident in the SDS –PAGE as the profile differs under reducing and non-reducing conditions (Tan *et al.* 2014). The composition and nomenclature of soya bean storage globulins are shown in Fig 2.3. The protein band distributions of some legumes are shown in fig 2.4. The protein isolates from mung-bean, black-bean and bambara were compared using SDS page. The major protein band of bambara had molecular weight 58 and 66 KDa. The polypeptide bands with MW 58 and 66 KDa were observed under both conditions of reducing and non-reducing (Adebowale *et al.* 2011; Kudre *et al.* 2013). These bands were considered as 7S vicillin proteins which are widely present in seed proteins and devoid of disulphide bonds between some units. However, no further research was carried out on the 7S vicilin by these authors to establish vicilin as the major storage protein in bambara. Kudre *et al.* (2013), reported that some subunits of mung bean were being stabilized by disulphide bonds as new bands emerges under reducing condition. This shows that the protein compositions may vary in size, type and bond involved in stabilizing the protein structure depending on different isolate.

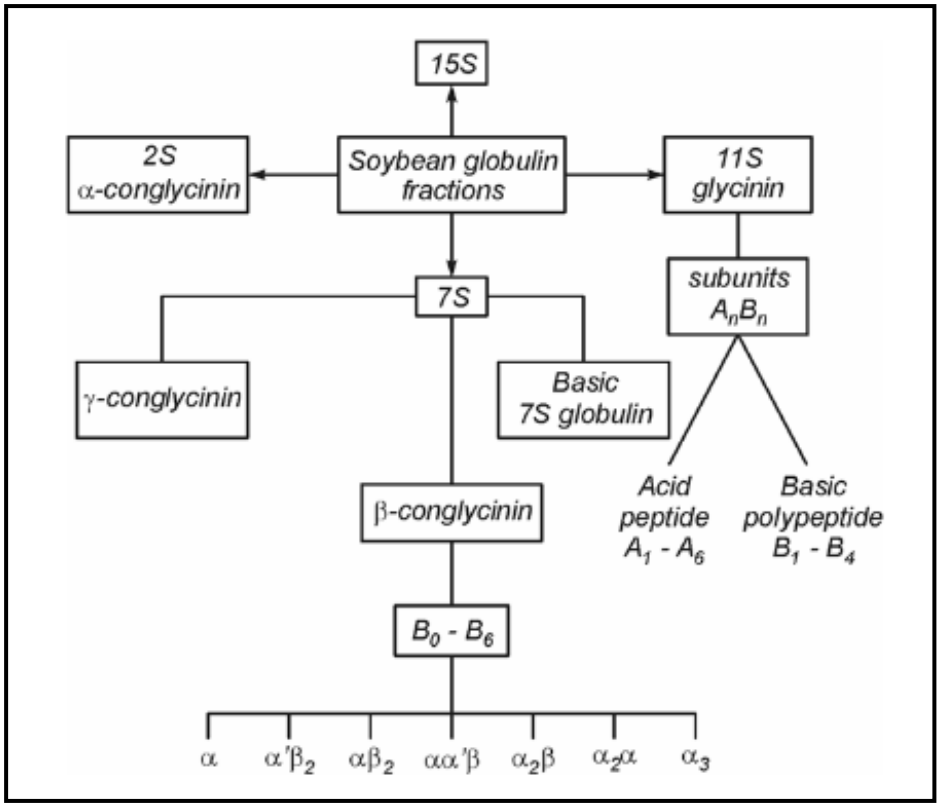
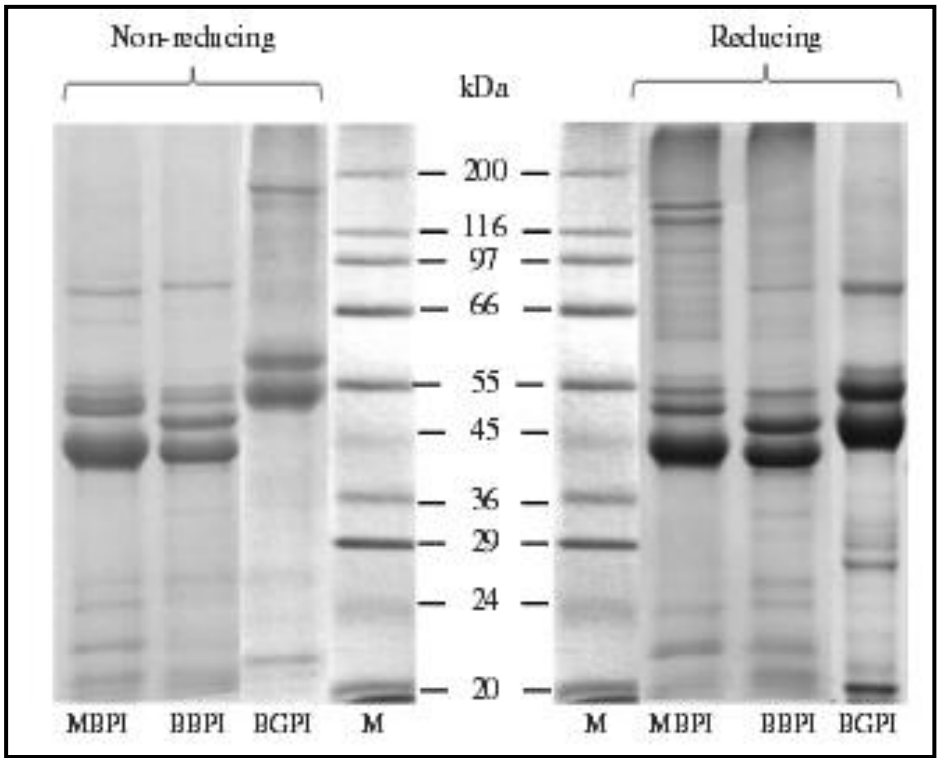


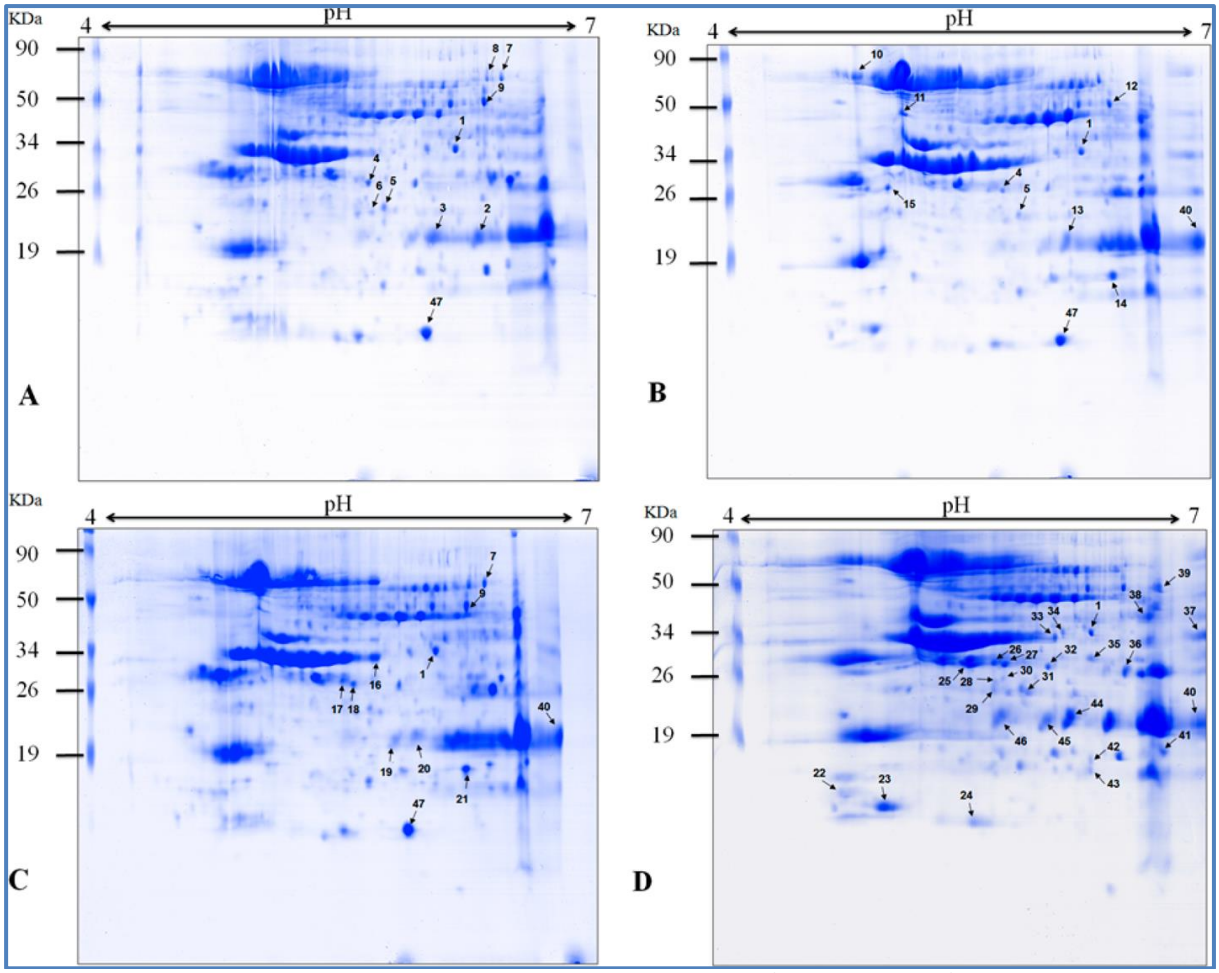
Fig.2.3: The nomenclature and composition of reserved soybean globulins (Source:Barać and Stanojević (2005))



M; marker; MBPI, Mung bean protein isolate; BBPI, Black bean protein isolate;
BGPI, bambara groundnut protein isolate Source: (Kudre *et al*. 2013)

Fig.2.4: SDS-PAGE patterns of various legumes seed protein isolates under non – reducing and reducing conditions.

The use of two dimensional maps of seed proteomics can be useful in both fundamental and applied research. The technologies of proteomic have been used increasingly to determine protein profile of some legumes such as common bean (Natarajan *et al.* 2013), pea seed (Dziuba *et al.* 2014), soya bean seed and marama bean (Amonsou *et al.* 2012). The principle of the two-dimensional polyacrylamide gel electrophoresis (2D –PAGE) is separation of proteins based on their isoelectric point (PI) in the first dimension and subsequently based on molecular weight (MW) in the second dimension. For instance, Gomes *et al.* (2014) used 2D to analyse four conventional soya bean (Fig. 2.5). The author discovered that the four conventional soya bean seeds exhibit different protein profiles which could have an impact on their functionality. In addition, 2D-PAGE was used to analyse common bean, 141 protein spots were identified. This information will be useful to scientists wishing to improve the protein content of common beans (Natarajan *et al.* 2013). Furthermore, 2D gel electrophoresis of albumins and globulins of pea seed revealed the presence of 4 and 2 spots , respectively representing proteins with allergenic potential (Dziuba *et al.* 2014). However, the protein expression of many existing indigenous legumes such as bambara grain are not known and this information would be required to gain an understanding of their function in food. In addition, a comparative study of protein expression among different landraces of bambara will provide information needed for the particular functional property of the particular landrace.



Two-dimensional electrophoresis was carried out using a narrow-range 18 cm IPG strip of pH 4−7: analyses of the proteins extracted using a thiourea/urea protocol from Glycine max cultivars (A) BRS 257, (B) BRS 258, (C) Embrapa 48, and (D) BRS 267. Identical numbers Source (Gomes *et al.* 2014)

Fig.2.5 : Two-dimensional electrophoresis of soya beans

2.5. Extraction of Legumes Protein

The extraction methods used for most plant protein isolates are assumed to contribute to the physicochemical, digestibility, functional and nutritional properties of different protein isolate fractions. Selection of the appropriate technology and conditions for protein extraction is essential in food processing as these can influence the functional and nutritional properties of the finished product (Boye *et al.* 2010). The different extraction methods that have been used in extracting plant proteins are discussed below.

2.5.1. Water- and salt- soluble protein extraction

The salt extraction principle of protein isolation is based on the ionic strength to dissolve and fractionate the two major storage (globulin and albumin) proteins found in legumes (Boye *et al.* 2010). The high glycoprotein (up to 45%) found in albumin increases its solubility in water by enhancing the protein-water interaction. On the other hand, the low glycoprotein (3.9%) present in globulin may contribute to its insolubility in water (Mundi and Aluko

2012). The salt extraction method has been used to isolate protein from plant sources such as canola and flaxseed (Karaca *et al.* 2011), kidney bean (Mundi 2012), soya bean and bambara (Adebowale *et al.* 2011). Dialysis of the salt extract against water followed by centrifugation, yields a globulin-rich precipitate and an albumin-rich supernatant (Mundi and Aluko 2012).

2.5.2. Isoelectric protein precipitation

The most common method of extracting protein from legumes involves the use of aqueous alkali extraction followed by isoelectric precipitation (IEP) (Boye *et al.* 2010). This technique takes advantage of the differential solubility of legumes proteins which is high at alkaline pH thereby necessitating solubilisation of the proteins and low at their isoelectric point usually at pH 4-5 (Boye *et al.* 2010). In some cases, the isoelectric precipitated protein may have a high protein content. For instance, Adebowale *et al.* (2011), reported protein content of 90% and 92% for bambara and soya bean isoelectric precipitated isolates respectively. However, the protein recovery is incomplete which may be as a result of inadequate solubilisation or loss in the supernatant during centrifugation of the precipitated protein (Malomo 2015). Another limitation is poor functional properties, Arise *et al.* (2015), reported lower foaming, emulsifying and water holding capacity for isoelectric precipitated bambara protein isolate in comparison to the micellised isolate. An additional limitation is that isoelectric precipitated isolates contain high amounts of ash generated during the acid-base neutralization procedure (Yin *et al.* 2011; Malomo 2015).

2.5.3. Acid extraction

The acid extraction principle is similar to that of alkaline extraction except that the initial protein extraction is conducted under acidic conditions. Since the legume protein solubility is also high at very high acid conditions (pH< 4.0). Thus low pH is used to solubilise the protein then followed by isoelectric precipitation, cryo-precipitation or membrane separation (Boye *et al.* 2010). Past studies reported about 95.7% protein for kidney bean (Alli *et al.* 1993), 91.2% and 91.9% protein for faba and pea bean (Vose 1980) protein isolates, respectively when citric acid solution or direct acidification method was used.

2.5.4. Ultrafiltration membrane

Ultrafiltration, a pressure-driven barrier membrane separation, is frequently used as an alternative to isoelectric precipitation. The supernatant obtained by either alkaline or acid extraction is subjected to ultrafiltration (Boye *et al.* 2010). In order to enable retention of

proteins or peptides of interest, the membrane is usually carefully selected with specific molecular cut-offs. The efficiency of separation of the protein can be affected by the molecular weight cut-offs, type of membrane used and the volume concentration ratio (Mundi 2012). Previous studies also reported a higher protein contents of 94.1% and 89.5% for faba bean and pea protein isolates, respectively using ultrafiltration membrane (Vose 1980).

2.6. Functional Properties of Legume Protein

Functional properties are the intrinsic physicochemical properties that affect the behaviour of proteins in food systems during processing, preparation, manufacturing and storage (Amonsou 2010; Mundi 2012). These properties include solubility, water and oil absorption, emulsifying, foaming properties and rheological properties (Amonsou 2010). Legumes, including bambara, are been exploited as functional ingredients for the preparation of various meals either on their own or as ingredients in other food materials because of their high nutritional value and availability. Various studies have exploited the protein functionalities of legume crops such as soya bean protein (De la Caba *et al*. 2012; Rebholz *et al*. 2012), bambara (Adebowale *et al*. 2011), kidney bean (Wani *et al*. 2013), pea (Barac *et al*. 2015) and fenugreek (Feyzi *et al*. 2015).

2.6.1. Protein solubility

The determinant factor for protein functionality in food processing and application has always been solubility in an aqueous solution (Karaca *et al.* 2011). The amount of water contained in food material that can be extracted by water or suitable solvent under specific conditions is its protein solubility. Protein solubility depends on a number of factors such as ionic strength, pH, food matrix medium and temperature. Another critical factor is the isoelectric point of the protein. This is the point at which the protein carries no net electrical charge (Mundi and Aluko 2012). Solubility is usually least near or at the isoelectric point. This is because the amphoteric nature (containing both acidic and basic functional groups) of the protein causes precipitation at the pH corresponding to the isoelectric point, thereby leading to minimum protein solubility (Malomo 2015).

Furthermore, the type or variety of plant protein source may have an impact in determining the solubility and thus affect the functional properties of the final isolates (Barac *et al.* 2015). However, the solubility profile of bambara grown in Southern Africa has not been determined. Apart from the isoelectric point, the pH of the environment is also an important determining factor of protein solubility. For example, the minimum solubility reported for

two varieties of bambara protein isolates occur at pH 5.0 (Adebowale *et al.* 2011), pH 4.8 was reported for kidney bean globulin (mundi and Aluko) while pH 4-5 was reported for mung bean and black bean protein isolates (Kudre *et al.* 2013).

2.6.2. Water and oil-holding capacity

Water holding capacity (WHC) and oil holding capacity (OHC) is the ability to physically and physicochemically retain water or oil against gravity (Zayas 1997). It is also an index of protein interaction with water or oil in food system. The WHC depends on several parameters such as size, shape and conformational characteristics (Malomo *et al.* 2014). WHC is also affected by the polar and non-polar amino acid balance of the protein molecules, presence of lipids, physicochemical environment (pH, ionic strength) and solubility with reference to hydrophillic amino groups which are the primary sites of protein-water interaction (Malomo *et al.* 2014). The poor water holding capacity of some proteins could possibly result from their low hydrophilic properties as determined by the proportion of hydrophilic to hydrophobic amino acids (Nosenko *et al.* 2014). For instance, rapeseed proteins with higher hydrophobic amino acids in comparison to soya bean protein had lower WHC than soya bean protein (Nosenko *et al.* 2014). The method of extraction was also observed to have effect on WHC, a study carried out by Adebowale *et al.* (2011) reported a higher WHC for bambara protein isolate extracted through micellisation method than the isoelectric precipitated isolate. This may be as a result of the exposure of the hydrophillic and ionic group with higher affinity for the surrounding water by means of hydrogen bonding. In addition, micellised technique of extraction favours conformational changes in the protein molecules which expose previously buried side chains, thereby making them available to interact with water (Adebowale *et al.* 2011).

The OHC is the ability of fat to bind the hydrophobic (non polar) side of proteins. It is related to emulsifying ability, a functional property that deals with hydrophobicity (Malomo 2015). The method of extraction, protein species and sources determine the oil holding capacity of proteins. For example, Nosenko *et al.* (2014), reported ~30% higher OHC for spring rapeseed in comparison to winter rapeseed protein isolate. Also, the micellisation method produces higher OHC in pigeon pea (Paredes-López *et al.* 1991), cowpea (Mwasaru *et al.* 1999) and bambara protein isolate than their isoelectric precipitated isolates (Adebowale *et al.* 2011). While the protein with higher amounts of hydrophilic groups near the surface will hold onto more water, the protein with higher amounts of hydrophobic groups near the

surface holds more oil. The oil holding capacity is a crucial determinant on the use of proteins as potential functional ingredients in foods such as high-fat bakery products, doughnuts and emulsion-type foods (Liu *et al.* 2013; Malomo 2015).

2.6.3. Foaming properties

Foams are two phase systems that are made up of air bubbles surrounded by a continuous liquid lamellar phase (Tan *et al.* 2011). They are formed when proteins form an interface that keeps air bubbles in suspension and prevents their collapse. Good foam is characterized by protein molecular flexibility, structural disorder and metastability; thus once formed, it is thermodynamically stable over a period of time (Ptaszek 2013). Generally, foaming properties are measured by foaming capacity and stability. Foaming capacity is the ability of a protein, under certain conditions (such as concentration, pH, temperature, salt concentration) to form a foam, while foaming stability indicates how well protein can retain the foam volume over a period of time (Stone *et al.* 2015). Certain factors such as protein concentration, pH, high pressure, thermal treatment, foam formation procedure, nature and behaviour of the interface (protein-protein interactions, denaturation) as well as their interaction with other food ingredients has been reported to influence foaming properties of protein isolates (Stone *et al.* 2015). At high alkaline pH values, very low foam stability is obtained because of increased net charge-induced weak protein-protein interactions, which reduce the ability of the protein to form strong interfacial membranes at the air-water interface (Tan *et al.* 2011). The method of extraction, as well as the drying method, has also been found to affect foaming properties. Stone *et al.* (2015) reported better foaming capacity for salt extracted spray-dried pea protein isolate than the isoelectric precipitated isolates. Also better foaming capacity was reported for micellised bambara isolates in comparison to the isoelectric precipitated isolate (Adebowale *et al.* 2011). However, foam stability is influenced by various factors like the protein adsorption at the water-air interface, the surface rheological properties, diffusion of the air out and into foam cells, size distributions of the cells, liquid surface tension, external pressure and temperature (Hojilla-Evangelista *et al.* 2014). Several plant protein isolates have been studied in the past for their foaming property, which is one of the desirable protein functional properties in food processing industries such as milk and dairies, breweries and confectioneries (Mundi and Aluko 2012; Hojilla-Evangelista *et al.* 2014; Barac *et al.* 2015).

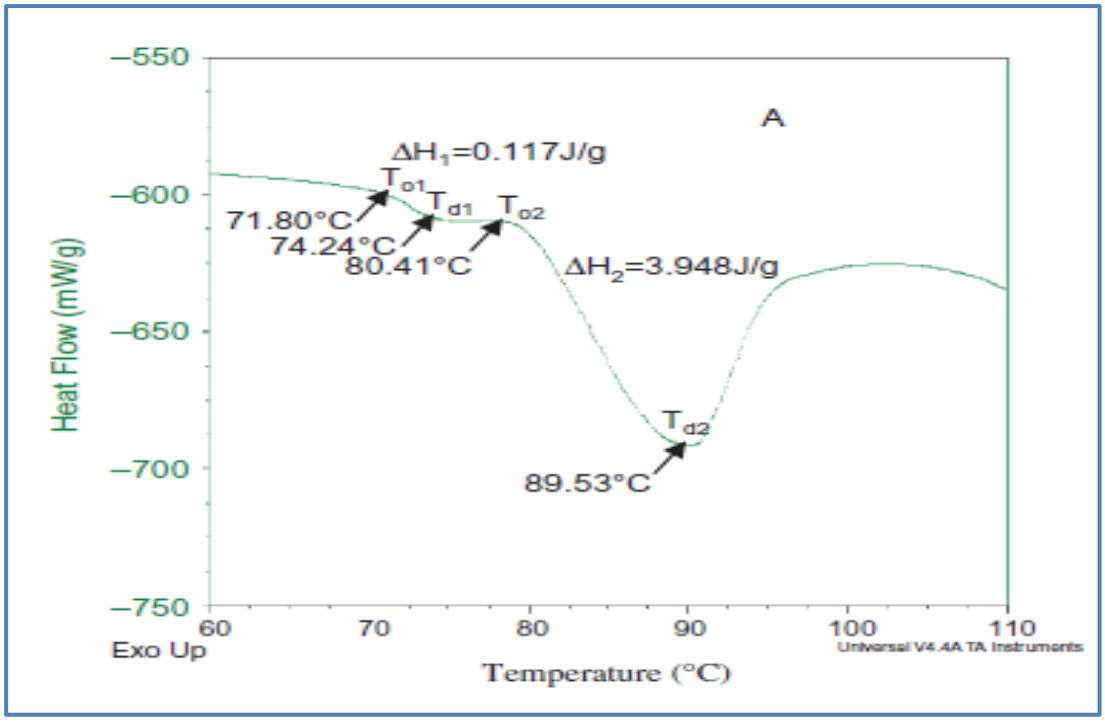
2.6.4. Emulsifying properties

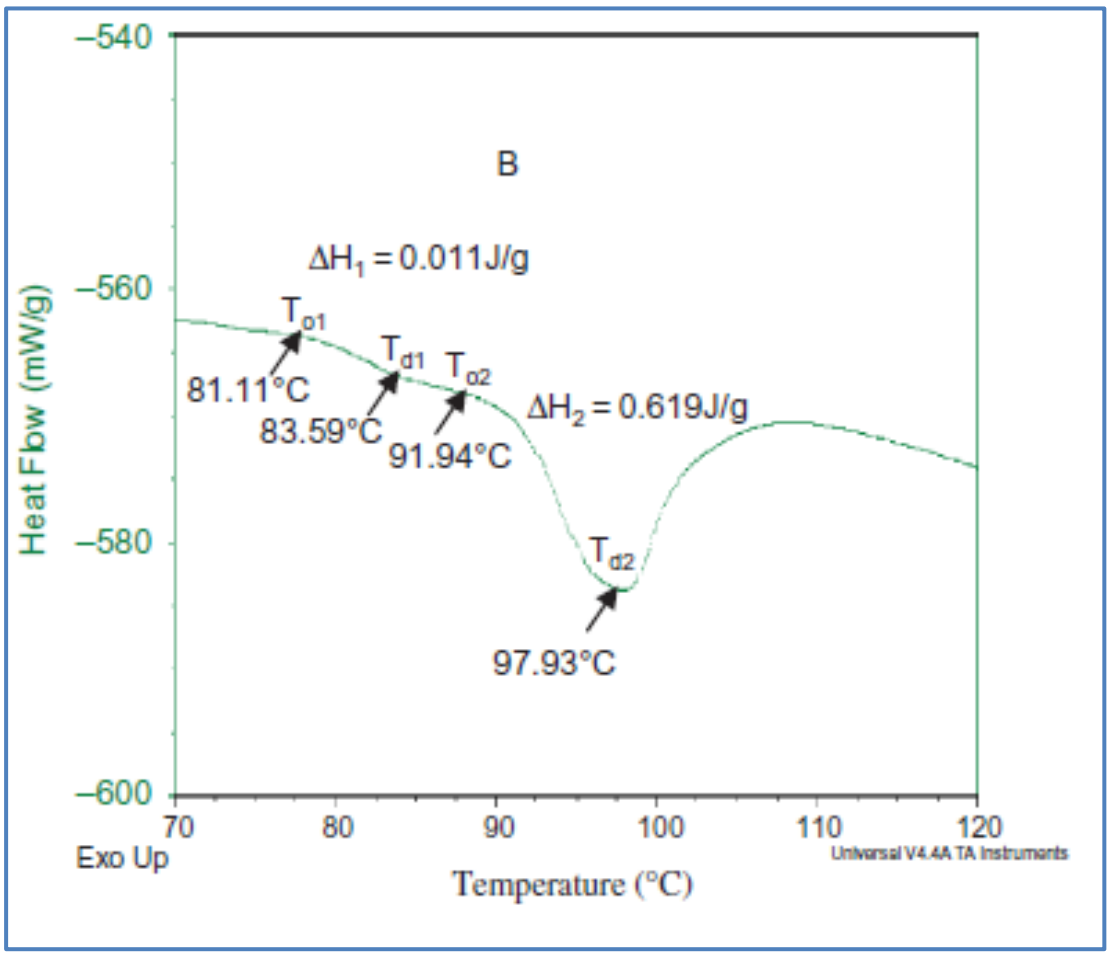
Emulsifying capacity can be defined as the ability of proteins to act as the intermediary agent that facilitates mixing of the two surfaces (water and oil). This is essential because interactions between water and oils are not possible in food systems. Thus, there is a need for an intermediary agent (Malomo and Aluko 2015). Appropriateness of a protein isolate as an emulsifier depends on the rate at which protein diffuses into the interface and on the deformability of its conformation under the influence of interfacial tension such as surface denaturation (Barac *et al.* 2015). The capacity of the hydrophilic part to interact with water and the hydrophobic part to interact with oil would help to form stable emulsions and enhanced formed-oil-water-phases of most formulated foods. This makes emulsifying capacity very important in the food processing industry and human health applications. Proteins with ideal qualities for an emulsion would have a relatively low molecular weight, a balanced amino acid composition (in terms of charged, polar or non-polar), good water solubility, well developed surface hydrophobicity and relatively stable conformation (Barac *et al.* 2015). The emulsifying properties of proteins are often expressed as emulsifying activity index (EAI) and emulsifying stability index (ESI) (Boye *et al.* 2010). The EAI measures the maximum surface area created per unit protein i.e. the amount of oil that can be emulsified per unit of protein, whereas ESI evaluates the ability of the emulsion to resist changes to its structure over a defined time period, which defines the ability of a protein to stabilize an emulsion (Boye *et al.* 2010). A number of studies reported that the emulsifying ability of legume protein concentrates or isolates is affected by the type of beans or the method applied for their preparation. For example, Adebowale *et al.* (2011), reported higher ESI for bambara micellised isolate than the isoelectric precipitated isolates. Also, the pH of the extraction medium has been reported to have an effect on emulsifying activities. Pedroche *et al.* (2004), studied the effect of extraction pH on the emulsifying properties of acid-precipitated protein isolates. They found that protein isolates extracted at alkaline (either pH 10, 11 or 12) have lower emulsifying properties than its meal. The emulsifying properties both (emulsifying capacity and stability) decreased as the extraction pH increased. This is in contrast with the results of Aluko and McIntosh (2001), where the ESI of the acid-precipitated was higher than that of its meal. The difference could be as a result of differences in cultivars and method of extraction used (Tan *et al.* 2011).

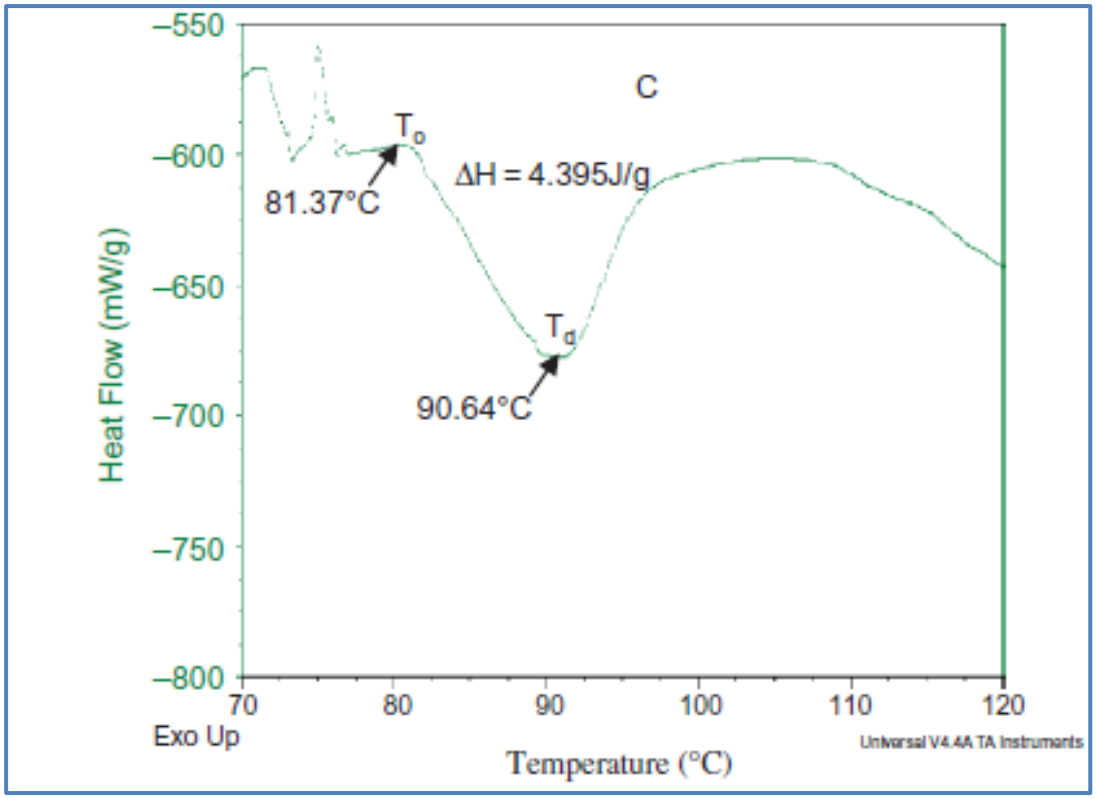
2.6.5. Thermal properties

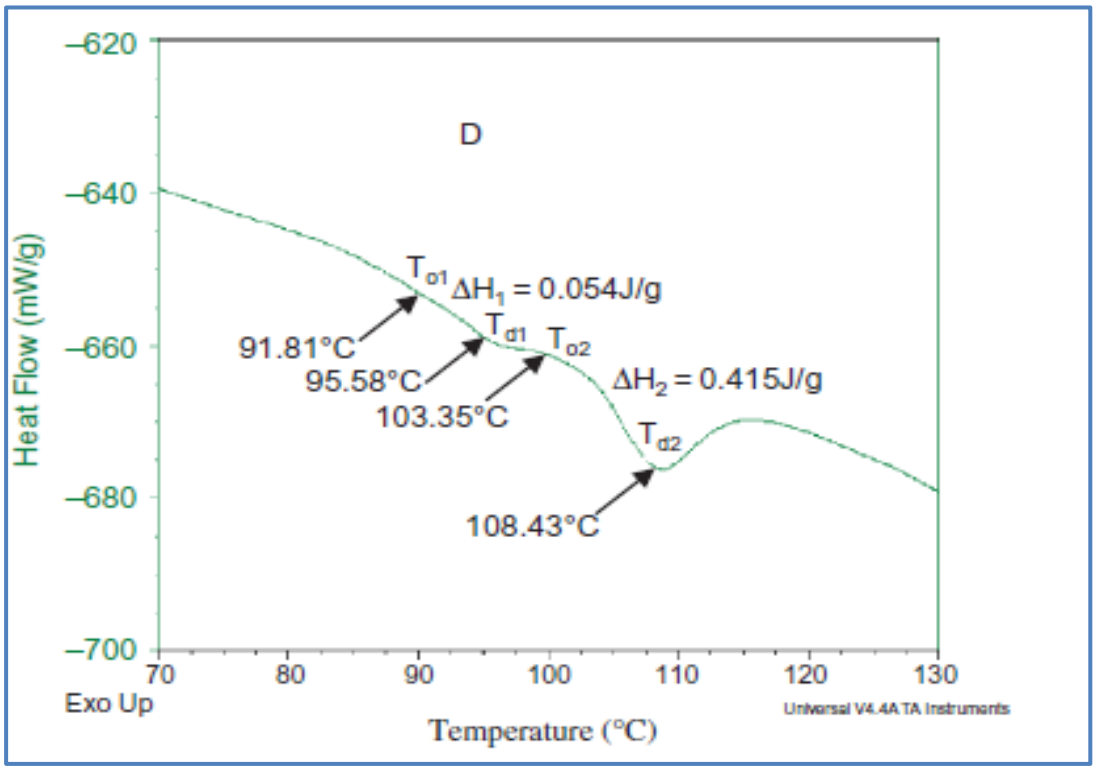
Heating causes denaturation of protein by disrupting the bonds that are involved in formation and maintenance of the protein structure (Tan *et al.* 2011). The temperature needed and the extent of these changes have been determined by the thermal stability of the protein. This can be studied from the endothermic peaks of their differential scanning calorimetry (DSC) profiles. The temperature of denaturation (T_d) and enthalpy (ΔH) are the two parameters commonly used to describe thermal characteristics of legume proteins (Amonsou 2010). These parameters provide information on the type of protein structure (simple or complex). The heat flow into the protein is measured by the enthalpy. Greater heat flow means greater state of nativity which results in a more complex protein structure (Amonsou 2010).

A study on the thermal denaturation of mung bean, black bean and bambara protein isolates by DSC revealed two major endothermic peaks, which could be attributed to thermal denaturation of the 7S protein at lower temperature and the 11S proteins at the higher temperature (Kudre *et al.* 2013). The thermal stability of legume protein has been reported to be affected by a large number of factors such as protein structure, amino acid composition, binding of metals and other prosthetic groups, intramolecular interactions, protein-protein contacts, linkages and environmental factors (Tan *et al.* 2011). In addition, thermal stability of bambara protein has been reported to be affected by extraction methods. Adebowale *et al.* (2011), reported lower denaturation temperature (T_d) for isoelectric isolates in comparison to the micellised isolates. The relatively low T_d value of isoelectric isolate was linked to the high pH used during the isolation of bambara protein. The extraction at alkaline pH (9.0) and subsequent precipitation at the isoelectric point (pH 5.0) may have caused the structural rearrangements (Adebowale *et al.* 2011). Furthermore, the micellised protein had well-defined narrow and symmetrical peaks (Fig. 2.7), which showed a homogeneous, and less denatured protein population. High T_d and T_o is associated with the denaturation of protein groups which are less heat stable. Partial denaturation of protein isolate usually results in decreased enthalpy (ΔH), while completely denatured proteins will show no endothermic transition.


A
ΔH1=0.117J/g
To1
Td1
To2
71.80°C
74.24°C
80.41°C
ΔH2=3.948J/g
Td2
89.53°C
Heat Flow (mW/g)
−550
−600
−650
−700
−750
60
70
80
90
100
110
Exo Up
Universal V4.4A TA Instruments
Temperature (°C)


B
ΔH1 = 0.011J/g
To1
Td1
To2
81.11°C
83.59°C
91.94°C
ΔH2 = 0.619J/g
Td2
97.93°C
Heat Flow (mW/g)
−540
−560
−580
−600
70
80
90
100
110
120
Exo Up
Universal V4.4A TA Instruments
Temperature (°C)





The isolates were produced by : (A) Isoelectric precipitation (white variety): (B) micellisation (white variety): (C) Isoelectric precipitation (brown variety): (D) micellised (brown variety**) Source : (Adebowale *et al.* 2011)**

Fig.2.6: DSC thermograms of bambara groundnut protein isolates

2.7. Structural Characterisation of Legume Protein

Globular proteins have compact, tightly folded structures in their native state. This structure can be altered by change in environment or by subjecting them to extremes pH, high temperatures, detergents and organic solvent. Such exposure will cause the protein to unfold (Mundi 2012). The physicochemical properties of the unfolded proteins are usually different than those of the folded molecules. It is therefore pertinent to study the structure of protein isolates in order to understand how proteins fold into biologically active states and how these states are stabilised. These studies are to be performed under the condition in which the legume protein actually operate (generally in solution) as well as under other conditions and to provide measures of the rates of structural changes of proteins, which are often essential to their biological function (Mundi 2012). Spectroscopic methods such as surface hydrophobicity, circular dichroism (CD), and fluorescence spectroscopy (FS) are powerful analytical tools that can measure conformational changes that may occur (at the molecular level) due to changes in the protein environment (Pain 2001; Kelly *et al.* 2005).

2.7.1. Surface hydrophobicity

In order to extensively study protein conformation, determination of intrinsic and surface hydrophobicity is very essential. This is simply based on fluorescence properties of the aromatic amino acid (i.e. tyrosine, phenylanine and tryptophan). Most of the intrinsic fluorescence studies involve the determination of tyrosine and tryptophan emission, which is due to their high molar absorptivity, with tryptophan being the most dominant (Malomo 2015). The use of florescence spectroscopy in various protein research works is common. This is because of its high sensitivity level, rapid data acquisition, wide dynamic range and convenient instrumentation (Szabo 2000). It has been reported that hydrophobic interactions of native protein structures are solution-dependent as the hydrophobic side chain amino acid residues that are buried in the protein interior need to be exposed to the surface in order to measure hydrophobicity (Malomo 2015). Therefore, in order to determine the position of the aromatic amino acids and dictate the magnitude of the measured hydrophobicity, the solution properties such as pH, salt and temperature will be essential. The distribution of polar and hydrophobic groups of amino acid side chains has been suggested to determine protein solubility since these two groups always have opposite interactions in solution (He *et al.* 2013).

2.7.2. Circular dichroism (CD) spectroscopy

Functional and biological activities of protein structural conformations are dependent on the primary structure (amino acid sequence), secondary structure (α-helices and β-sheets stabilized largely by hydrogen bonds), tertiary structure (3-D organisation of secondary structures stabilised by disulphide bonds) and quaternary structure of the proteins (Forman-Kay and Mittag 2013; Marsh and Teichmann 2014). Therefore, any alteration in the secondary, tertiary and quaternary structures of the protein could lead to denaturation. This takes effect when hydrogen, ionic or hydrophobic bonds are disrupted due to changes in temperature, pH, interfacial area and/or presence of organic compounds (Malomo 2015). Proteins are linear polymeric compounds composed of well-defined amino acid sequences that become folded in required specific conformations for functional and biological activities (Malomo 2015). CD is being increasingly recognised as a valuable technique for examining protein structure in solution, the stability of the designed protein fragment and the extent and rate of structural changes that occurred within the protein structure (Kelly *et al.* 2005). During extraction, isolation, digestion and characterisation, the structural integrity of protein can be altered, therefore the CD technique is needed to assess conformational changes in such protein (Malomo 2015). CD spectroscopy has good precision for assessing changes in both secondary (α-helix, β-sheet, β-turns and unordered) and tertiary protein structures. CD technology is based on the differential absorption of left and right circularly polarised radiation by chromophores of the protein samples when placed in either intrinsic chirality or chiral environments. This would give an empirical gauge of the structural arrangement and conformation of the protein (Kelly *et al.* 2005). The standard unit of reporting CD spectroscopy measurement is mean residue ellipticity, which is the molar ellipticity of the protein divided by the number of residues. The peptide bond absorption in the protein chromophores gives rise to the CD signals at the far ultraviolet (UV) region of 240-180 nm to represent the contents of regular secondary structural features (Kelly *et al.* 2005). The other spectrum in the near UV region at 320-250 nm reflects the aromatic amino acid side chain environments to give information about the tertiary structure of the protein (Kelly *et al.* 2005).

2.7.3. Fluorescence spectroscopy

The determination of intrinsic hydrophobicity is very essential in order to extensively study protein conformation. This is simply based on fluorescence properties of the aromatic amino

acid (i.e. tyrosine, phenylalanine and tryptophan). Most of the intrinsic fluorescence studies involve the determination of tyrosine and tryptophan emission, which is due to their high molar absorptivity, with tryptophan being the most dominant (Malomo 2015). Fluorescence emission is observed when an excited electron returns from the first excited state back to the ground state. Proteins are capable of emitting fluorescence which can be measured as a signal when excited with ultraviolet light (Mundi 2012). Thus, florescence is an excellent spectroscopic probe to investigate conformational changes of protein. Protein fluorescence can reveal a variety of information such as the extent of rotational freedom, the exposure of amino acid side chains to quenchers and intramolecular distances (Mundi 2012). Therefore, changes in intrinsic fluorescence can be used to monitor structural conformations of proteins. The intrinsic fluorescence is usually by the contribution of the tryptophan residues in proteins that contain all the three aromatic amino acids. This is because their absorbance at the wavelength of excitation and their quantum yield of emission are considerably greater than the respective values for tyrosine and phenylalanine. The other factor in fluorescence is transfer of energy between residues, for instance, phenylalanine fluorescence is barely observed because energy transfer to that of tyrosine and tryptophan efficiently quenches its emission. The main advantage of fluorescence is that very limited quantities of materials (Nano mole) of the analyte is required (Kelly *et al.* 2005).

2.8. Biological Activities of Proteins from Legumes

Food proteins and peptides exhibit specific biological activities in addition to their established nutritional value (Mine *et al.* 2010; Hernández-Álvarez *et al.* 2013). Bioactive peptides are small amino acid sequences derived from food proteins that possess potential physiological properties beyond their normal and adequate nutrition (Udenigwe and Aluko 2012). These peptides are inactive when they are within the precursor proteins but once released they can display various biological activities (Udenigwe and Aluko 2012; López-Barrios *et al.* 2014). Therefore, proteolysis is necessary to release the peptide from the parent protein. This may occur through enzymatic digestion of the precursor protein either *in vivo* or *in vitro.* The *in vitro* protein digestibility of plant protein determines the level of digestion (hydrolysis) by the proteinase used to produce the bioactive peptides (Marambe *et al.* 2013). Consequently, for successful *in vitro* protein digestion, environmental conditions such as pH, temperature, time and concentration must be given major and adequate consideration (Moyano *et al.* 2015). Another factor to be considered is the specificity of the digestive proteases and the targeted enzyme that are responsible for the pathogenesis of the chronic diseases. This specificity

(whether open or folded conformation) determines their activity and interactions with the substrates (plant proteins) and bioactive peptides (in the case of disease-targeted enzymes) in a competitive or non-competitive manner. Thus, enzymatic protein digestion involves breaking down the long polypeptide chains into shorter chains that could fit into the specific active site of the disease-related metabolic enzymes. The potential and multi-functional health benefits of protein-derived bioactive peptides include anti-inflammatory (Malomo 2015), anti-hypertensive (Ajibola *et al.* 2013; Ruiz-ruiz *et al.* 2013), antimicrobial (Paul and Somkuti 2010), antioxidant (Alashi *et al.* 2014; Thammarat *et al.* 2015), antithrombotic (Sarmadi and Ismail 2010), antidiabetic, prevention of atherosclerosis and dyslipidaemia treatment (Malomo 2015). The bioactivity of food plant protein-derived peptides is dependent on factors such as degree of hydrolysis (Onuh *et al.* 2013), protein content (Mundi and Aluko 2014), protein solubility (He *et al.* 2013), structural conformation (He *et al.* 2013), hydrophobicity (He *et al.* 2013) and amino acid arrangement (Girgih *et al.* 2011).

2.8.1. Antioxidant effects of legumes

Biological combustion involved in the respiration process produces harmful intermediates known as reactive oxygen species (ROS•) examples of which are hydroxyl radicals (OH•), peroxyl radicals (OOR•), superoxide anion (O_2•) and peroxynitrite (ONOO). Excess ROS in the body can lead to cumulative damage in proteins, oxidation of membrane phospholipids, and modification in low density lipoproteins and mutations in DNA, thereby resulting in oxidative stress as shown in Figure 2.7 (Sarmadi and Ismail 2010).

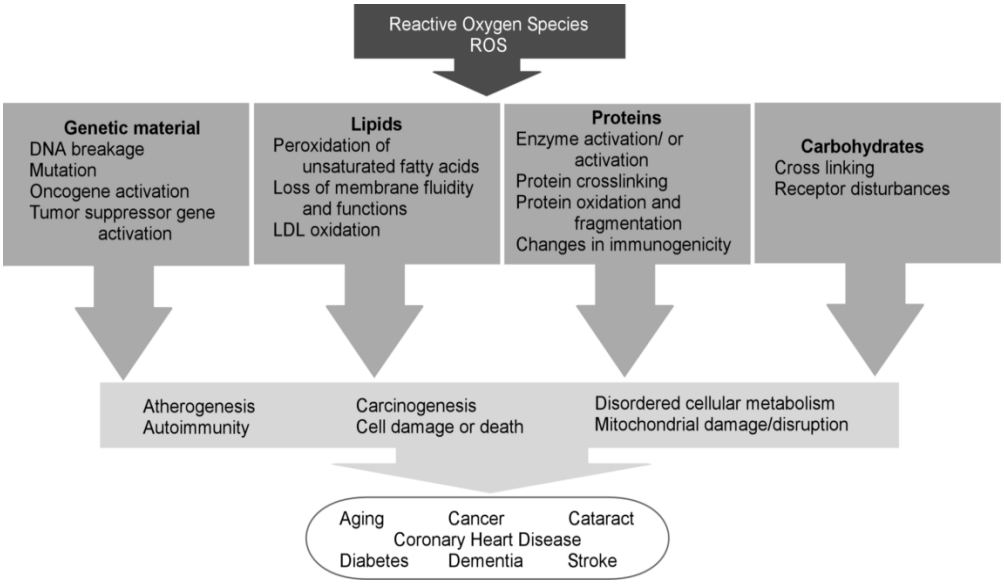


Fig.2.7: **Damage to biological molecules by reactive oxygen species (Modified from Sarmadi and Ismail (2010)**

Oxidative stress is the imbalance between oxidants and antioxidants in favour of the oxidants. This has been linked with the cause of aging, inflammatory bowel disease, and atherosclerosis (Mundi 2012). Hence, the balance between antioxidation and oxidation is believed to be a critical concept for maintaining a healthy biological system. Antioxidants are substances that when present at low concentrations compared to those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate. Since oxidative stress usually promotes the initiator of chronic diseases, there is need for synthetic and natural antioxidant to prevent oxidative stress and its deleterious effects (López-Barrios *et al.* 2014). Synthetic antioxidant has been found to be cost-effective and efficient but it displays some toxic and hazardous effects (Sarmadi and Ismail 2010). Thus, there is a need for natural antioxidants. Naturally occurring antioxidant peptides and those derived from protein hydrolysis are now considered as novel and potential dietary ingredients to promote human health (Alashi *et al.* 2014). However, the efficiency of an antioxidant peptide is usually affected by the type of protein substrate, the hydrolytic enzyme used, and the condition (pH, ionic strength, temperature, and preheat treatment) under which peptides are released from a given protein (López-Barrios *et al.* 2014). Many peptides, including those from legumes, have been prepared by enzymatic hydrolysis and have been shown to exhibit strong antioxidant activity (Table 2.5).

Generally, there remains insufficient data on role of legumes, especially bambara proteins and peptides as antioxidant agents. Therefore, there is need for further research in the production and potency evaluation of bambara protein antioxidant peptides. Although, Thammarat *et al.* (2015), studied the functional and antioxidative property of bambara, their study was limited to a hydrolysate produced using a single digestive protease. Furthermore, these authors did not study the effect of peptide fractions. The use of different proteases is important in order to determine the enzyme that produces peptides with the best antioxidant activities (Alashi *et al.* 2014; Mundi and Aluko 2014) . Also, by fractionating the hydrolysate, it is possible to identify the effect of peptide size on antioxidant activities (Ajibola *et al.* 2011). Therefore, there is a need to further study the antioxidative activities of bambara protein using different proteases and their peptide fractions.

Table 2.5: Antioxidant mechanisms of selected food protein hydrolysates

Source protein	Enzyme	Antioxidant mechanism	Reference
Canola	pepsin	Scavenging DPPH, superoxide, inhibition of linolenic acid oxidation	Alashi *et al.* (2014)
Rapeseed	alcalase	Scavenging DPPH , superoxide	He *et al.* (2013)
Bean	Pepsin and pancreatin	Scavenging of ABTS•, metal chelation	Carrasco-Castilla *et al.* (2012)
African yam bean	Alcalase	Scavenging DPPH, metal chelation, inhibition of lipid oxidation	Ajibola *et al.* (2011)
Peanut protein	Alcalase	Inhibition of linoleic acid oxidation, radical-scavenging activity, reducing power and inhibitor of liver lipid oxidation	Chen *et al.* (2007)

DPPH•: 2, 2-diphenyl-1-picrylhydrazyl radical; ABTS•+:2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

2.8.2. Blood pressure effect of legume

There is a high prevalence of high blood pressure (HBP) in most nations. This critical health condition accounts for approximately 45-51% of total global deaths (Malomo and Aluko 2015). Hypertension or HBP can occur as a result of several contributory factors which include, diabetes, kidney disease, and obesity among others (Aluko 2015). Usually, hypertension is said to be normal when systolic and diastolic blood pressure are 140 and 90 mm Hg respectively (Malomo *et al.* 2015). During normal blood regulation, renin converts angiotensinogen into inactive peptide fragment known as angiotensin I. A second renin-angiotensin system (RAS) enzyme known as angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II which binds to the vascular wall to cause blood vessel contractions (Aluko, 2015). Nevertheless, during metabolic disorder excessive functional operation of RAS leads to abnormally high blood levels of angiotensin II which is the main cause of hypertension (Fig. 2.8). ACE activity also contributes to hypertension through another reaction where it catalyses degradation and inactivation of bradykinin a powerful vasolidator (Fig. 2.8) Hence, one of the main approaches to hypertension treatment is inhibition of renin and ACE activities to produce homeostatic angiotensin II levels as illustrated in Fig. 2.8.

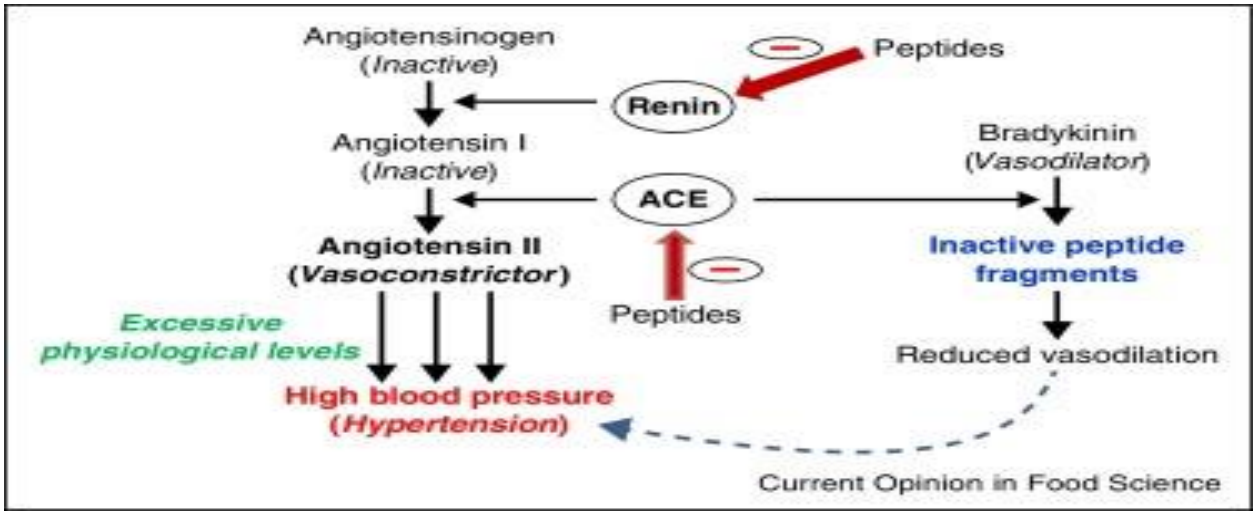


Fig.2.8: **The renin-angiotensin system-induced hypertension and enzyme targets for negative modulation by antihypertensive peptide**s **Source:(Aluko 2015)**

Suppression of angiotensin II production through ACE inhibition will be of great importance because once angiotensin II is prevented from forming there would be no hypertension. ACE has also been reported to contain four functional amino acid residues of Tyr, Arg, GLu and Lys at the active site and three hydrophobic binding sub-sites which can be blocked by small peptides with high affinity for active sites (Mundi and Aluko 2014). Based on this, small peptides are targeted for developing antihypertensive food products (Mundi and Aluko 2014). Renin inhibitors can serve as effective blood pressure lowering agents. This is because ACE catalysed reaction is wholly dependent on renin activity to obtain angiotensin I that is needed for angiotensin II productions. Therefore, a decrease in renin activity will lead to decrease in angiotensin II levels and concurrently lower vascular contractile activity (Aluko 2015). It has been reported that negative modulation of RAS enzyme activities was achieved using several synthetic compounds such as Capoten (Captopril), Prinivil (Lisinopril), Vasotec (Enalapril), and Altace (Ramipril) that act as ACE-inhibitory drugs (Aluko, 2015). However, ACE-inhibitory drugs were not very effective in several patients due to two main reasons. First, apart from ACE, there are other enzymes such as chymase, trypsin, tonin, kallikrein, and cathepsin G that can generate angiotensin II (Belova 2000). Second, ACE inhibition usually leads to increased renal renin secretion hence, high plasma angiotensin I molecules, which are then converted via non-ACE routes to cause elevated angiotensin II levels (Malomo *et al.* 2015). Recently, Aliskiren (Tekturna) became the only renin-inhibitory drug approved for treatment of human hypertension. Since single enzyme (renin) is responsible for conversion of angiotensinogen to angiotensin I, then renin inhibitors may provide better blood pressure modulation than ACE inhibition (Aluko 2015). The regular use of ACE-inhibitory and renin-

inhibitory drugs has been associated with negative side effects such as dry cough, edema, diarrhoea, fatigue, dizziness, headache and erectile dysfunction (Aluko 2015). The results of side effects include reduction in patient's compliance with the treatment dosage, worsening disease conditions and in extreme cases, preventable loss of life (He *et al.* 2013). Therefore, there is an urgent need for development and production of natural food protein-derived peptides that could replace or complement antihypertensive drugs. This is because naturally produced peptides seem to have none or lesser negative side effects when compared to synthetic compounds (He *et al.* 2013; He *et al.* 2013; Alashi *et al.* 2014).

Recently, studies have shown antihypertensive activities of some legumes. For instance canola protein hydrolysate produced by various proteases (alcalase, chymotrypsin, pepsin, trypsin and pancreatin) inhibited in vitro activities of renin and ACE in addition to in vivo lowering of systolic blood pressure in spontaneous hypertensive rats (Alashi *et al.* 2014). Furthermore, the <1 and 5-10 kDa peptide fractions of kidney bean protein hydrolysate exhibited significantly higher renin inhibition than other fractions (Mundi and Aluko 2014). In addition, hemp seed protein digest was also found to show in vitro ACE and renin inhibition (Malomo *et al.* 2015). Despite the abundance of information on the RAS-inhibitory and antihypertensive bioactive peptides from plant proteins, there exists little or no information on ACE-inhibitory properties of bambara proteins and peptides as antihypertensive agents. Therefore, there is need for further research in the production and potency evaluation of bambara protein antihypertensive peptides.

2.9. Conclusions

The protein composition and structure of legumes varies depending on legume type, genotype and growing conditions. The proteomic maps of most indigenous legumes including bambara have not been established. Also, the protein composition and structure in terms of effect of pH on the circular dichroism (CD) and fluorescence spectroscopy properties of bambara protein has not been reported. In addition, to our knowledge, different studies performed by different researchers have not reported the antioxidant and antihypertensive potencies of protein hydrolysates and peptides from bambara protein. This information is necessary to determine the potential usage of bambara protein in food and health product processing.

RESEARCH CHAPTERS

These chapters are organised into four chapters based on specific objectives of this study.

The chapters are presented as follows:

Chapter 3: Influence of extraction methods on bambara protein functionality

Chapter 4: Composition of bambara protein

Chapter 5: Antioxidant properties of bambara protein hydrolysates and fractions

Chapter 6: Antihypertensive properties of bambara protein hydrolysates and fractions

CHAPTER THREE

3. Influence of extraction methods on functional properties of protein concentrates prepared from South African bambara groundnut landraces

ABSTRACT

Functional properties of protein concentrates prepared from three bambara groundnut landraces using acid precipitation and salt solubilisation methods were evaluated. The protein content of bambara grains (26–27%) was similar for the three landraces. The acid precipitation gave a much higher yield of protein concentrates (52%), which were also high in protein (79%) compared to the salt solubilisation method (yield: 25%, protein content: 57%). Functional properties of proteins were more influenced by the methods of preparation rather than the landraces. Protein concentrate prepared by salt solubilisation method showed higher emulsifying (63–66%), foaming (53–57%), water (1.4–2.0 mg mL^{1}) and oil absorption properties (2.2–2.6 mg mL^{1}) than the acid-precipitated concentrates (53–57%, 63–66%, 2.0–2.7 mg mL^{1}, 1.4–1.7 mg mL^{1}). The foaming capacity and stability of all the protein concentrates decreased with increasing pH from 3 to 8. Salt solubilisation may be the most appropriate method for the enhanced functionality and utilisation of bambara groundnuts' protein concentrates.

3.1. Introduction

Bambara groundnut (*Vigna subterranea* L. Verdc) is an underutilised legume of the African origin (Adegbola and Bamishaiye 2011). It is the third most important legume after groundnut (*Arachis hypogeal*) and cowpea (*Vigna unguiculata*) in Africa (Adegbola and Bamishaiye 2011). Bambara is indigenous to South Africa and is grown mainly in Limpopo, Mpumalanga and KwaZulu-Natal provinces of South Africa (Mabhaudhi and Modi 2013). The protein content of bambara grain may vary between 15% and 25% (Adegbola and Bamishaiye 2011; Hillocks *et al.* 2012; Murevanhema and Jideani 2013). This is similar to cowpea and slightly lower when compared to that of soya bean (Adegbola and Bamishaiye 2011). Furthermore, bambara groundnut is highly drought tolerant and produces good yield under harsh agronomic conditions, which gives it an advantage over other legume grains such as groundnut and soybean (Mazahib *et al.* 2013). Despite these attributes, the agro-ecological, genetic potential

as well as the nutritional importance of bambara groundnuts has not been fully researched (Boateng *et al.* 2013). The crop is still cultivated from local landraces in South Africa.

Plant protein concentrates may be utilised in foods for the improvement of both nutritional and functional quality of the food products. The yield, composition and functionality of proteins may vary depending on the grain varieties and method of extraction (Zayas 1997). The functional properties of soya bean protein and concentrates have been extensively researched (Kwon *et al.* 2010; De la Caba *et al.* 2012; Rebholz *et al.* 2012). For example, the protein yield of soya bean concentrate (16.2%) prepared by micellisation method was found to be substantially low, three to four times that obtained by isoelectric precipitation. However, soya bean protein concentrate obtained by isoelectric precipitation showed lower foaming capacity compared to soya bean concentrate extracted using the micellisation method (Adebowale *et al.* 2011). This suggests that methods of extraction may have an influence on the functionality of proteins and, consequently, their application in foods. A comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut cultivated in Thailand was carried out by Kudre *et al.* (2013). Findings show that all protein isolates contain substantial amount of lysine. However, by DSC, mung bean and bambara isolates were characterised by two endothermic peaks, whereas three peaks were found for black bean isolates, suggesting some differences in thermal stability and structural composition. In a recent study, (Kudre and Benjakul 2014) investigated further the effects of heat treatment (50–80 °C) in combination with ethylenediaminetetraacetic acid (EDTA) on functional and sensory properties of bambara protein isolates. Results show that bambara protein isolates prepared in the presence of EDTA exhibited higher emulsion activity and stability indices as well as higher foam expansion and stability than those prepared in the absence of EDTA, regardless of the heating temperature at 95% level of significance. Although diverse studies on bambara landraces have been reported in Africa, particularly in Nigeria (Adebowale *et al.* 2011; Adegunwa *et al.* 2013), results have revealed some differences in their functional properties. However, as stated above, bambara from Southern Africa remains underutilised. Due to the increasing interest in alternative protein sources for human nutrition and functional applications in foods, the knowledge of the physicochemical properties of South African bambara protein may be required to facilitate utilisation and value addition. Therefore, this study aims to investigate the functional properties of protein concentrate extracted from South African bambara groundnut landrace using acid precipitation and salt solubilisation methods.

3.2. Materials and Methods

3.2.1. Materials

Three cultivated landraces of bambara groundnuts were obtained from Josini, KwaZulu-Natal province of South Africa. These were identified based on the seed coat colour as red, maroon and cream.

Fig. 3.1: Bambara Landraces (A : Red, B: Maroon and C: Cream)

3.2.2. Preparation of defatted flours

Bambara groundnuts were dehulled manually using a mortar and pestle. Dehulled grains were thereafter ground in a Warring laboratory mill blender (HGBTWTS3, Torrington, CT, USA) and sieved through a screen mesh of 355 μm to obtain fine flour. Bambara flour was defatted using n-hexane in the ratio 1:5 (flour: solvent) for 3 h on a magnetic stirrer at speed of 198 g.

3.2.3. Preparation of concentrates

Acid precipitation and salt solubilisation methods were followed for the preparation of bambara groundnut concentrates. For acid precipitation, the method utilised by (Adebowale *et al.* 2007) was adopted with some modifications. Briefly, defatted flour was suspended in water at 1:10 (flour to water ratio) and pH was adjusted to 8.0 with 1 M NaOH, to ehance protein solubilisation. The suspension was stirred for 4 h at 32°C in a shaking water bath (Scientific, 132A, Pretoria, South Africa). Centrifugation was thereafter carried out (Eppendorf 5810R, Hamburg, Germany). The centrifuge was set at 4000 g for 30 minutes at 4°C. After centrifugation and recovery of the supernatant, the precipitate was resuspended in half the volume of initial water and extraction was carried out as described above. The supernatants were collected and pH adjusted to 4.0 with 0.5 M HCL to precipitate the protein concentrates, which were recovered by centrifugation at 5000 g for 30 minutes at 4°C. The

protein concentrate was freeze-dried (model 22KBTES_55ZIRBUS technology, Bad Grund, Germany) and kept at 4°C until required.

Protein concentrates were prepared using the method of (Teixeira *et al.* 2013), with some modifications. Defatted flours were suspended in 0.5 M NaCl (1:10 w/v). The suspension was stirred in a shaking water bath for 4 h at 32°C. The suspension was centrifuged at 12 000 g for 20 minutes at 4°C. The clear supernatant was dialysed (cut-off 10 kDa) against distilled water for 48 h. The dialysed extract was freeze-dried and stored at 4°C until required.

3.3. Analysis

3.3.1 Grain composition

Moisture, crude fat and total ash contents were determined using AOAC methods (AOAC, 2000) with analytical numbers 950.46, 960.39 and 920.153, respectively. The crude protein content (N x 6.25) was determined by Kjeldahl method with No. 928.08. Total carbohydrate was calculated by difference.

3.3.2 Protein yield and protein content

Yield of protein concentrate was determined as the dry weight of protein concentrate after precipitation and solubilisation, respectively, per weight of the defatted flour as shown below (Qayyum *et al.* 2012). The protein content (N x 6.25) of the defatted flour and the protein content of the concentrates were determined by Kjeldahl method (AOAC 2000).

$$Yield(\%) = \frac{Protein\ concentrate\ recovery \times protein\ content\ of\ concentrate\ (\%) \times 100}{Protein\ content\ of\ defatted\ flour(\%)}$$

3.3.3 Colour

Colour measurement of the concentrates was carried out using colour flex (A60-1014-593; Hunter Associates Laboratory, Reston, VA, USA) on the basis of lightness (L*), red-green (a*) and yellow-blue (b*) values. Soya bean isolate was used as reference. The instrument was calibrated against white and black colour tiles before colour measurement. Total colour difference (DE) was calculated as shown below $\Delta E = [\ (\Delta L^*)^2 + (\Delta a^*) + (\Delta b^*)^2]^{1/2}$.

3.3.4 Water and oil absorption capacity

The water absorption capacity and oil absorption capacity of protein concentrates obtained by acid precipitation and salt solubilisation were measured as previously described by Mundi & Aluko (2012), with some adjustments. One gram of each sample was dissolved in 10 mL of distilled water (or sunflower oil) in a 50 mL pre-weighed centrifuge tube. The dispersion was

vortexed for one minute, allowed to stand for thirty minutes and then centrifuged at 4000 g for 30 minutes at ambient temperature. The supernatant was emptied, excess water (or oil) in the upper phase was drained for 15 minutes. In order to determine the amount of water or oil retained per gram of the sample, the weight of the tube comprising the residue was taken again.

3.3.5 Foam capacity

Foams were formed as prescribed by Aluko et al. (2009), with some modifications. Suspensions were prepared by dispersing 300 mg in 5 mL of 0.1 M phosphate buffer at pH 7.0. Sample suspensions were homogenised at 3600 g for 1 minute using Polytron homogenizer (PT 210; Fisher Scientific, Water Side, UK). Volumes of the suspension were recorded before and after homogenisation. Foaming capacity was determined as follows using the mean of three measurements.

$$Foam\ capacity\ (FC) = \frac{Volume\ after\ homogenisation - volume\ before\ homogenisation\ \times 100}{volume\ before\ homogenisation}$$

Foam stability was determined as the volume of foam that remained after 8 h at room temperature expressed as percentage of the initial volume.

The effect of pH on foaming properties was carried out by adjusting the suspension to the desired pH 3.0, 6.5 and 8.0 using either 1 M HCl or 1 M NaOH. Homogenisation was thereafter carried out as described above. The final volume after the addition of HCl or NaOH was used as the volume before homogenisation for the calculation of the foaming capacity.

3.3.6 Emulsifying activity and stability

Emulsifying activity and stability were determined using the method described by Lawal et al. (2007). Five millilitre portions of protein solution were homogenised with five mL of sunflower oil. The emulsions were centrifuged at 1100 g for five minutes. The height of the emulsified layer and that of the total contents in the tube was determined. The emulsifying activity (EA) was calculated using the expression below.

$$EA\ (\%) = \frac{Height\ of\ emulsified\ layer\ in\ the\ tube\ \times 100}{Height\ of\ the\ total\ content\ in\ the\ tube}$$

Emulsion stability was determined by heating the emulsion at 80 °C for 30 minutes after which it was centrifuged at 1100 g for 5 minutes.

$$ES\ (\%) = \frac{Height\ of\ emulsified\ layer\ after\ heating\ \times 100}{Height\ of\ emulsified\ layer\ before\ heating}$$

3.3.7 Statistical analysis

Experiments were conducted in triplicate. Mean scores of some of the results and their standard deviation were reported. Data were subjected to analysis of variances, and Duncan multiple range (Duncan, 1995) test was used to separate the means.

3.4. Results and Discussion

3.4.1. Grain composition

The carbohydrate, protein, fat, ash and moisture content of the three landraces of bambara grain were found to be similar (Table 3.1). The protein contents (26–27%) of bambara landraces observed in this study appeared slightly higher than (23-24%) those previously reported for the previous research (Mahala and Mohammed 2010; Adegbola and Bamishaiye 2011).The protein contents of bambara landraces in comparison with other legumes were seemingly higher than those of cowpea and chickpea (Hillocks *et al.* 2012), but similar to that of kidney bean (Wani *et al.* 2013). Carbohydrate was the major nutrient in bambara, with approximately 56%, which was within the values reported for bambara groundnut flour (Adegbola and Bamishaiye 2011; Hillocks *et al.* 2012; Murevanhema and Jideani 2013). The carbohydrate contents of the landraces grains appeared slightly lower than those of cowpea, faba bean and chickpea (Hillocks *et al.* 2012). Bambara landraces contained low fat contents, which are within the range reported by (Hillocks *et al.* 2012).

Table 3.1: Proximate composition of the three landraces bambara groundnut grains

Samples	Moisture	Protein	Fat	Ash	*Carbohydrate
Red	$8.6^{b} \pm 0.2$	$26.0^{a} \pm 0.3$	$6.6^{c} \pm 0.0$	$3.0^{a} \pm 0.0$	$55.8^{a} \pm 0.2$
Maroon	$8.0^{a} \pm 0.9$	$27.3^{c} \pm 0.2$	$5.3^{a} \pm 0.1$	$3.3^{c} \pm 0.0$	$56.0^{a} \pm 0.0$
Cream	$8.1^{bc} \pm 0.3$	$26.4^{b} \pm 0.1$	$5.7^{b} \pm 0.0$	$3.2^{b} \pm 0.0$	$56.6^{b} \pm 0.1$

Mean ± SD. Means values followed by different superscripts in each column are significant different at $P \leq 0.05$.

*Carbohydrates by difference

3.4.2. Protein content and protein yield of the concentrate

Protein content and yield varied with the method of extraction rather than the landraces (Table 3.2). Concentrates prepared by acid precipitation gave much higher protein contents and yields (79% and 52%), respectively, compared to that obtained by salt solubilisation. The variations in protein contents and yields as a function of the extraction methods seem to be in agreement with literature (Boye *et al.* 2010). Previous studies conducted on soya and other

legumes such as chickpea, broad bean and kidney bean have found acid precipitation method with relatively high protein content (Castel *et al.* 2012; Qayyum *et al.* 2012). Therefore, the protein content of the concentrates obtained through acid precipitation in this research is in agreement with the result reported for pea, chickpea, lentil, broad bean and kidney bean (Castel *et al.* 2012; Qayyum *et al.* 2012). Similarly, the higher protein yield obtained in this study may be attributed to the adjustment in pH using NaOH (pH 8), which may have enhanced the extractability of the protein. Proteins have been reported to exhibit higher solubility at pH above their isoelectric point (Adebowale *et al.* 2011). Okezie and Bello (1988), found that a change in pH of the extracting medium resulted in greater protein extractability of winged bean flour. Castel *et al.* (2012), reported high protein yield for Amaranth mantegazzianus protein concentrate when acid precipitation method was used. Similar studies have found salt solubilisation method with lower protein yield and content. Adebowale *et al.* (2007) reported lower yield for mucuna bean protein concentrate prepared by salt solubilisation when compared to the acid precipitation method. The protein content of the concentrates extracted through salt solubilisation in this study is similar to those previously reported for bambara protein concentrates and Bauhinia cheilantha seeds (Boateng *et al.* 2013; Teixeira *et al.* 2013).

Table 3.2: Protein yield and content of bambara groundnut concentrates obtained by acid precipitation and salt solubilisation

Extraction Method	Landrace	Protein Content (%)	Protein Yield (%)
Acid Precipitation	Red	$78.8^{de} \pm 0.4$	$51.2^{c} \pm 1.1$
	Maroon	$77.9^{d} \pm 0.5$	$54.5^{d} \pm 0.7$
	Cream	$79.8^{e} \pm 0.4$	$49.5^{c} \pm 0.8$
Salt Solubilisation	Red	$57.3^{b} \pm 0.8$	$25.5^{b} \pm 0.7$
	Maroon	$59.1^{c} \pm 0.8$	$23.5^{a} \pm 0.7$
	Cream	$55.1^{a} \pm 0.8$	$24.5^{b} \pm 0.7$

Mean ± SD. Means values followed by different superscripts in each column are significantly different at $P \leq 0.05$

3.4.3. Colour

The colours of bambara protein concentrates were significantly influenced by the method of extraction (Table 3.3). The protein concentrates prepared by acid precipitation appeared slightly brownish in colour with low lightness (L*) and high redness (a*) and yellowness (b*) values compared to the concentrates prepared by salt solubilisation. The variation in colour indices was reflected in the total colour difference (ΔE). The colours of the salt solubilisation protein concentrates were much similar to that of the reference soya protein. The use of acid produced slightly brownish concentrates. The browning of the acid-precipitated isolate may

be due to changes in pH during the preparation. Similar findings have been reported for kidney bean when the same method was used for the preparation of its isolates (Wani *et al.* 2015) and lentil protein isolates (Joshi *et al.* 2011). Unlike the method of extraction, the colour of the grain coats did not seem to have any major effect on the colour of protein concentrates after preparation.

Table 3.3: Colour values of concentrates prepared by acid precipitation and salt solubilisation of bambara landraces

Extraction Method	Landrace	L*	a*	b*	ΔE
Acid Preparation	Red	84.3^{b}	0.5^{a}	13.2^{e}	14.5^{c}
	Maroon	84.9^{e}	1.3^{d}	14.6^{d}	15.3^{d}
	Cream	80.5^{a}	2.8^{e}	14.8^{e}	18.7^{e}
Salt Solubilisation	Red	90.7^{e}	0.6^{b}	8.4^{b}	6.9^{a}
	Maroon	89.3^{d}	0.9^{e}	8.2^{a}	7.6^{b}
	Cream	90.7^{e}	0.6^{b}	8.3^{b}	6.9^{a}

Mean ± SD. Means values followed by different superscripts in each column significant different at $P \leq 0.05$

3.4.4. Water and oil absorption capacity

Water absorption capacity (WAC) was influenced by the landrace and extraction methods (Fig. 3.2). Protein concentrate prepared through salt solubilisation absorbed slightly more water (2.4 mL g^{1}) than those prepared through acid precipitation. For the acid precipitation method, the cream landraces showed a slightly high water absorption capacity (2.0 mL g^{1}) than the red and maroon landraces. On the other hand, the red landraces showed higher values of water absorption capacity than other landraces in the concentrates obtained through the salt solubilisation concentrate. The higher water absorption capacity value for concentrates obtained through salt solubilisation is in agreement with the result reported for chickpea protein when a similar method of extraction is used (Boye *et al.* 2010). Albumin and globulin are the major storage proteins in the concentrate obtained by salt solubilisation. These proteins may have been responsible for the high WAC. Bambara concentrates obtained by salt solubilisation method recorded a low protein compared to that obtained by acid precipitation (Table 3.2). Possibly, the presence of non-protein contents in salt-solubilised concentrates may have contributed to its high WAC when compared to the acid precipitated concentrates. Water absorption capacity of protein concentrates in this study is similar to isolates from kidney bean and Ginkgo biloba seeds (Deng *et al.* 2011; Shevkani *et al.* 2014).

Previous reports have recommended water absorption capacity ranging from 1.49 to 4.72 Mlg^{1} for use in viscous food (Mundi and Aluko 2012). The high water absorption capacity reported in this study, especially for the concentrates prepared by salt solubilisation, falls

within the stated range. Therefore, this suggests that bambara concentrates may be used in the formulation of some foods such as dough, soups, processed cheese and baked products.

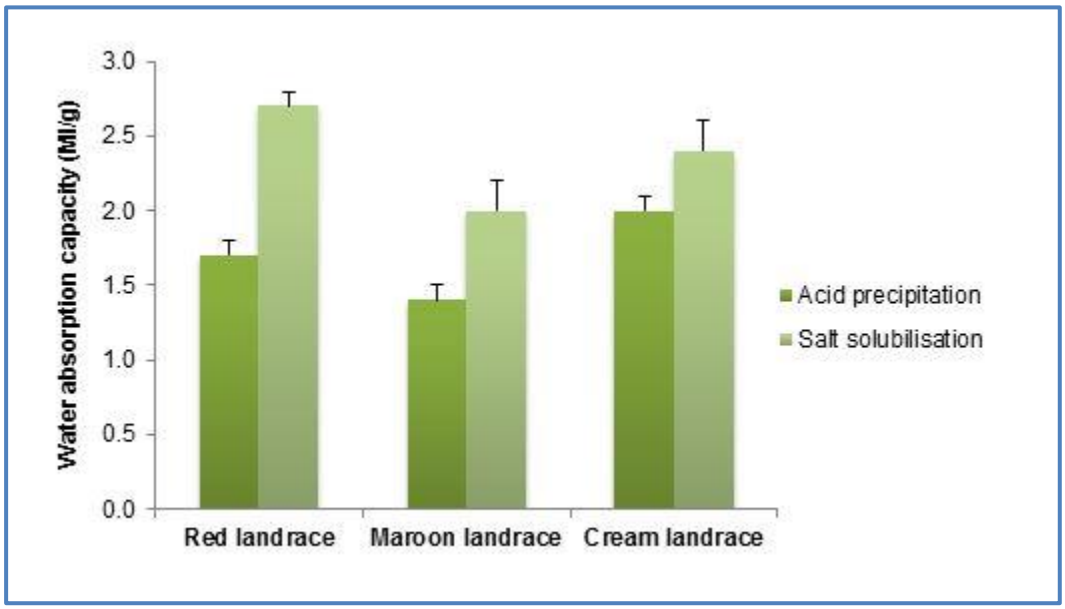


Fig. 3.2: Water absorption capacity of acid precipitation and salt solubilisation concentrates of three bambara groundnut landraces

Oil absorption capacity (OAC) was influenced by both landrace and extraction methods (Fig. 3.3). OAC of the protein concentrates prepared by salt solubilisation for the three landraces was higher (red: 2.2 mg mL^1; maroon: 2.6 mL g^1; and cream: 2.2 mL g^1). These values were almost twice the values of acid precipitation concentrate (red: 1.7 mg mL^1; maroon: 1.4 mL g^1; and cream: 1.5 mL g^1). This result is similar to findings earlier reported for chickpea protein isolate. Boye *et al.* (2010) reported a higher oil absorption capacity for chickpea protein isolate extracted using salt when compared to isoelectric precipitation. A similar result was reported by Mwasaru *et al.* (1999) for cowpea and pigeon pea concentrate in which both salt solubilisation and acid precipitation method were used for extraction. The oil absorption capacity is crucial and of great importance from an industrial point of view. This is due to its influence on the emulsifying capacity, a highly desirable characteristic in products such as mayonnaise (Mundi and Aluko 2012). The oil absorption capacity of the concentrates obtained by salt solubilisation in this study is in agreement with that reported for chickpea protein isolate (Boye *et al.* 2010), but higher than that of soya bean protein isolates (Shevkani *et al.* 2014). Findings from this study suggest that bambara protein concentrates prepared through salt solubilisation could be used in retaning flavour, improvement of palatability and extension of shelf life.

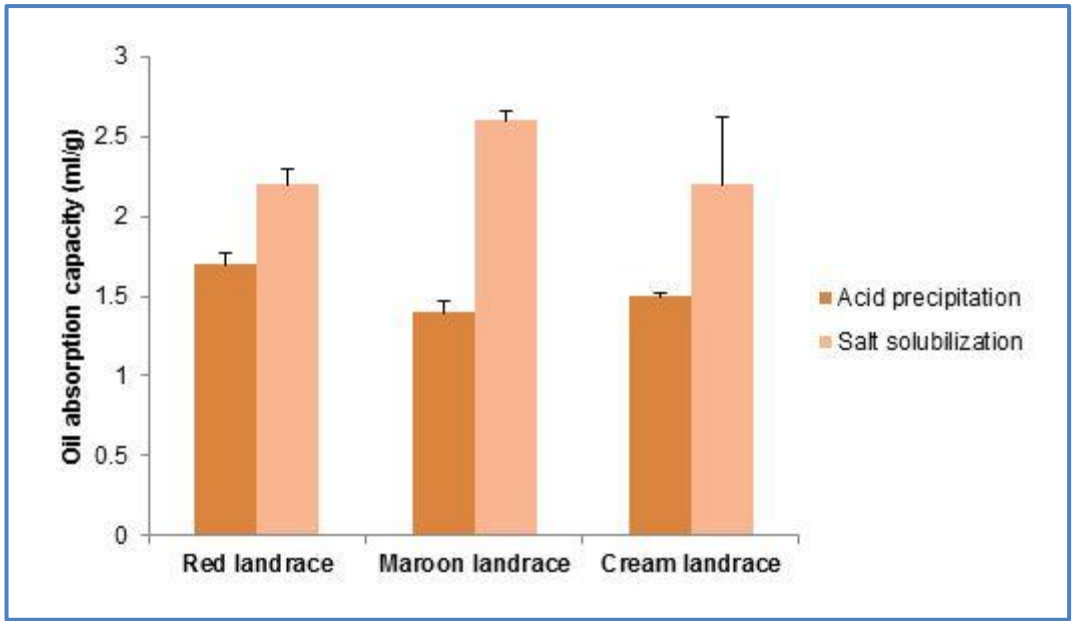


Fig. 3.3: Oil absorption capacity of the acid precipitation and salt solubilisation concentrates from three landraces bambara groundnut

3.4.5. Foaming capacity

The foaming capacity of the three landraces decreased with increased pH (Fig. 3.4). All the three landraces showed similar foaming capacity at pH 6.5 and 8.0. However, at pH 3.0, the maroon landrace showed a slightly higher foaming capacity compared to other landraces. Furthermore, the foaming capacity of the concentrates extracted by salt solubilisation was higher (approximately 1.3 times) than the concentrates obtained by acid precipitation method.

Decrease in foaming capacity with increase in pH has been reported for both mucuna bean and cowpea (Aluko and Yada 1995; Lawal *et al.* 2007). Foaming indicates the ability of protein to form films around gas bubbles in water. The solubility of protein in aqueous and its ability to diffuse at the air–water interface, unfold, form a cohesive and strong film to prevent rupture and coalescence are important factors in foaming capacity (Damodaran and Parkin 2008). Graham and Phillips (1976), linked good foaming capacity with flexible protein molecules that can reduce surface tension, while highly ordered globular proteins, which are relatively difficult to surface-denature, gave low foaming capacity. Hence, one may suggest that bambara groundnut proteins may be high in flexible protein at acidic pH. The highest foaming capacity at the acidic pH 3 could be due to the decrease in attractive hydrophobic forces among the protein molecules, which occur at acidic regions. This development leads to repulsion, which facilitates the flexibility of the protein molecules, making them diffuse more rapidly in the air–water interface to encapsulate air particles, leading to high foaming capacity (Adebowale and Lawal 2003). The results of the foaming capacity obtained in this study for

both methods of extraction are in agreement with values reported for pigeon pea and mucuna bean concentrate when similar extraction methods were used (Adebowale and Lawal 2003). The higher foaming capacity of the salt solubilisation may be due to the presence of albumin, which has weakened the hydrophobic interaction. This may result in an increase in solubility and flexibility that allows for a wider spread on the air-water interface, better encapsulation of air particles, and consequently, an increase in foam formation (Shevkani *et al.* 2014). Furthermore, the salt used in the preparation of the salt solubilisation concentrate may be responsible for its higher foaming capacity. This is because there is higher solubility of vegetable protein in salt solutions, coupled with the ability of the salt to aid diffusion and spread at the interface (Akintayo *et al.* 1999).

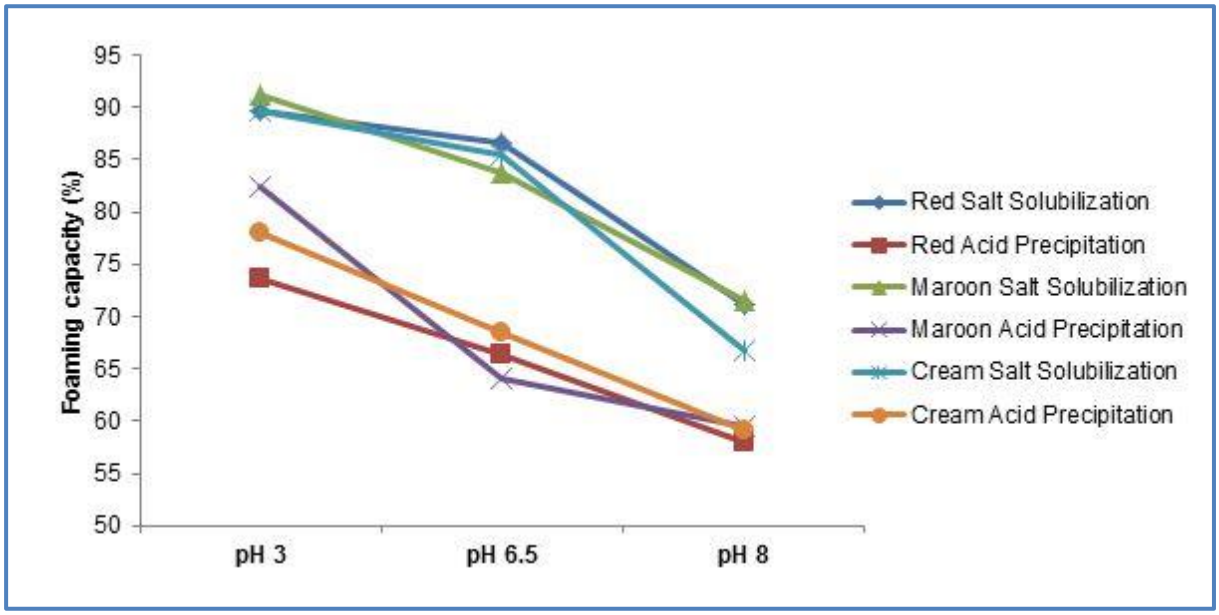


Fig. 3.4: Effect of pH on foaming capacity of the landrace acid precipitation and salt solubilisation concentrate

3.4.6. Foaming stability

The foaming stability decreased as pH increased for all the landraces. Also, the same trend was observed for the extraction methods (Table 3.4). After 8 h, the highest foaming stability was observed at pH 3 for all the landraces. However, concentrates obtained by salt solubilisation had better foaming stability than the concentrates obtained through acid precipitation at all pH. Similar results were reported for soybean protein extracted by salt solubilisation and acid precipitation (Adebowale *et al.* 2011). Boye *et al.* (2010) also reported lower foaming stability for pea proteins obtained through acid precipitation. The maroon landrace concentrate obtained by acid precipitation had the highest (83%) foaming stability compared to other landrace protein. The highest foaming stability observed at pH 3 could be

as a result of formation of more stable molecular layers in the air–water interface of the foams. Protein adsorption and viscoelasticity at an air–water interface are higher near or at isoelectric pH because protein is weakly resisted. Higher foaming stability at low pH 4 has been previously reported for mucuna protein concentrate (Adebowale and Lawal 2003). The results obtained for the foaming activity and stability in this study indicates that the concentrates prepared through salt solubilisation could serve as replacements of better-known proteins in food applications such as whipping, toppings and ice cream.

Table 3.4: Foaming stability (%) of bambara landrace protein concentrates extracted at different pH

Extraction method	Landrace	pH 3	pH 6.5	pH 8
Acid precipitation	Red	73.6a ± 2.0	66.3a ± 1.0	58.0a ± 0.5
	Maroon	82.5c ± 1.5	64.1a ± 3.1	59.5a ± 2.0
	Cream	78.0b ± 0.3	68.5a ± 2.5	59.2a ± 0.7
Salt solubilisation	Red	89.7d ± 0.4	86.6b ± 2.4	71.1c ± 0.4
	Maroon	91.2d ± 2.1	83.8b ± 1.9	71.5c ± 2.1
	Cream	89.7d ± 0.8	85.5b ± 0.8	66.7b ± 0.0

Mean ± SD. Means values followed by different superscripts in each column significant different at $P \leq 0.05$

3.4.7. Emulsion properties

The emulsifying activity and stability of the cream landrace were higher compared to the red and maroon landraces (Figs 3.5 and 3.6). The maroon landrace concentrates obtained through acid precipitation had the lowest emulsion stability in comparison with other landraces.

The methods of extraction adopted had an influence on the emulsifying activity and stability of the concentrates. The emulsifying activity of concentrates obtained through salt solubilisation (approximately 64%) was slightly higher than the concentrate obtained through acid precipitation method (approximately 55%). The higher emulsifying activity and stability observed for the salt solubilisation concentrates may be attributed to its lower protein content (Table 2) compared to the acid precipitated concentrate. This may be due to the fact that the emulsifying capacity of proteins tends to decrease as protein concentration increase (Mao and Hua 2012). Boye *et al.* (2010) reported higher emulsifying properties for micellised pea, chickpea and lentil protein concentrate compared to the isoelectric concentrate. Generally, the high emulsifying activity and stability of bambara protein concentrate obtained in this study, especially concentrates prepared through salt solubilisation, could serve as a potential ingredient in formulations of foods such as ice creams, mayonnaises and sausages.

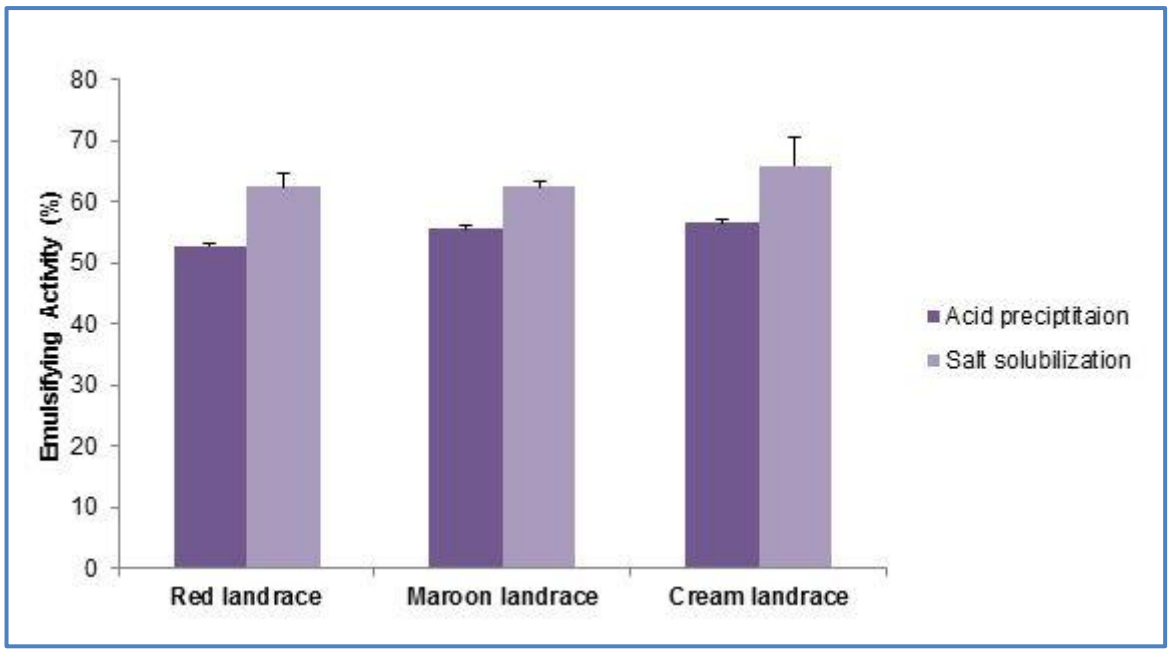


Fig. 3.5: Emulsifying activity of acid precipitated and salt solubilised concentrate of bambara landraces

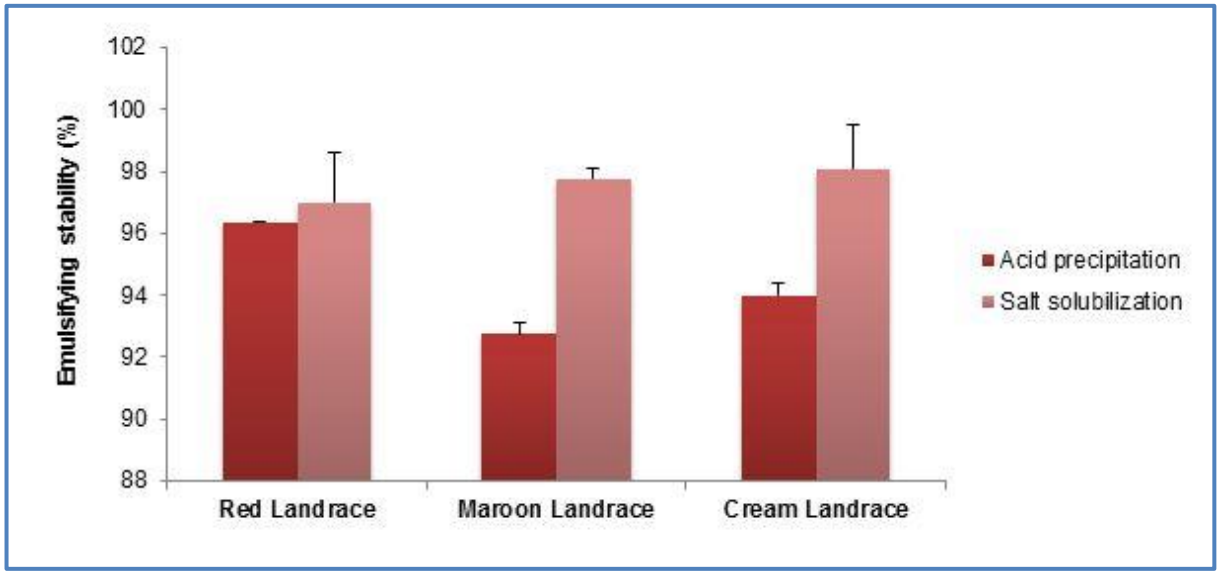


Fig. 3.6: Emulsifying stability of Acid precipitated and salt solubilised concentrate from landrace bambara groundnut

3.5. Conclusions

Bambara groundnut landraces are good sources of proteins and carbohydrates. Acid precipitation produces bambara concentrates with high protein content and yield compared to concentrates prepared by salt solubilisation. Protein concentrates prepared through salt solubilisation method exhibited better functional properties in terms of water absorption capacity, oil absorption capacity, foaming capacity, foaming stability and emulsion activities

when compared to concentrates obtained through acid precipitation. This study suggests that salt solubilisation may be more appropriate method for the enhanced functionality and utilisation of bambara groundnuts' protein concentrates.

3.6. Research outputs

a) Journal article

1. **Abimbola K. Arise**, Eric O. Amonsou and Oluwatosin A. Ijabadeniyi (2015). Influence of extraction methods on functional properties of protein concentrates prepared from South African bambara groundnut landraces. International Journal of Food Science and Technology, 50: 1095-1101.

CHAPTER FOUR

4. Composition, structural and functional properties of protein isolates from bambara groundnut (*Vigna subterranea*) landraces

ABSTRACT

The structural and functional properties of protein isolates from three bambara grains (red, maroon and cream) were determined. The protein contents (approx. 91%) were similar for the 3 isolates while glutamic and aspartic acids were the most abundant amino acids. SDS-PAGE revealed four major protein bands: one broad band at 55 kDa, two medium bands at 62 kDa and 80 kDa and a high molecular (HMW) protein at 141 kDa. Bambara seed proteins contained two subunits (62 and 55 kDa) of the vicilin-like protein. Under reducing conditions, the HMW protein was absent and two new protein bands (26 kDa and 18 kDa) emerged, thus suggesting the presence of disulphide bonds. Proteomic map of bambara protein revealed different spots across the landraces. Bambara seed proteins showed two endothermic peaks: 64-69^{o}C and 76-90^{o}C from differential scanning calorimetry analysis. The result from intrinsic fluorescence and circular dichroism show that bambara seed protein isolates had well-defined tertiary and secondary structures, respectively at pH 3.0, which decreased slightly at higher pH values. Foaming capacity and emulsion ability were similar for all the landraces and are pH dependent. Bambara protein will serve as an excellent ingredient for food foam and emulsions formulation.

4.1. Introduction

Bambara groundnut (*Vigna subterranea L.verdc*) is an indigenous African legume. It is a good source of protein (15-27%), similar to cowpea and peanuts (Adegbola and Bamishaiye 2011; Kudre *et al.* 2013; Arise *et al.* 2015). In Southern Africa, bambara is grown mainly in Limpopo, Mpumalanga and KwaZulu-Natal provinces of South Africa (Arise *et al.* 2015). It is a drought- tolerant legume, hence has great potential as an alternative legume seed to peanut and soybean, which cannot withstand harsh agronomic conditions (Mazahib *et al.* 2013). A recent effort to develop improved genotypes has been initiated by Shegro *et al.* (2013). These authors, using morphological quantitative markers, reported a wide genetic variability among 20 bambara groundnut accessions in South Africa. However, extensive breeding research is required before the new bambara varieties can be released to farmers. Currently, cultivated bambara varieties are predominantly the landrace types, which are

under-researched and underutilised. Traditionally, bambara grain is processed and consumed as snacks and porridges by the people living in areas where it is grown.

Knowledge of protein composition and structure may be important to explain functionality and application of legumes. The major storage proteins in legume seeds are the globulins. However, some storage proteins, especially among the dry beans (e.g mucuna and faba bean) have been found to contain more albumin fractions than the globulins (Adebowale *et al.* 2007; Rui *et al.* 2011). Globulin may contain vicilin (7S) and legumin (11S) protein fractions. The relative amount of 7S and 11S protein fractions have been found to vary and this variation may have a significant effect on protein functionality (Barać and Stanojević 2005). For instance, Bora *et al.* (1994), reported high gelation for pea vicilin whereas legumin did not gel under the same condition. Also, the vicilin in cowpea protein displayed better emulsifying properties than did its legumin (Rangel *et al.* 2003). In addition, the vicilin in soybean has been reported to be a trimeric glycoprotein consisting of three types of subunits (α, $\alpha^1$and β)in which only the α-subunit has allergenic reactions (Natarajan *et al.* 2006). Environmental factors which includes pH, temperature and ionic strength plays an important role in determining the structure-function relationships of proteins. These environmental factors are frequently encountered in food systems at numerous stages including food preservation, processing and storage. Other factors related to growth environment and grain variety can also introduce significant changes in composition, structure and functionality of grain protein.

Physicochemical characteristics of plant proteins such as hemp seed (Malomo *et al.* 2014), pinto bean and soybean (Tan *et al.* 2014) have been well studied. So far, very limited studies have been conducted on bambara grain protein composition and structural characterisation, especially those grown in Southern Africa. Our recent study evaluated the functional properties of protein concentrates (Arise *et al.* 2015). According to our findings, bambara protein concentrate prepared by salt solubilisation showed better foaming and emulsifying properties compared to the concentrates obtained by isoelectric precipitation. However, the isoelectric precipitation gave a higher yield of concentrates.

The knowledge of protein composition may be important to better understand functionality and facilitate the utilization of bambara protein. Hence, the objective of this study was to determine the physicochemical properties of protein isolates from different bambara landraces.

4.2. Materials and Methods

4.2.1. Materials

Three cultivated bambara landraces were obtained from Josini, KwaZulu-Natal province of South Africa. These were identified based on the seed coat colour as red, maroon and cream. The bambara seeds were stored at 4^{o}C until use.

Low-molecular mass protein calibration kits were from Thermo Scientific laboratories (EU, Lithuania). Precast (4-15%) gradient, polyacrylamide Tris-HCl gels and Coomassie brilliant blue R-250 were obtained from Bio-Rad laboratories (Hercules, CA). All other analytical-grade chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2. Preparation of defatted flours

Bambara seeds were de-hulled manually using a mortar and pestle. De-hulled seed were milled in a Warring laboratory mill blender (HGBTWTS3, Torrington, CT, USA) and sieved through a 355 μm screen mesh to obtain fine flour. The resulting bambara seed flours were each defatted with n-hexane in the ratio 1:5 (flour: solvent) for 3 h. In order to remove the remaining hexane, defatted flours were placed in a fume hood overnight. The fat content after defatting using soxhlet extractor was less than 0.01%. Protein content (N x 6.25) of the defatted flours were determined by Kjeldahl method (AOAC 2000).

4.2.3. Storage protein extraction

Protein was extracted from defatted bambara seed flour using a modified isoelectric precipitation procedure (Boye *et al.* 2010) . Briefly, the defatted flour was dispersed in Milli-Q water at ratio 1:10 (w:v) ratio. This was followed by adjustment to pH 9.0 with 1.0 M NaOH. The resulting mixture was stirred at 1,000 rpm for 1 hr and centrifuged at 5,000 x g for 20 minutes at 4^{o}C. The supernatant was recovered for later use and the process was repeated with a 1 to 5 (w: v). Supernatants from both extractions were pooled together and pH was adjusted to 4.6 using 1.0 M HCl. The precipitate was collected through centrifugation (5000 x g, 20 minutes at 4^{o}C),; washed with 25 mL of water, frozen at -80^{o}C and then freeze dried (model 22KBTES_55, ZIRBUS technology, Bad Grund, Germany) to yield a free flowing powder. Protein concentration was determined using the Bradford method. Protein isolates were stored in a sealed tube at 4^{o}C until analysed.

4.2.4. Vicilin extraction

The vicilin extraction procedure was carried out according to the method of Rubio *et al.* (2014) with some modifications. Briefly, defatted flour was extracted (1:10 w/v) with 0.2 M borate buffer (pH 9) containing 0.5 M NaCl and centrifuged (20,100 x g, 30 minutes, 4^{o}C). The supernatant was retained (supernatant A) and the borate insoluble sediment was re-extracted as above and centrifuged to obtain supernatant B. Supernatants A and B were combined and adjusted to pH 4.6 with glacial acetic acid in the cold, stirred for 30 min and centrifuged (20,100 x g, 30 minutes, 4^{o}C). The supernatant was dialysed for 4 days against distilled water and centrifuged under the same condition. The sediment obtained was freeze-dried as the 7S Vicilin.

4.2.5. Total protein extraction

TCA/ acetone preparation method as described by Natarajan *et al.* (2005) was employed for the extraction of total protein. The protein precipitate was dissolved in the rehydration buffer which consists of 7 M urea, 2 M thiourea, 2% (w/v) chaps. Protein solutions were kept at -20^{o}C until further use. Protein concentrations were estimated based on Bradford method (Bradford 1976).

4.3. Analyses

4.3.1. Protein content and yield

The protein content of the isolates was estimated using Bradford (1976) method while protein yield was determined as the dry weight of protein isolate per weight of the defatted flour as shown below (Arise *et al.* 2015). The crude protein content (N x 6.25) of the defatted flours were determined by Kjeldahl method (AOAC 2000).

$$Yield\ (\%) = \frac{Protein\ content\ of\ isolate\ (\%) \times 100}{Protein\ content\ of\ defatted\ flour\ (\%)}$$

4.3.2. Amino acid composition

The amino acid contents of the protein isolates were estimated using Pico-tag method (Bidlingmeyer *et al.* 1984). This method is based on the principle of reverse phase chromatography with pre-column derivatization following acid digestion. Protein samples were hydrolysed with 6 M HCl at 116^{o}C for 24 h prior to chromatographic analysis.

4.3.3. Protein solubility

The pH solubility profiles of protein isolates were determined as described by Kudre *et al.* (2013), with some modifications. Protein isolates (100 mg) were dispersed in de- ionized water, adjusted to different values (pH 2-10) with either 1 M HCl or 1 M NaOH and adjusted to a final 10 mL volume. The resulting mixture was stirred for 1 hour at room temperature and centrifuged at 8,000 x g for 15 minutes. Protein content of the supernatant was determined using the Bradford (1976) method. Protein solubility was calculated as follows:

$$Protein\ solubility\ (\%) = \frac{Total\ amount\ of\ protein\ in\ supernatant\ \times 100}{Total\ amount\ of\ protein\ in\ protein\ isolate}$$

4.3.4. SDS-PAGE

SDS-PAGE of bambara seed proteins was done as described by Mundi and Aluko (2012). This was carried out under both reducing (with mercapthoethanol) and non-reducing conditions. Pre-cast (4-12%) gradient polyacrylamide Tris - HCl gels were used with a Bio-Rad Criterion cell under a constant voltage of 200 V. A mixture of standard proteins (10-250 kDa) was used as the molecular weight markers. Gels were stained with Coomassie Brilliant R -250.

4.3.5. Proteomic Analysis

4.3.5.1. IPG strip rehydration

Protein samples were solubilised in 150 µL of 2% rehydration buffer (20 mM dithiothreitol (DTT), 8 M urea, 2% CHAPS; cholamido-propyl-dimethylammonio-propane sulfonate, 0.5% (v/v) IPG buffer and 0.002% bromophenol blue, pH 3-10. Subsequently IPG gel strips with a linear pH range (3–10) were rehydrated for at least 10 h at 20°C into the strip holder. An amount of 50-100 µg of protein was loaded on each individual gel and a total volume of 125 µL was loaded per IPG strip.

4.3.5.2. Isoelectric focusing (IEF)

IEF was carried out using 13 cm IPG strips (pH 3-6 non-linear, NL). The strips were focused on a gradient at 150 v for 2 h, 500 v for 1 h, 1000 v for 1 h and 8000 v for 3 h using the Ettan IPGphor II system (Amersham Biosciences, Uppsala , Sweden).

4.3.5.3. IPG Strip Equilibration and Running Gels

After isoelectric focusing, the IPG gel strip was prepared for transfer to the second dimension by soaking with gentle agitation for 15 minutes in an equilibration buffer 1 which contains DDT and then in equilibration buffer 2 which contains iodoacetamide each for 15 minutes. The equilibrated IPG gel strip was embedded at the top of SDS-PAGE gel carried out in a Hoefer SE 600 Ruby electrophoresis unit (Amersham Biosciences) at 30 mA/gels, voltage set at maximum. The 2D gel was stained with coomassie blue. Gel images were attained using a Versa Doc Documentation system (Bio-Rad, Hercules , Canada). These gel were analysed with Decon Delta 2 D software. Master gels were produced for each sample based on the highest number of spots. SDS-PAGE gels (12.5%) were run at 50-60 V per gel for 90-120 minutes.

4.3.5.4. In-gel digestion

2D SDS-PAGE gels were placed on a clean glass plate and spots were removed with a 200 µl pipette tip, spots were transferred to individual labeled and corresponding 2 ml sterile Eppendorf tubes. Gel spots were destained with 200 µl of 50% acetonitrile containing 25 mM ammonium bicarbonate until clear. Samples were then dehydrated and desiccated with 100 µl acetonitrile (ACN) followed by overnight trypsinisation at 37°C with 10 µl trypsin solution. Peptides were then extracted with 10 µl 30% ACN; 0.1% trifluoroacetic acid (TFA) for 60 minutes at room temperature.

4.3.5.5. Mass spectrometry analysis

MALDI-TOF MS and LIFT MS/MS was performed using a UltrafleXtreme MALDI ToF/ToF system (Bruker Daltonics, Bremen, Germany) with instrument control through Flex control 3.4. Approximately 0.5 µl of each digest were spot onto a 800 µm MALDI Anchor chip target plate for peptide mass fingerprinting. Peptides were ionized with a 337 nm laser and spectra acquired in reflector positive mode at 28kV using 500 laser shots per spectrum with a scan range of m/z =700 -4000. Spectra were internally calibrated using peptide calibration standard II (Bruker Daltonics, Bremen, Germany). This calibration method provided a mass accuracy of 50 ppm across the mass range 700 Da to 4000 Da. Peptide spectra of accumulated 3,000 shots were automatically processed using Protein Scape software (Bruker Daltonics, Bremen, Germany). Database interrogation was performed with the Mascot algorithm using the NCBInr database and SwissProt database on a Protein Scape 3.0 workstation.

4.3.6. Intrinsic fluorescence emission

Intrinsic fluorescence spectra on a Jasco FP-6300 spectrofluorimeter (Jasco, Tokyo, Japan) was recorded at 25^{o}C using a 1 cm path length cuvette as described by Li and Aluko (2006). Protein stock solutions were prepared in 0.1 M sodium phosphate buffer, pH 3.0, 5.0, 7.0 or 9.0; these buffers were used to dilute the respective stock solution to 0.002% (w/v) and fluorescence spectra recorded at excitation wavelengths of 275 nm (tyrosine and tryptophan) with emission recorded from 280 to 450 nm. Fluorescence spectra of the sample was obtained by subtracting emissions of the buffer from the respective samples.

4.3.7. DSC

Thermal properties of all protein isolates were studied using a differential scanning calorimeter (SDT Q600, USA) as per method of Kudre *et al.* (2013), with some modifications. Protein isolates (10% w/v) were dispersed in 0.05 M sodium phosphate buffer (pH 7). The mixtures were left to stand for 12 h at 4^{o}C. The samples (20 µL) were precisely measured into 40 µL aluminium pans and hermetically sealed and heated from 20 to 120^{o}C at 10^{o}C /minute. A sealed empty pan was used as reference. On-set temperature (T_o), peak transition or denaturation temperature (T_d) and enthalpy change of the endotherm (ΔH) were computed from the thermograms.

4.3.8. Measurements of circular dichroism (CD) spectra

CD spectra of samples were measured at 25^{o}C in a J-810 spectropolarimeter (Jasco, Tokyo, Japan) using the method described by Omoni and Aluko (2006). Tertiary structure was determined using spectral range of 250-320 nm (near-UV) while range of 190-240 nm (far-UV) was used for secondary structure determinations. Protein stock solutions were diluted to required concentration in 10 mM phosphate and the secondary structure determined using a cuvette with path length of 0.05 cm containing 1 mg/mL protein solution. All the CD spectra were obtained as the average of three consecutive scans with automatic subtraction of the buffer spectra.

4.3.9. Foaming capacity (FC)

FC was carried out according to the method of Adebiyi and Aluko (2011), with some modifications. Briefly, suspensions were prepared by dispersing 300 mg in 5 mL of 0.1 M phosphate buffer pH 3.0, 7.0 and 9.0. Sample suspensions were homogenised at 3600 g for 1 min using Polytron homogenizer (PT210; Fisher Scientific, Water Side, UK). Volumes of the

suspensions were recorded before and after homogenisation. Foaming capacity was determined as follows using the mean of three measurements.

$$Foaming\ capacity\ (\%) = \frac{Vol.after\ homogenisation - Vol.before\ homogenisation\ X\ 100}{Vol.before\ homogenization}$$

Foam stability was calculated as the volume of foam that remained after 8 h at room temperature expressed as percentage of the initial volume.

4.3.10. Emulsifying activity and stability

Emulsifying activity and stability were determined using the method described by Arise *et al.* (2015) with some modifications. Protein samples (300 mg) were measured into 5 mL of 0.1 M phosphate buffer pH 3.0, 7.0 and 9.0 respectively. The resulting suspensions were homogenised with 5 mL of sunflower oil. The emulsions were centrifuged at 1100 g for 5 minutes. The height of the emulsified layer and that of the total contents in the tube was measured. The emulsifying activity (EA) was calculated using the expression below.

$$EA\ (\%) = \frac{Height\ of\ emulsified\ layer\ in\ the\ tube\ X\ 100}{Height\ of\ total\ content\ in\ the\ tube}$$

Emulsion stability (ES) was determined by heating the emulsion at 80 °C for 30 minutes after which it was centrifuged at 1100 g for 5 minutes.

$$ES\ (\%) = \frac{Height\ of\ emulsified\ layer\ after\ heating\ \times 100}{Height\ of\ emulsified\ layer\ before\ heating}$$

4.3.11. Statistical analysis

All experiments were conducted in triplicate. Data were analysed using analysis of variance (ANOVA) and means were compared using Fischer's Least Significant Difference Test ($p< 0.05$).

4.4. Results and Discussion

4.4.1. Protein content and yield of Bambara isolate

The protein content (approx. 90.7%) and yield (51.2%) of the bambara extract were similar across landraces (Table 4.1). The protein contents of the isolates in this study were higher than those previously reported by Mwasaru *et al.* (1999) for pigeon (82.4%) and Malomo *et al.* (2014) hemp seed (84.2%) obtained by isoelectric precipitated method. But the results are similar to 89.5% protein content that was reported for cowpea seed protein isolate

(Mwasaru *et al.* 1999). Therefore, the results suggest that the bambara protein isolates are of relatively high purity. Also, the protein yield obtained for the isolates (51.2%) is higher than that of hemp seed (37.9%), but similar to 51% reported for cowpea protein isolates.

Table 4.1. Protein content and yield of bambara protein isolates

Seed type	Protein content (%)	Protein yield (%)
Red	$91.7^{a} \pm 0.5$	$49.6^{a} \pm 0.3$
Maroon	$89.8^{a} \pm 0.3$	$51.5^{a} \pm 0.4$
Cream	$90.8^{a} \pm 0.4$	$52.5^{a} \pm 0.2$

Mean ± SD. Means values followed by different superscripts in each column significant different at $P \leq 0.05$. [x]carbohydrates by difference

4.4.2. Amino acid composition

Amino acid composition of the three bambara protein landraces showed similar pattern (Table 4.2). Glutamic acid and aspartic acid, which may include glutamine and asparagine respectively, were the major amino acids in bambara protein. These amino acids are also the major amino acids found in other legume seeds such as soybean (Adebowale *et al.* 2011), fenugreek (Feyzi *et al.* 2015), mung bean and black bean (Kudre *et al.* 2013). The lysine content of bambara protein is high (5.8- 6.0 g/100 g protein) and similar among landraces. The lysine content of bambara protein compares favourably to reported values for soybean protein (Adebowale *et al.* 2011; Amonsou *et al.* 2012). Based on FAO/WHO (2007) recommended pattern, bambara proteins may be considered good sources of lysine for pre-school children and adults. Therefore, bambara protein could offer some potential in food formulation where it could be used to complement cereal proteins which generally lacks lysine.

4.4.3. Protein solubility

Bambara proteins exhibited U- shaped solubility curves in the pH 2-10 range (Fig.4.1). Minimum solubility (approx. 12%) was observed around pH 4-5. This could be attributed to the intermolecular attraction of protein molecules in the isoelectric zones (Ali *et al.* 2010). Protein solubility increased when the pH increased gradually from 4 to 10 or reduced from 4 to 2 reaching a maximum of approximately 79%. This is because at high acidic or high alkaline pH values, proteins carry net positive or negative charges respectively and thus, electrostatic repulsion and ionic hydration could have promoted protein solubilisation (El Nasri and El Tinay 2007). The results obtained in this study are similar to that reported for kidney bean protein isolate, chickpea and cowpea which showed increase in protein solubility

at pH values above and below their isoelectric point (Sanjeewa *et al.* 2010; Mundi and Aluko 2012).

Table 4.2. Amino acid (g/100 g protein) composition of Bambara seed protein isolates

Amino acid	Red	Maroon	Cream isolate	[b]FAO/WHO children (2007)	FAO/WHO Adult (2007)
Isoleucine[a]	2.97	3.16	2.83	2.8	1.3
Leucine[a]	6.71	6.96	6.69	6.6	1.9
Lysine[a]	5.95	6.03	5.81	5.8	1.6
Methionine[a]	1.26	1.26	1.1	2.7	1.7
Phenylalanine[a]	5.35	5.97	4.83	6.3	1.9
Threonine[a]	5.11	5.13	4.61	3.4	0.9
Valine[a]	4.09	4.45	3.82	3.5	1.5
Histidine	2.41	2.43	2.39	1.9	1.6
Arginine[a]	6.61	7.31	5.9		
Tyrosine	2.73	2.99	2.56		
Alanine	3.57	3.92	3.23		
Aspartic acid	9.32	10.72	8.49		
Glutamic acid	15.66	15.91	14.93		
Glycine	2.63	2.81	1.87		
Serine	3.99	4.48	3.17		
Proline	3.08	3.41	2.42		

[a]Essential amino acids; [b]FAO (2007)

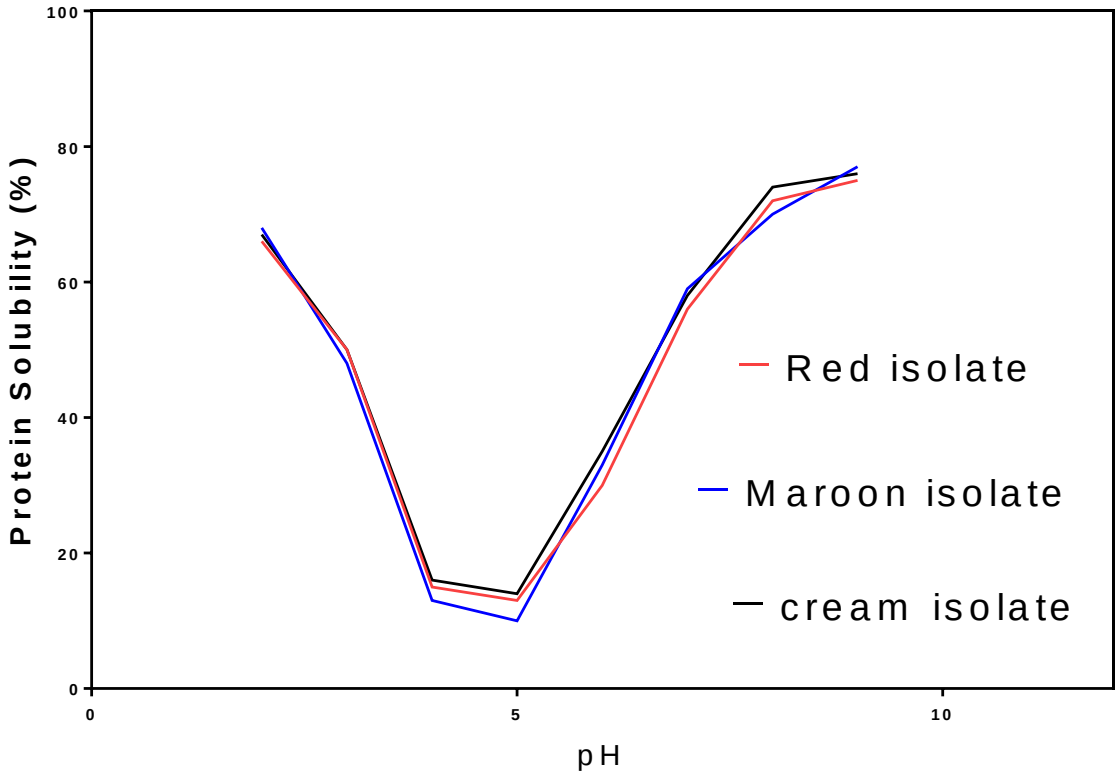


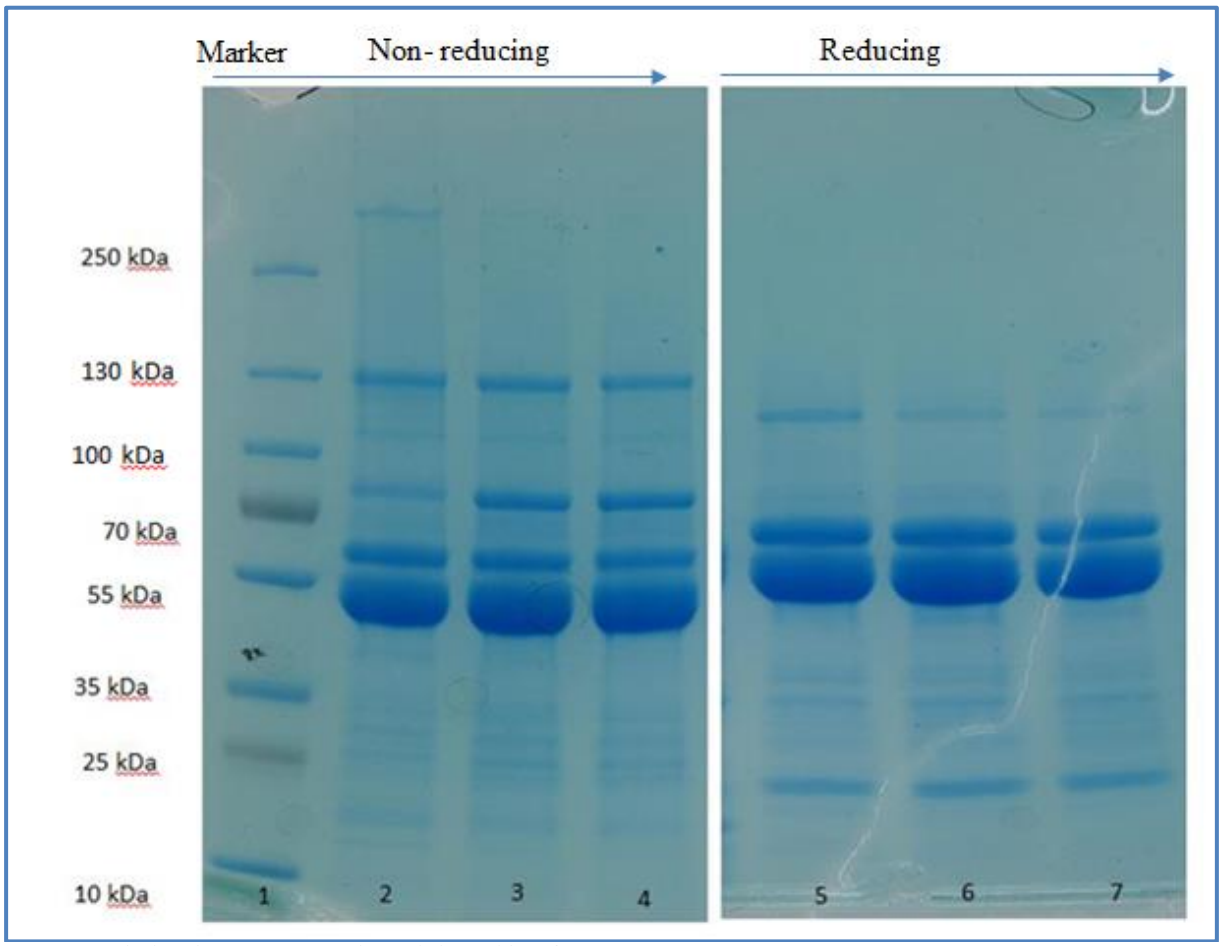
Fig. 4.1: **Effects of different pH on solubility of bambara groundnut protein isolate**

4.4.4. SDS -PAGE

Bambara seed proteins showed four major bands: A broad band at 55 kDa, two medium bands at 62 kDa and 80 kDa and a high molecular weight (HMW) protein at 141 kDa. The HMW protein was reduced by mercapthoethanol indicating the presence of disulphide bonds linkage within its constituent polypeptides (Fig. 4.2). Minor bands emerged at 26 kDa and 18 kDa under reducing condition for all the isolates. HMW proteins have similarly been reported for pulses such as kidney bean (150 kDa) and field pea (155 kDa) (Shevkani *et al.* 2015).The proteins bands 55 kDa and 62 kDa may correspond to 7S vicilin subunits (Boye *et al.* 2010; Rui *et al.* 2011; Kudre *et al.* 2013; Shevkani *et al.* 2015). Similar protein pattern observed in this research has been reported for pea (Park *et al.* 2010), mung bean, and black bean isolate (Kudre *et al.* 2013) with major broad band at 54, 51, 54 and 52 kDa respectively. A medium intensity protein band of approx. 62 kDa has also been reported for cowpea (Rangel *et al.* 2003).

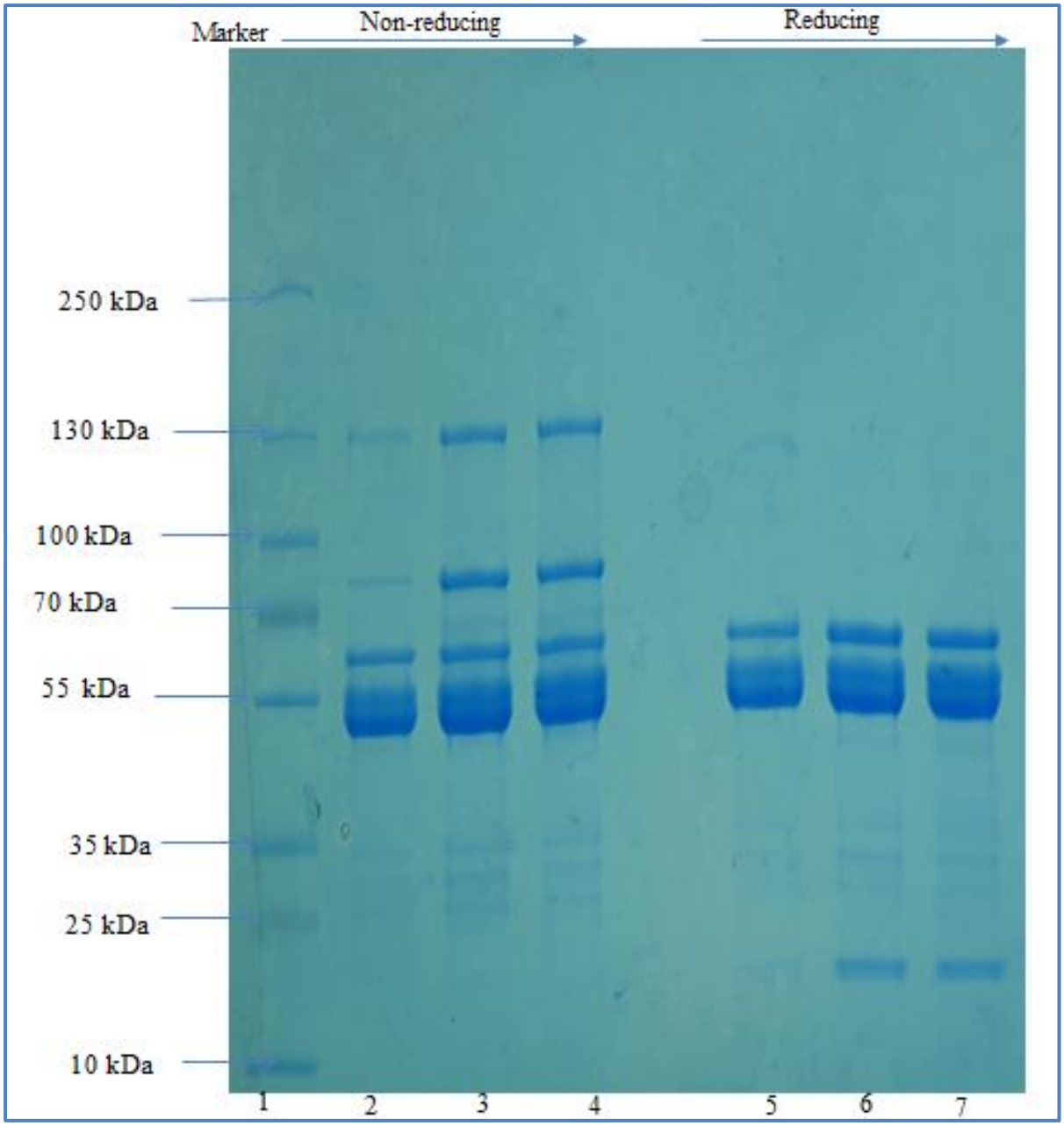
To confirm the major protein fraction in bambara, the vicilin was extracted from bambara grains and analysed by electrophoresis. Interestingly, SDS–Page of vicilin under non-reducing and reducing conditions did not differ from those obtained for the protein isolate

(Fig.4.3). The two vicilin subunits appeared in the gels under reducing and non-reducing conditions. This further confirms that vicilin of bambara seed contains two sub units and may be the major protein fraction of bambara storage proteins.



Lane 1 – standard marker. Lanes 2, 3, and 4 carried out without β-mecarptoethanol. Lane 2 Red isolate, lane 3 Maroon isolate, Lane 4 Cream isolate. Lanes 5, 6 and 7 carried out with β-mecarptoethanol. Lane 5 Red isolate, Lane 6 cream isolate and lane 7 maroon isolate.

Fig. 4.2 : SDS-PAGE patterns of bambara groundnut protein isolates.



Vicilin isolates. Lane 1 – standard marker. Lanes 2, 3, and 4 carried out without β-mercapthoethanol. Lane 2 Red isolate, lane 3 Maroon isolate, Lane 4 Cream isolate. Lanes 5, 6 and 7 carried out with β-mercapthoethanol. Lane 5 Red isolate, Lane 6 cream isolate and lane 7 maroon isolate

Fig. 4.3: SDS-PAGE patterns of bambara groundnut protein

4.4.5. Two-dimensional gel electrophoresis

Bambara proteins contain different numbers of spots across the landrace (Fig. 4.4 and Table 4.3). Cream Isolate has the highest number of spot (162) while the red Isolate contain the lowest amount of spots (126). In order to determine the matching spots, cream isolate was use as the master gel, it was observed that there are 77 spots matches between the red and cream isolates and also 77 spots matches between marron and cream isolates (Table 4.3). This indicates that 77 spots may possibly be the matching spots across the landraces.The

differences and similarities obtained for spot among the landraces in this study is in line according to result obtained for four conventional Soya bean (Gomes *et al.* 2014). In general, acidic polypeptides are the major protein across the landraces; this can be linked to the fact that bambara major proteins are 7S vicllin which are acidic in nature. Also based on the amino acid composition of bambara protein, acidic amino acid were more abundant. Thus, the proteome pattern of bambara appears to be associated to its amino acid composition.

Table 4.3: Polypeptide distribution of bambara landraces, matches and unmatched spots using pH range 4-7

Samples	Red Isolate	Cream Isolate	Maroon Isolate
Protein spots	126 ± 5	162 ± 7	157 ± 1
Tentativespot matches	77 ± 6		77 ± 4
Unmatchtentative spots	49 ± 4	85 ± 6	81 3

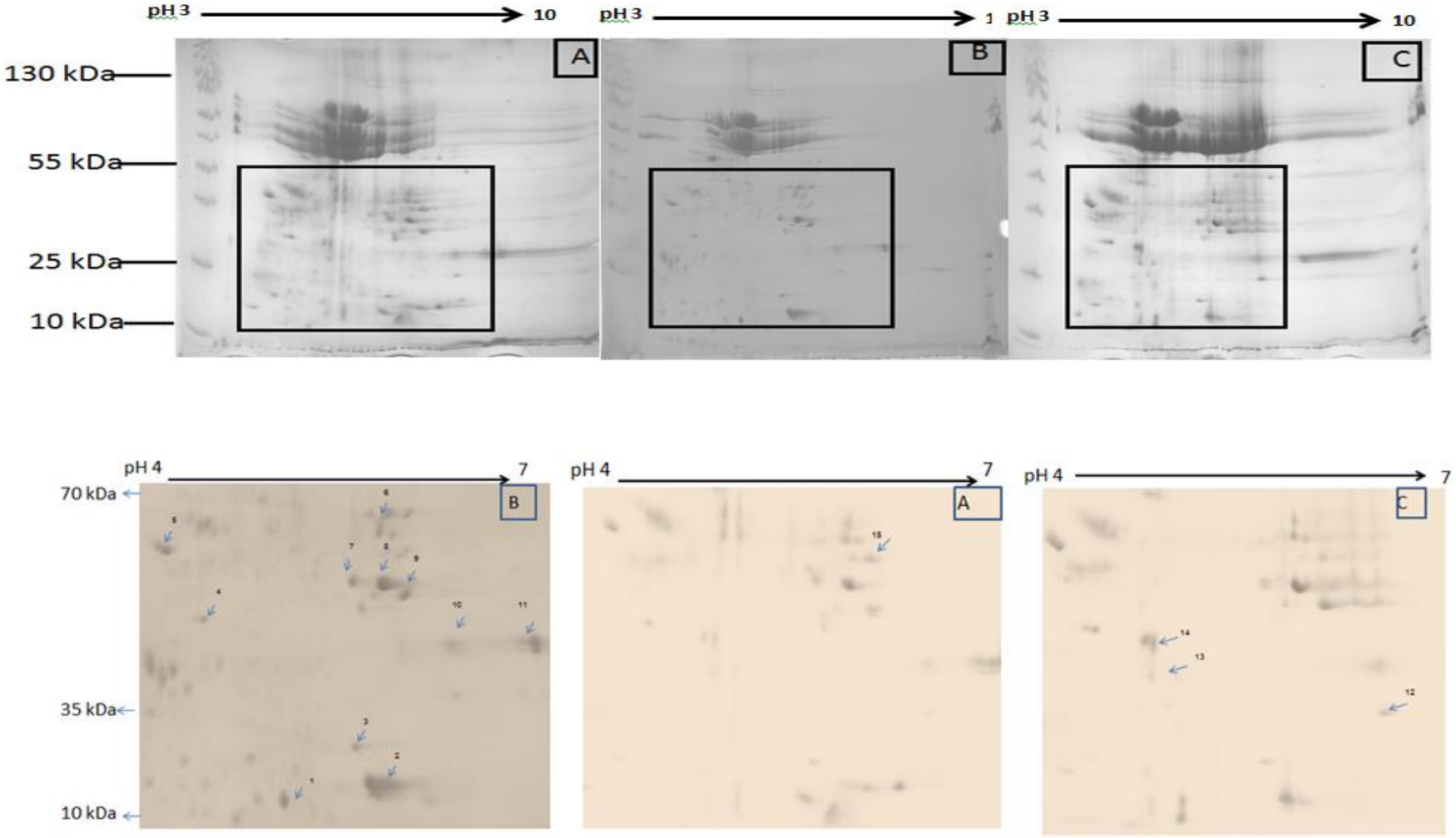


Fig. 4.4: Two dimensional maps of bambara (ABC) using immobilized pH gradient (IPG) strips (3-10 and 4-7 non linear,NL).

A: Red colour bambara, B: Maroon colour bambara and C: cream colour bambara

4.4.6. Peptide identification

In order to qualitatively survey the protein visualized by 2D- PAGE, a total of 15 spots of more abundant protein around the vicilin region were excised from the 2D-PAGE gels. The peptide fragments were extracted and analyzed by MALDI-TOF-MS. 11match spots were chosen upon superimposition of the 3 gels using the 2D delta software while 4 unmatched spots were also analyzed. Among the 15 spots processed, 13 proteins were successfully identified by querying NCBInr and SwissProt data base using the Mascot search engine (Table 4.4). Data in Table 1 include an assigned protein spot number, theoretical isoelectric point (pI), molecular weight (Mr),protein identity and its original species, number of peptides matched, percentage sequence coverage, MOWSE score, and accession number. Storage protein (phaseolin), defense related protein (proteinase inhibitor), stress related protein (heat shock protein and superoxide dismutase) and proteins involved in metabolism (5OS ribosomal protein) were present in bambara protein. These classes of protein have been reported for common bean and soya bean (Natarajan *et al.* 2005; Natarajan *et al.* 2013). The data further confirm that most of the storage protein of bambara is present at the acidic region and vicilin is the major storage protein (Fig. 4.5). It is interesting to find that among the landraces, the heat shock protein is present only in the cream bambara protein. The heat shock protein which is stress related protein, is synthesized in response to elevated temperature and during various developmental processes which include seed maturation. This protein is present in soya bean and functions as molecular chaperones associated with protein folding. Protein translocation and degradation (Natarajan *et al.* 2013). Heat shock protein is also involved in protective functions throughout germination in pea seed (Wang *et al.* 2004).

Table 4.4. Proteins identified from bambara landraces by MALDI-TOF/TOF

S/N	Protein identification	M_r	pI	MO/score	PM	SC(%)	Accession No	Database	ID method
1	hypothetical protein LR48_Vigan11g112500 [Vigna angularis]	10.9	5.9	491.2	5	73.7	gi|920718894	NCBInr	MALDI TOF/TOF
2	Bowman-Birk type proteinase inhibitor OS=Phaseolus angularis PE=1 SV=1	9.1	4.8	128	3	25.6	P01058	swissprot	MALDI TOF/TOF
3	superoxide dismutase 4A [Zea mays]	15.1	5.4	368.8	4	31.6	gi|1885354	NCBInr	MALDI TOF/TOF
4	seed storage protein A [Vigna luteola]	49.8	5	55.2	1	3.4	gi|70672850	NCBInr	MALDI TOF/TOF
5	Phosphoglucomutase, cytoplasmic OS=Bromus inermis GN=PGM1 PE=2 SV=1	62.6	5.2	29.1	1	2.8	Q9SNX2	swissprot	MALDI TOF/TOF
6	beta-conglycinin, beta chain-like precursor [Vigna radiata]	51.8	5.7	410	7	21	gi|955079824	NCBInr	MALDI TOF/TOF
7	Vicilin protein [Vigna unguiculata]	49.7	5.2	368.6	6	16.2	gi|160332746	NCBInr	MALDI TOF/TOF
8	hypothetical protein LR48_Vigan10g020300 [Vigna angularis]	49.8	5.9	119.4	2	9.5	gi|920714223	NCBInr	MALDI TOF/TOF
9	Serine carboxypeptidase II-2 (Fragment) OS=Hordeum vulgare GN=CXP;2-2 PE=1 SV=1	48.9	6	33.2	1	2.3	P55748	swissprot	MALDI TOF/TOF
10	PREDICTED: glycinin G4-like [Vigna radiata var. radiata]	69.5	5.1	161.7	2	5.1	gi|951056419	NCBInr	MALDI TOF/TOF
11	PREDICTED: glycinin G4-like [Vigna radiata var. radiata]	69.5	5.1	161.7	2	5.1	gi|951056419	NCBInr	MALDI TOF/TOF
12	18.2 kDa class I heat shock protein OS=Medicago sativa GN=HSP18.2 PE=2 SV=1	18.2	5.7	179.7	5	25.9	P27880	swissprot	MALDI TOF/TOF
13	Seed storage protein A (*vigna luteola*)	49.8	5	185.9	3	8.5	gi|951056419	NCBInr	MALDI TOF/TOF

S/N-Spot no: SC (%)- Sequence coverage :PM- peptide match : M_r –molecular weight: pI- isoelectric point.

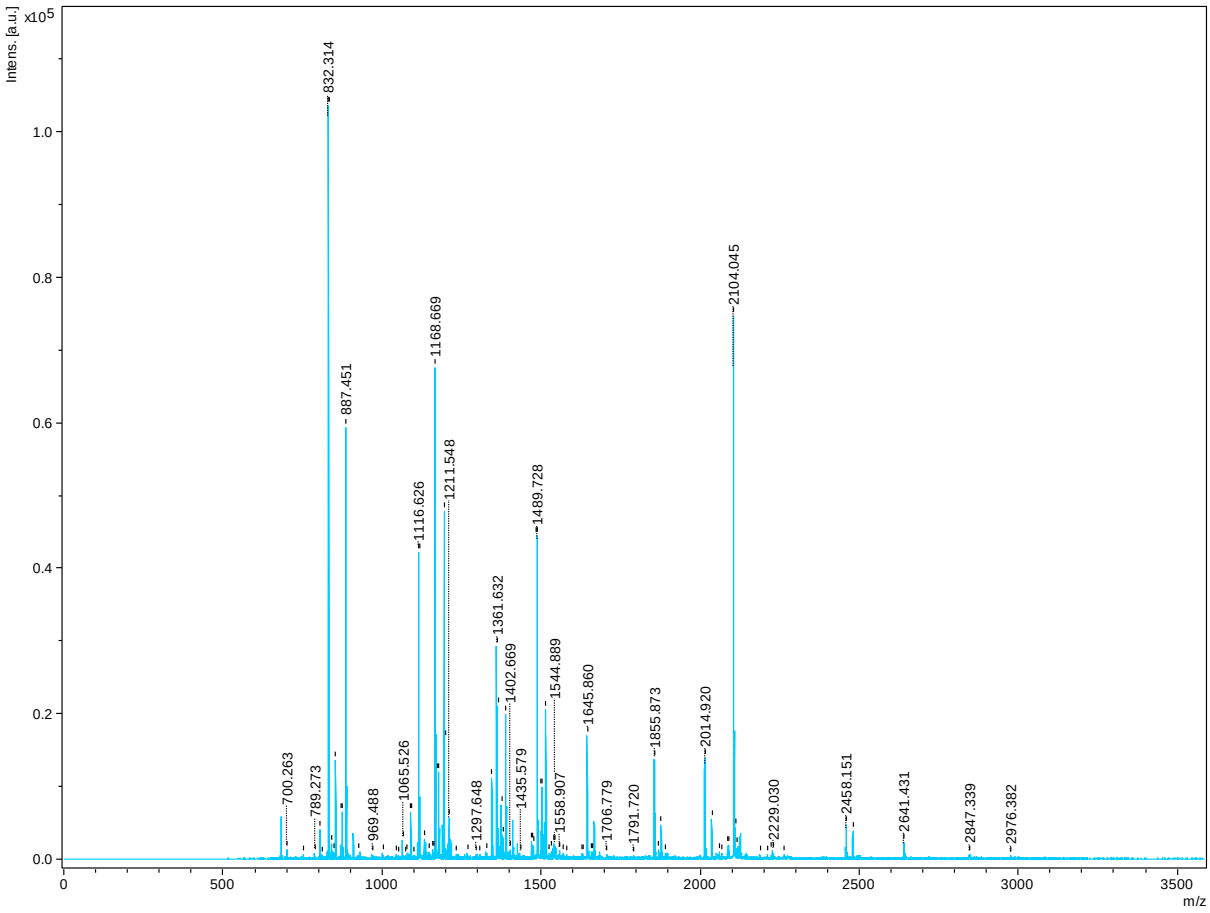


Fig. 4.5 : A typical spectral of bambara vicilin protein spot (Spot 7)

4.4.7. Intrinsic Florescence Emission

Intrinsic emission fluorescence spectroscopy technique reveals the conformational changes of bambara proteins at different pH values (Fig.4.4). At pH 9.0, bambara proteins showed a maximum fluorescence wavelength (λ_{max}) of 338 nm. Thus, revealing a characteristic fluorescence profile of tryptophan residues which are mostly exposed within a hydrophilic environment (Mundi and Aluko 2013). However, at pH 3.0 and 5.0, the λ_{max} (332 nm) showed a blue shift that suggests relocation of tryptophan residues to a more hydrophobic pocket. Further, at pH 9.0 there were slight increases in the F_{max} values obtained for tryptophan peaks which indicate structural rearrangement that improved tryptophan - tryptophan interactions. The observed λ_{max} at high pH value in this study is in line with previous reports on quinoa protein isolates (Abugoch *et al.* 2008) and hemp seed isolates (Malomo *et al.* 2014).

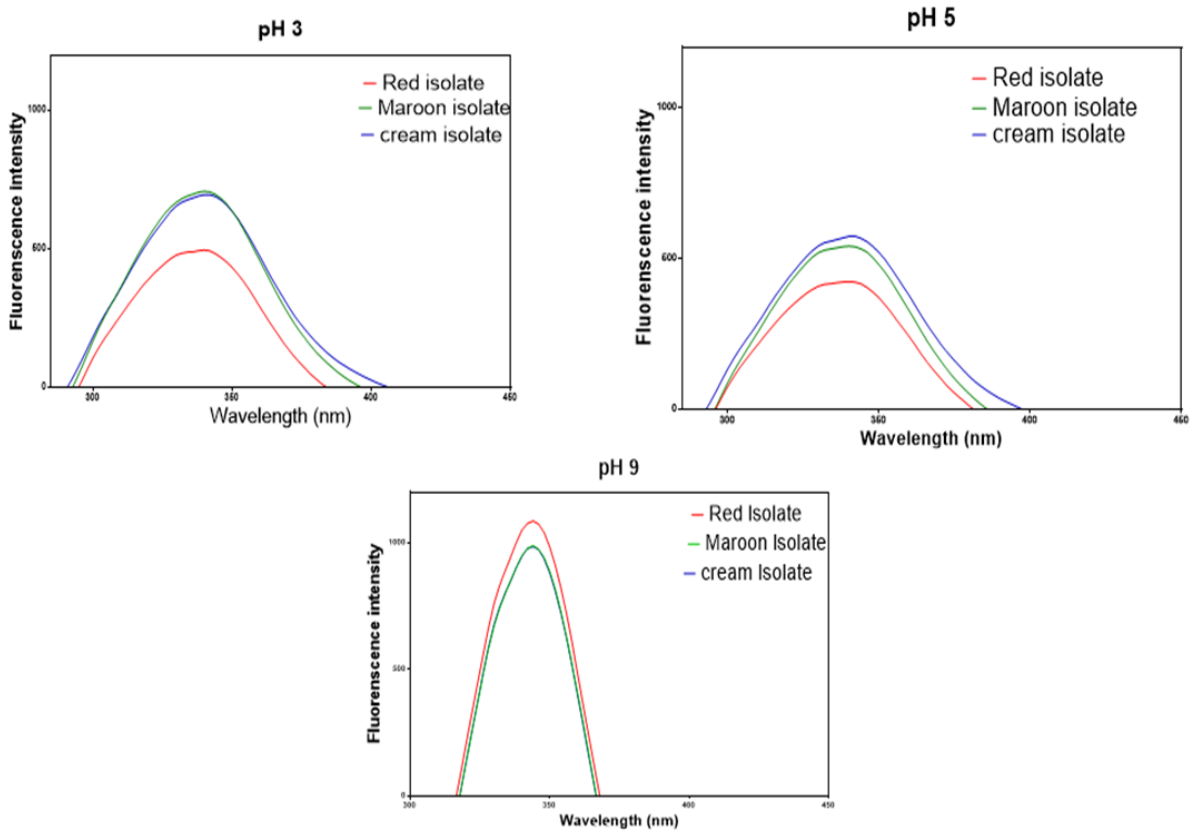


Fig. 4.6: Intrinsic Fluorescence intensity (arbitrary units) of bambara seed protein at different pH values

4.4.8. Differential scanning calorimetry (DSC)

The structure of bambara seed protein was further characterised by DSC (Table 4.3). The protein showed two endothermic peaks at 64 - 69^{o}C and 76 - 90^{o}C. These two peaks most likely represent the denaturation of the 7S protein at lower temperature (64-69^{o}C) and HMW protein at higher temperature (76-90^{o}C) as indicated by SDS-PAGE (Fig.4.2). Also, the two endothermic peaks could be ascribed to independent transitions in different subunits structure of vicilin. A similar trend of two endothermic peaks have been reported for cowpea isolates with denaturing temperatures (T_d) of 83-84^{o}C and 91-93^{o}C respectively (Avanza *et al.* 2013). The high thermal denaturation temperature of the cowpea seed proteins were linked to a high proportion of β-sheet structure (Hirano *et al.* 1992). The structure of bambara protein was further analysed by CD.

Table 4. 5. Thermal properties of bambara seed proteins from different landraces

Samples	Peak	T_o (^oC)	Td (^oC)	ΔH (J/g)
	I	64.2 ± 0.1	71.0 ± 0.6	6.6 ± 0.1
Red Isolate	II	78.5 ± 0.5	84.3 ± 0.4	6.8 ± 0.4
	I	62.1 ± 0.1	68.7 ± 0.2	6.4 ± 0.3
Maroon Isolate	II	78.1 ± 0.2	90.4 ± 0.1	11.7 ± 0.5
	I	66.9 ± 0.7	69.9 ± 0.5	6.5 ± 0.1
Cream Isolate	II	76.0 ± 0.1	78.9 ± 0.7	6.7 ± 0.7

T_0: Onset temperature; T_d : Peak denaturation temperature; ΔH : Enthalpy of denaturation

4.4.9. CD spectra

CD determination was not possible at pH5.0 in this study. This is as a result of very low protein solubility at the isoelectric point. The CD spectrum of the isolates at pH 3.0 in the far-UV region showed the dominance of a negative peak at about 210 and 220 nm, a weak positive peak in the vicinity of 195 nm and a broad shoulder that extends from 220 to 240 nm (Fig.4.5). These features of the spectrum are sufficient indicators of highly ordered structure, including α-helix and β-types (Yang *et al.* 1986; Yin *et al.* 2011; Anwer *et al.* 2016). The isolates revealed a strong secondary structure majorly controlled by α-helix conformation. This is revealed by intense ellipticity between 210 and 220 nm. All the isolates showed similar ellipticity shape but the cream isolate had less intensity in comparison to red and maroon isolates. In addition, the ellipticity at 195 nm was stronger for red and maroon isolates, which indicates the presence of more β-sheet structure in comparison to cream isolate. The higher level of β sheet structure specifies a more open conformation. The far-UV CD data are in line with the florescence intensity result that revealed reduced F_{max} and higher λ_{max}. This revealed that the degree of exposure of aromatic groups to the hydrophilic environment is higher at pH 3.0. At pH 7.0, the maroon isolate had a higher ellipticity values between 210 and 220 nm specifying the existence of more α- helix than at pH 3.0. At pH 7.0, the result of far- UV CD also support the fluorescence emission data (Fig.4.4) that revealed a lower F_{max} value and a red shift in λ_{max} in comparison to pH 3.0. This is an indication of increased interactions with the hydrophilic environment. At pH 9.0, the ellipticity at 195 nm for the cream isolate became more intense when compared to that obtained at pH 3.0 and 7.0. However, for the α-helix there is reduction in intensity for all the isolates at pH 9.0. This indicates that increased charge at alkaline pH 9.0 may have generated better protein -protein electrostatic repulsion resulting in more disorganised structure in comparison to pH 7.0. This result is in contrast to what Mundi and Aluko (2013), reported for kidney bean vicilin. The

authors reported that kidney bean vicilin showed no defined structure at pH 3.0. However, the present results are consistent with previous reports that protein secondary structure conformation can be changed as a result of pH-dependent variations in electrostatic interactions (Yin *et al.* 2011; Malomo *et al.* 2014; Malomo *et al.* 2015).

The near-UV CD spectra shows that the structural conformation of bambara seed proteins were significantly affected by changing the pH conditions (Figure 4.6). For example, at pH 3.0, the proteins had peak ellipticity at 276 nm, which is in accordance with tyrosine residues within a hydrophobic environment. At pH 7.0, there was an increased ellipticity in comparison to pH 3.0. This may be as a result of a shift in the aromatic amino acid residues into the more hydrophobic interior as the exterior became more hydrophilic. This is revealed in a greater ellipticity values at pH 9.0 for all the isolates. The result is in line with the report of Kelly *et al.* (2005). According to these authors, many factors including number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups and polarizability) and their spatial disposition in the protein may influence the actual shape and magnitude of the near-UV CD spectrum of a protein. In this study, as the environment became negatively charged, the hydrophobic groups, especially tyrosine were pushed into the protein core, which enhanced ellipticity values. The results further suggest that ionization of amino acid side chains at pH values above the isoelectric point had greater effects in increasing the bambara seed protein rigidity when compared to the positively charged environment at pH 3.0. Similar increase in ellipticity as pH increased has been reported for hemp seed protein (Malomo and Aluko 2015).

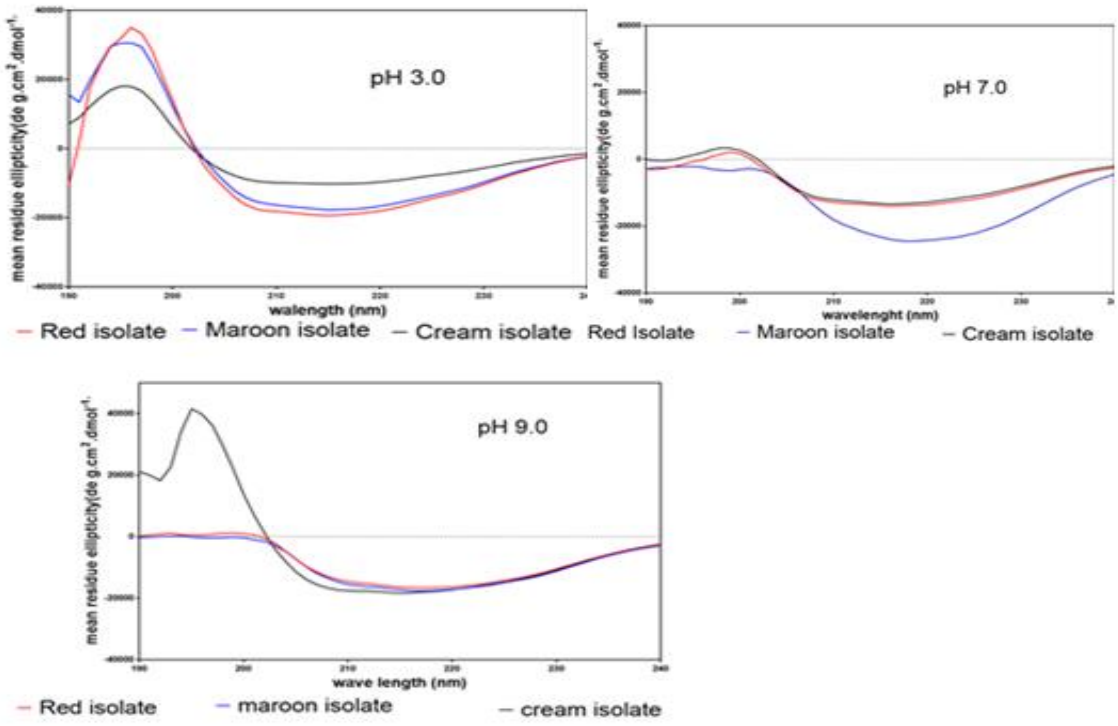


Fig. 4.7: Far- UV circular dichroism spectra of bambara protein isolates at different pH values.

Results are presented as mean ± standard deviation, For each row, mean values that contain different alphabets are significantly different at $p<0.05$

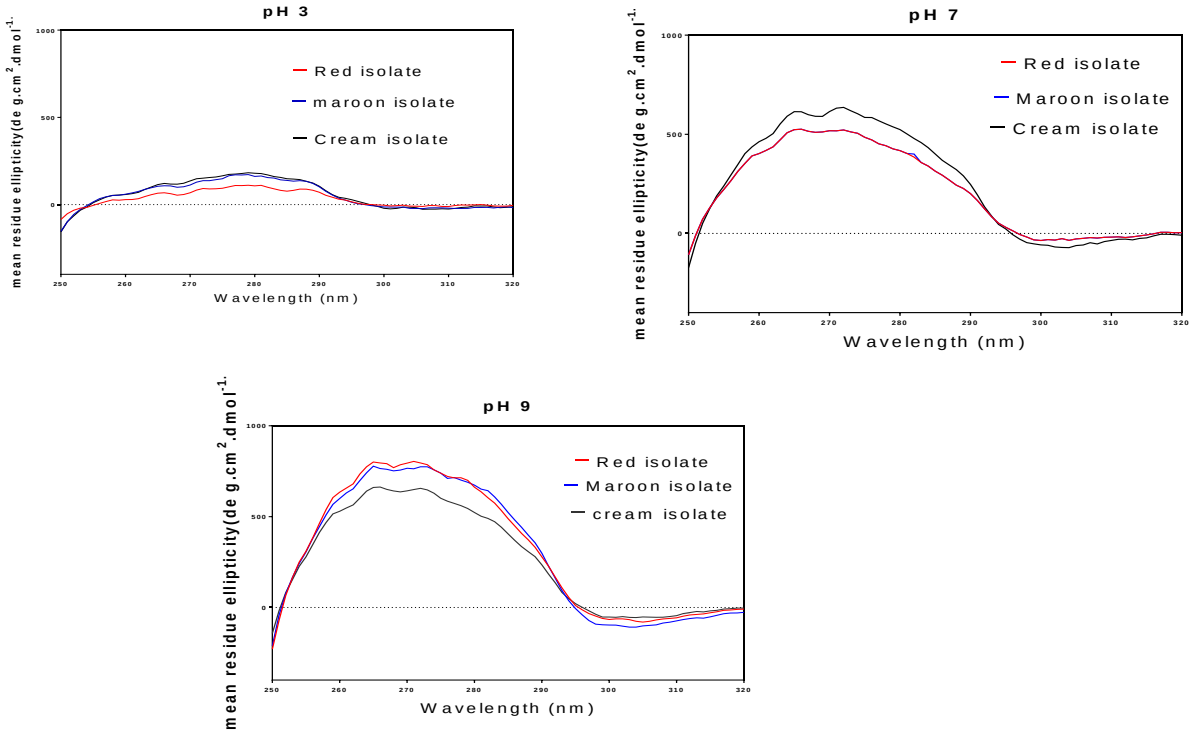


Fig. 4.8: Near- UV circular dichroism spectra of bambara seed protein at different pH values

4.4.10. Foaming capacity and foam stability

The foaming capacities of bambara proteins were dependent on pH with maximum percentage FC observed at pH 3.0 (Fig.4.7). FC profiles were similar among landrace. The degree of protein solubility signifcantly affects foaming capaicty of proteins (Malomo *et al.* 2014). Consequently, the high FC observed at pH 3.0 for all bambara protein isolates may be due to increased solubility in more acidic region (Fig.4.1). In addition, the results are in accordance with the better secondary structure conformation obtained for all the isolates by circular dichroism at pH 3.0 (Fig.4.5). Further, higher FC suggests better interfaces with the aqueous phase which improve the capacity of the protein molecules to encapsulate air particles. Since at acidic region, there is decrease in attractive hydrophobic forces among the molecules which will enhance protein unfolding and as a result leads to high FC (Adebowale and Lawal 2003; Arise *et al.* 2015). Minimum FC was observed at pH 7.0 for all the isolates. This could be due to the fact that the balance of protein conformation and net charge probably limit air encapsulation ability. The results obtained in this study is in agreement with the report for hemp seed (Malomo *et al.* 2014), kidney bean (Mundi and Aluko 2012) and cowpea protein isolates (Ragab *et al.* 2004).

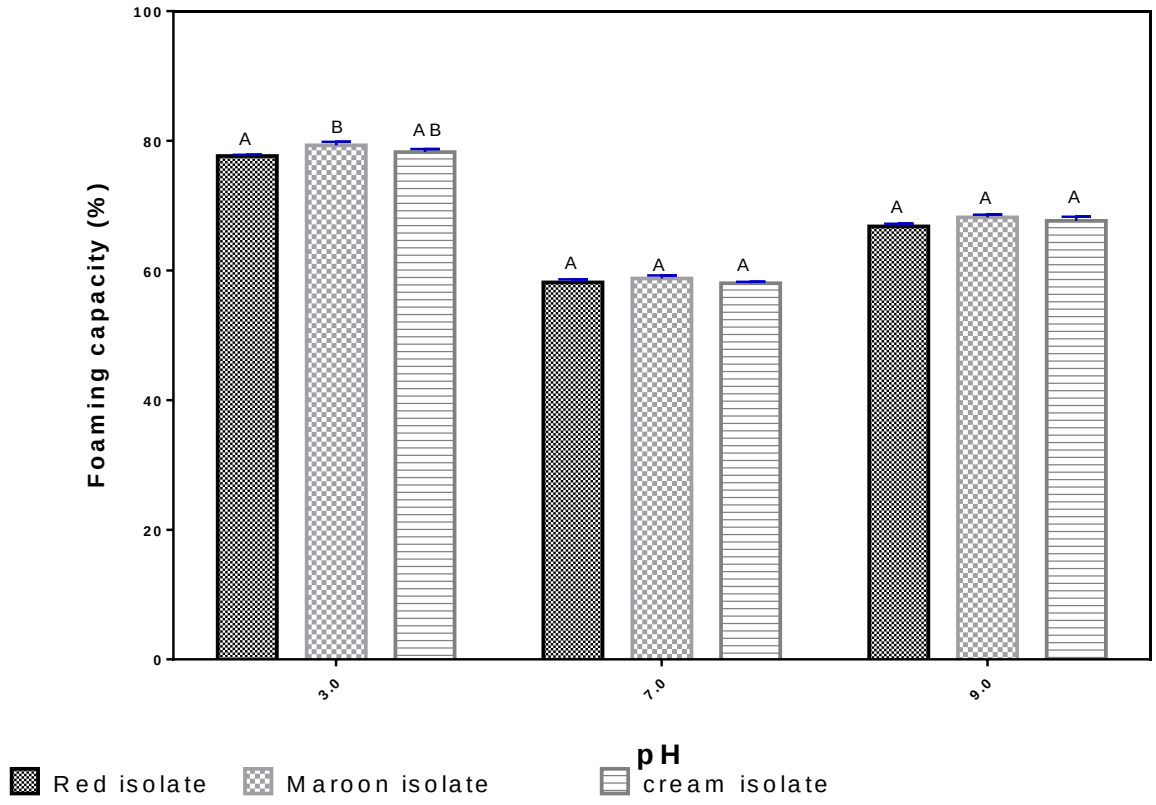


Fig. 4.9: Foam capacity of bambara protein isolates at different pH values

The foaming stability revealed higher FS at pH 3.0 for all the isolates (Fig. 4.8). This may occur as a result of formation of stable molecular layers in the air-water interface of the foams. Higher FS at low pH have been previously reported for mucuna protein (Adebowale and Lawal 2003) and hemp seed protein (Malomo and Aluko 2015).

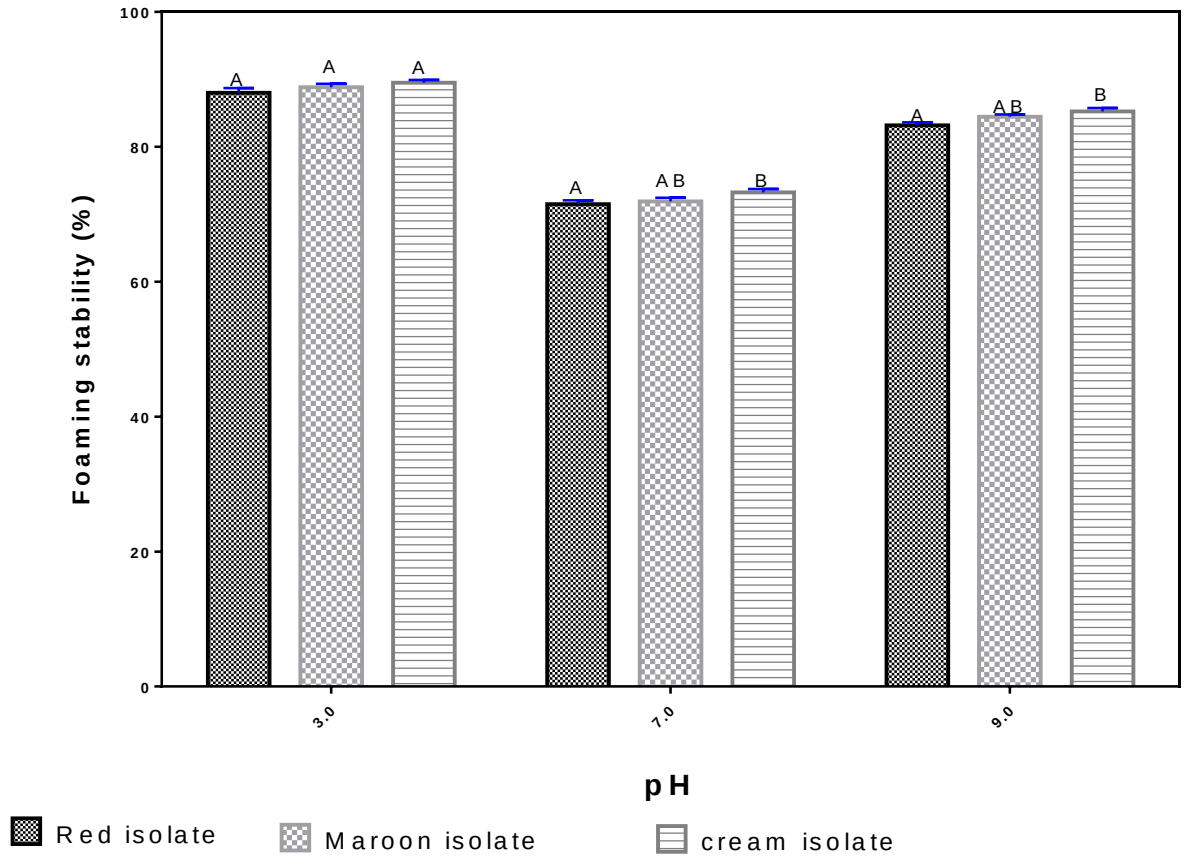


Fig. 4.10: **Foam stability of bambara protein isolates at different pH values**

4.4.11. Emulsion activity (EA) and stability (ES)

The emulsion activity increased with increasing pH for all the isolates (Fig.4.9). Possibly, as the pH increases, protein-protein interactions would have caused better interfacial membrane formation and thus resulting in higher EA. This same trend was observed for *Ginko biloba* seeds (Deng *et al.* 2011) and kidney bean protein isolates (Mundi and Aluko 2012).

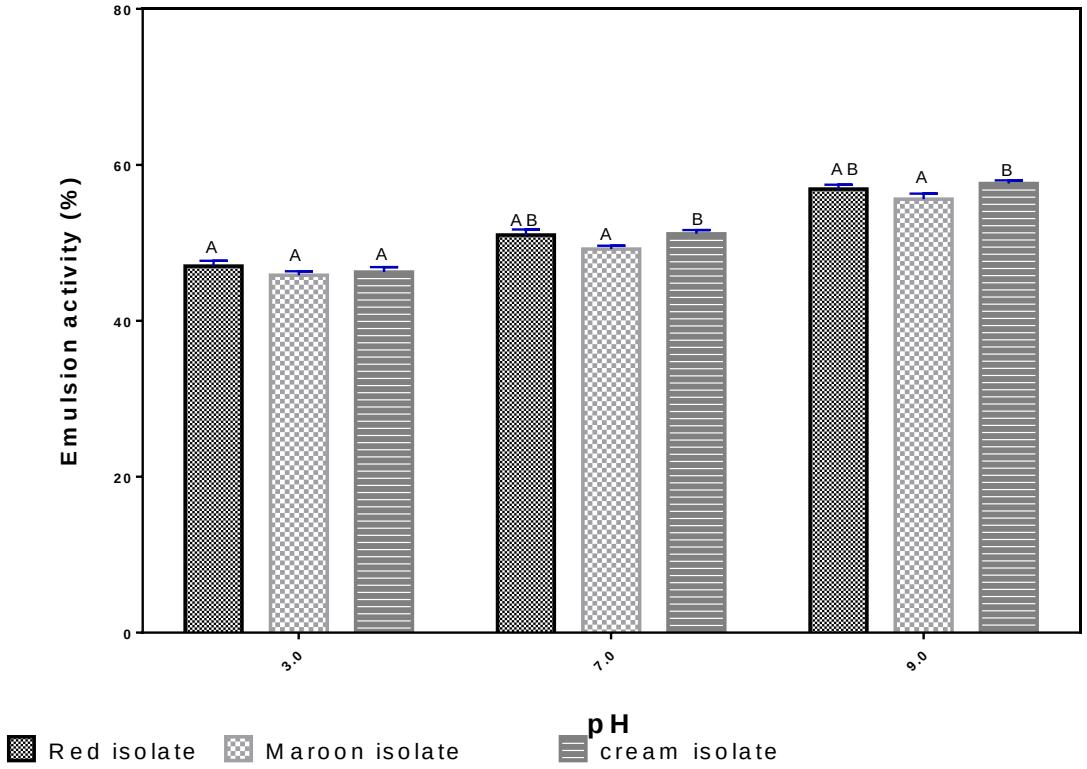


Fig. 4.11: Emulsion activity of bambara protein isolates at different pH values

The emulsion stability result revealed significant difference ($p<0.05$) for all the isolates at different pH values. ES is higher at acidic and alkaline pH in comparison to neutral pH. The lower value of ES observed at pH 7.0 suggest a weak interfacial membrane formation as protein-protein interaction are reduced at this pH. In general, the isolates were able to stabilise emulsion very well (> 70%) at all pH values which could be attributed to the high protein content of isolates (approx. 90.7%). The results obtained in this study are comparable to that reported for pea, chickpea and lentil protein isolated by Boye *et al.* (2010).

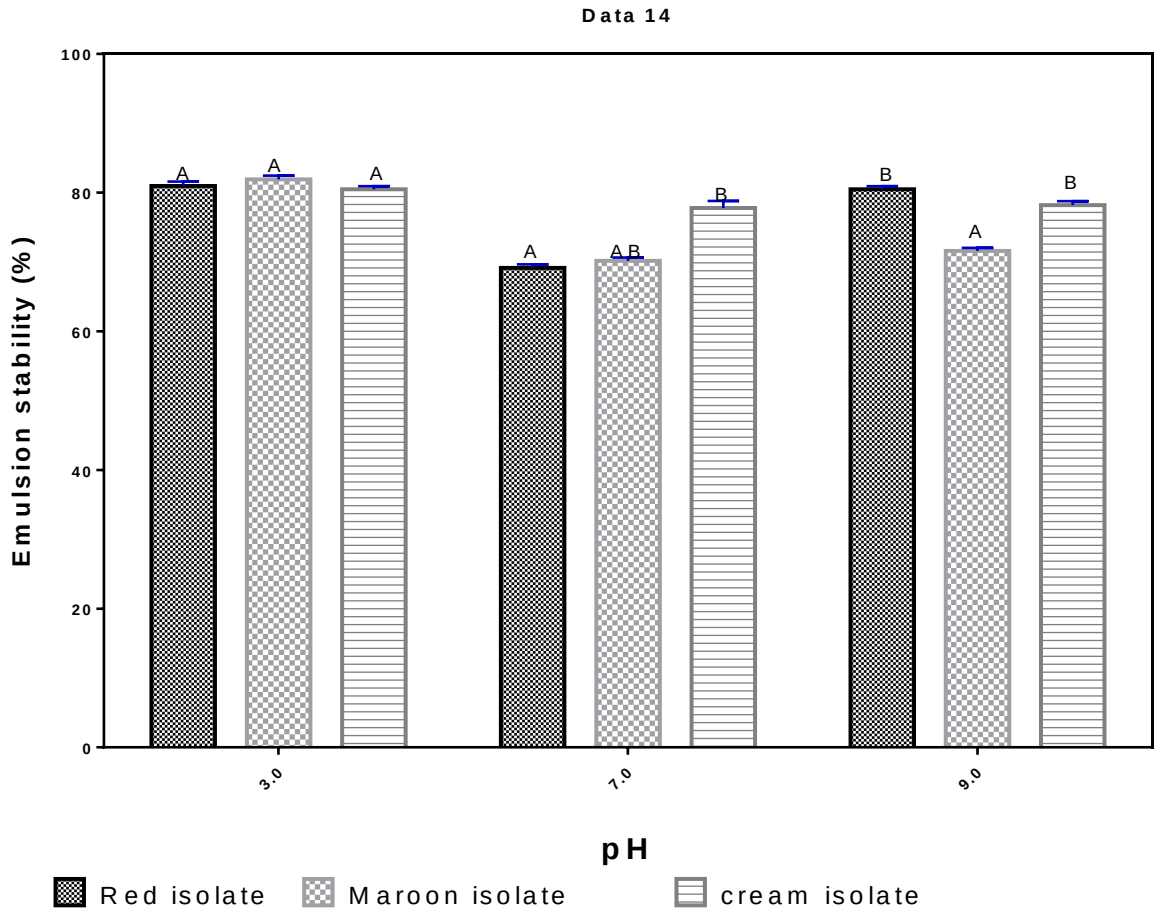


Fig. 4.12: Emulsion stability of bambara protein isolates at different pH values

4.5. Conclusions

Bambara seed represents a good source of protein that are rich in lysine. Thus, bambara proteins can be used to supplement cereals and represent a viable source of protein raw material for the food industry. All bambara protein isolates investigated in this work exhibited similar molecular structure, intrinsic fluorescence, circular dichroism, thermal stability, solubility, foaming capacity and emulsion activity. Molecular structure revealed two subunit of vicilin as the major storage proteins in bambara seed. Intrinsic fluorescence studies showed that tryptophan interacted less with the hydrophilic environment at pH 3.0, 5.0 and 9.0 as evident in the tryptophan 332-338 nm λ_{max} values. Circular dichroism data revealed that bambara proteins existed in different conformational states when assessed at various pH values but with well- defined structure at pH 3.0. In addition, the high thermal stability of bambara seed proteins suggests that the protein could be used as an ingredient in food systems in which heat treatment (but not protein denaturation) is a required process.

Furthermore, bambara protein are possibly good ingredients for the production of food foams and emulsions

4.6. Research outputs

a) Journal articles

1. **Abimbola K. Arise**, Ifeanyi D. Nwachukwu , Rotimi E. Aluko and Eric .O. Amonsou. Composition, structural and functional properties of protein isolates from bambara groundnut (*Vigna subterranea*) landraces . Under review in Food Hydrocolloids

b) Conference Papers

2. **Arise A.K**., Ijabadeniyi O.A and Amonsou E.O . Composition, Solubility profile and thermal properties of protein isolates from bambara groundnut (*vigna subterranea*) landraces. South African Association of Food Scientist and Technologist (SAAFOST), Durban, September 2015.

3. **Arise A.K**. and Amonsou E.O. Physicochemical properties of protein isolates from bambara groundnut (*Vigna subterranea*) landraces. 29th EFFoST Conference on Food Science Research and Innovation: Delivery sustainable solutions to the global economy and society. 10th -12th November, 2015, Greece.

CHAPTER FIVE

5. Antioxidant activities of bambara groundnut (*Vigna subterranea*) protein hydrolysates and their membrane ultrafiltration fractions

ABSTRACT

In this study, bambara protein isolate (BPI) was digested with three proteases: (Alcalase, trypsin and pepsin), to produce bambara protein hydrolysates (BPHs). These hydrolysates were passed through ultrafiltration membranes to get peptide fractions of different sizes (<1, 1-3, 3-5 and 5-10 kDa). The hydrolysates and their peptide fractions were investigated for antioxidant activities. Membrane fractions revealed that peptides with sizes < 3 kDa had significantly ($p<0.05$) reduced surface hydrophobicity in comparison with peptides >3 kDa. This is in agreement with the result obtained for ferric reducing power, metal chelating and hydroxyl radical scavenging activities where higher molecular weight peptides exhibited better activity ($p<0.05$) when compared to low molecular weight peptide fractions. However, for all the hydrolysates, the low molecular weight peptides were more effective diphenyl-1-picryhydradzyl (DPPH) radical scavengers but not superoxide radical when compared to the bigger peptides. In comparison to glutathione (GSH), BPHs and their membrane fractions had better ($p<0.05$) reducing power and ability to chelate metal ions except for the pepsin hydrolysate and its membrane fractions that did not show any metal chelating activity. However, the 5-10 kDa pepsin hydrolysate peptide fractions had greater (88%) hydroxyl scavenging activity than GSH, alcalase and trypsin hydrolysates (82%). These findings show potential use of BPHs and their peptide fraction as antioxidants in reducing food spoilage or management of oxidative stress - related metabolic disorders.

5.1. Introduction

Value addition to underutilized crops has become popular in recent time in order to maximize their potential use for human nutrition and health (Alashi *et al.* 2014). Bambara groundnut (*Vigna subterranea*), a scarcely studied crop of African origin, is the third most important legume seed after groundnut (*Arachis hypogeal)* and cowpea (*vigna unguiculata*) in Africa (Adegbola and Bamishaiye 2011; Arise *et al.* 2015). The protein content of bambara may vary between 15-27% (Adebowale *et al.* 2011; Adegbola and Bamishaiye 2011; Murevanhema and Jideani 2013; Arise *et al.* 2015), which is similar to that of cowpea

(Brough *et al.* 1993; Adegbola and Bamishaiye 2011; Hillocks *et al.* 2012) and slightly lower than the values reported for soya bean (Adebowale *et al.* 2011; Hillocks *et al.* 2012; Murevanhema and Jideani 2013). Bambara protein contains a high lysine content (6.5-6.8%) and reasonable amount of methionine (1.8 g/100 g) which is normally limiting in legumes (Ijarotimi and Esho 2009; Adebowale *et al.* 2011; Kudre *et al.* 2013). Other important attributes of bambara include tolerance to drought and poor soil conditions, resilience in the face of extreme weather conditions such as hot temperatures and heavy rainfall and resistance to pests and diseases (Thammarat *et al.* 2015). Despite these attributes, the use of bambara groundnut remains restricted to domestic food consumption (Adegbola and Bamishaiye 2011; Murevanhema and Jideani 2013). However, with further research, bambara could be used for the manufacturing of value- added products and its utilization may assist aid in solving the problem of food insecurity and poverty in developing countries.

In recent years, research has focused on the generation of bioactive peptides from food protein sources (Zhang *et al.* 2011; Durak *et al.* 2013). Bioactive peptides contain 2-20 amino acids per peptide as inactive sequences within large proteins. These peptides are released when the parent protein is hydrolysed by digestive enzymes (*in vitro* and *in vivo*), microbial enzymes or during food processing (Pownall *et al.* 2010). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides without adversely affecting the nutritive value (He *et al.* 2013; Thammarat *et al.* 2015). Peptides can be used in the formulation of functional foods and nutraceuticals to prevent damages related to oxidative stress in human disease conditions. Also, natural antioxidants are desirable because they can be used at higher concentrations without the toxic side effects associated with the use of synthetic equivalents (Li *et al.* 2008; Pownall *et al.* 2010). They also exhibit enhanced nutritional and functional properties in addition to their antioxidant activity (Xie *et al.* 2008; Alashi *et al.* 2014). To date, the antioxidant activities of enzymatic hydrolysates from plant food proteins including soy (Chen *et al.* 1995; Chen *et al.* 1998), African yam bean (Ajibola *et al.* 2011), canola protein (Alashi *et al.* 2014), hemp seed (Tang *et al.* 2009), peanut protein (Zhao *et al.* 2011) and chickpea protein hydrolysate (Li *et al.* 2008), have been widely investigated using many *in vitro* antioxidant evaluation systems. The antioxidant properties of these hydrolysates have been found to largely depend on protease specificity, degree of hydrolysis (DH) and nature of released peptides (molecular weight and amino acid composition) (Chen *et al.* 1995; Zhu *et al.* 2006; Ajibola *et al.* 2011; Girgih *et al.* 2011; Alashi *et al.* 2014). Therefore, in order to protect the human body against oxidative damage,

enzymatically modified proteins could be used as natural antioxidants. These protein hydrolysates may also serve as natural sources of antioxidants in functional foods to maintain freshness and extend shelf life. (Ajibola *et al.* 2011; Girgih *et al.* 2011; Alashi *et al.* 2014).

A review of available literature on previous research carried out on bambara revealed that there is scanty information on the antioxidant properties of bambara protein hydrolysate. A previous work by Thammarat *et al.* (2015), on the functional and antioxidative property of bambara, was limited to a hydrolysate produced using a single digestive protease. Further, these authors did not study the effect of peptide fractions. The use of different proteases is important in order to determine the enzyme that produces peptides with the best antioxidant activities. Also, by fractionating the hydrolysate, it is possible to identify the effect of peptide size on antioxidant activities. Therefore, the main aim of this study was to evaluate the *in vitro* antioxidant potential of bambara protein hydrolysates obtained using three proteases. The effect of peptide size on the measured antioxidant parameters was also evaluated.

5.2. Material and Methods

5.2.1. Materials

Bambara groundnut seeds were obtained from Josini, KwaZulu-Natal province of South Africa. Alcalase 2.4 L, trypsin, pepsin, 1,1 diphenyl-1-picryhydradzyl (DPPH), butylated hydroxyl toluene (BHT), glutathione (GSH) and other antioxidant reagents were obtained from Sigma (Sigma Chemicals, St. Louis, MO, USA) while 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 Bambara Seed Protein Isolate (BPI)

Bambara flour was defatted with n-hexane in the ratio 1:5 (flour: solvent) for 3 h using a magnetic stirrer at speed of 198 rpm. The defatted flour was placed in a fume hood overnight to remove the remaining hexane. The fat content of flour after defatting using a Soxhlet extractor was less than 0.01%. BPI was produced from defatted bambara flour using the method of Adebowale *et al.* (2011) with slight alterations. Concisely, bambara defatted flour was dispersed in deionized water (1:20, w/v), and the dispersion was adjusted to pH 10.0 with 2 M NaOH to solubilise the proteins. The resultant dispersion was stirred at 37^{o}C for 2 h followed by centrifugation (7000 x *g* at 4^{o}C) for 45 min. The residue was discarded and the supernatant filtered with cheesecloth and adjusted to pH 5.0 with 2 M HCl to precipitate most

of the proteins. Thereafter, the mixture was centrifuged (7000 x *g* at 4^{o}C) for 45 minutes. The resultant precipitate was re-dispersed in deionized water and adjusted to pH 7.0 with 2 M NaOH and freeze dried to obtain BPI powder, The protein content of the BPI was determined by the modified Lowry method (Markwell *et al.* 1978).

5.2.3. Preparation of bambara protein hydrolysates and peptide fractions

Proteolysis of the isolated bambara protein isolate was carried out using three enzymes with reaction conditions as described by He *et al.* (2013) with some modifications. Briefly, BPI (5%, w/v, protein basis) were dispensed in deionized water in a reaction vessel equipped with a stirrer, heated to the suitable temperature and adjusted to the suitable pH value before the addition of the proteolytic enzyme (Alcalase 50^{o}C, pH 8.0; Trypsin 37^{o}C, pH 8.0 or Pepsin 37^{o}C, pH 2.0). Each enzyme was added to the BPI slurry at an enzyme to substrate ratio (E/S) of 1:100, in accordance with the BPI protein content. Digestion was carried out for 4 h (pH was kept constant by addition of 1 M NaOH or 1 M HCl). At the end of the proteolysis period, the mixtures were heated in boiling water for 10 minutes to inactivate the action of the enzymes and by adjusting to pH 4.0 with 2 M HCl to precipitate the undigested proteins; thereafter, the hydrolysates were centrifuged at 8000 x *g* for 60 minutes to remove the undigested protein. The supernatant containing target peptides was collected, a part of the supernant was freeze dried to obtain BPH, while the remaining portion was passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1, 3, 5, 10 kDa in an Amicon stirred ultrafiltration cell. Supernatant was first passed through the 1 kDa membrane and the retentate passed through 3 kDa. The retentate from 3 kDa ultra membrane filtration was passed through a 5 kDa membrane whose retentate was then passed through a 10 kDa membrane. The permeate from each MWCO membrane (<1, 1–3, 3–5, and 5– 10 kDa, respectively) was collected, freeze-dried, and stored at -20^{o}C until required for further analysis. The protein contents of the freeze dried bambara protein hydrolysates (BPH) and membrane fractions were also estimated using the modified Lowry method (Markwell *et al.* 1978).

5.3. Analyses

5.3.1. Surface hydrophobicity (SO)

Surface hydrophobicity of BPHs and their membrane fractions were determined using an aromatic hydrophobicity fluorescence probe (ANS) as estimated by Wu *et al.* (1998) with some adjustments. Samples were serially diluted to a final concentration of 50–250 µg/mL in 0.01 M phosphate buffer (pH 7.0). A 20 µl ANS solution (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was added to 4 mL of each sample dilution and fluorescence intensity (FI) of the mixture was determined with a JASCO FP-6300 fluorescence spectrophotometer (JASCO, Tokyo, Japan) at excitation and emission wavelengths of 390 nm and 470 nm respectively.

5.3.2. DPPH radical scavenging assay

The scavenging activity of BPHs and their membrane fractions against DPPH was determined using the method of Alashi *et al.* (2014). BPH and peptide fractions were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. DPPH was dissolved in 95% methanol to a final concentration of 100 µM. The buffer was used as the blank assay while GSH served as the positive control. Suitable dilutions of the samples (100 µL) were mixed with 100 µL of DPPH solution in the 96-well plate to a final assay concentration of 2.5 mg/mL. Thereafter, the plate was covered with aluminium foil and incubated at ambient temperature in the dark for 30 minutes. Subsequently, the sample absorbance (As) and control absorbance (Ac) were read at 517 nm. The scavenging activity of the peptide fractions was compared to that of GSH (2.5 mg/mL). The percent scavenging activity was calculated using the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{Ab - As\ \times 100}{Ab}$$

Where Ab and As, are absorbance of the blank and sample respectively.

5.3.3. Superoxide radical scavenging assay (SRSA)

The method described by Alashi *et al.* (2014) was used to determine SRSA. Samples (1 mg/mL final concentration) were each dissolved in 50 mM Tris–HCl buffer, pH 8.3 containing 1 mM EDTA and 80 µL was transferred into a clear bottom microplate well. Subsequently, 80 µL of buffer was added to the blank well. This was followed by addition of 40 µL 1.5 mM pyrogallol (dissolved in 10 mM HCl) into each well in the dark and the change

in reaction rate was measured immediately at room temperature over a period of 4 minutes at a wavelength of 420 nm. The superoxide scavenging activity was calculated using the following equation:

$$Superoxide\ scavenging\ activity\ (\%) = \frac{\Delta A/\min blank - \Delta A/\min sample}{\Delta A/\min blank}$$

Where ΔA is change in absorbance

5.3.4. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was carried out with slight modifications as described by Ajibola *et al.* (2011). BPH, peptide fractions, GSH and 1,10-phenanthroline (3 mM) were separately dissolved in 0.1 M sodium phosphate buffer (pH 7.4). Whereas distilled water was used to dissolve $FeSO_4$ (3 mM) and 0.01% hydrogen peroxide. An aliquot (fifty µL) of BPH, peptide fractions or GSH (Equivalent to a final assay concentration of 1 mg/mL) or buffer (control) was first added to a clear, flat bottom ninety six-well plate followed by additions of fifty µL of 1, 10-phenanthroline and fifty µL of $FeSO_4$. To start the reaction in the wells, fifty µL of hydrogen peroxide (H_2O_2) solution was added to the mixture. The mixture was covered with aluminium foil and incubated at 37 °C for 1 h with constant shaking. After the incubation, the absorbance of the mixtures was measured at 536 nm every ten minutes for a period of 1 h. Blank absorbance (does not contain peptides or H_2O_2) and a control (did not contain peptides) were taken.

$$Hydroxyl\ radical\ scavenging\ activity\ (\%) = \frac{\Delta A/\min blank - \Delta A/\min sample}{\Delta A/\min blank}$$

Where ΔA is change in absorbance

5.3.5. Metal ion chelation activity

The metal chelating activity of BPHs and their peptides were determined using the method of Ajibola *et al.* (2011). BPH and peptide solutions or GSH (final concentration of 1 mg/mL) were each combined with 0.05 mL of $FeCl_2$ (2 mM) and 1.85 mL distilled water in a reaction tube. Thereafter, 0.1 mL of 5 mM Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4-disulfonic acid sodium salt] solution was added and mixed comprehensively. The mixture was allowed to stand at room temperature for 10 minutes. Thereafter, 200 µL aliquot of the reaction mixture was pipetted into a clear bottom 96-well plate. The control experiment contained all the reaction mixtures except that distilled water was used to replace the peptide sample. Absorbance of sample (As) and control (Ac) was measured using a

spectrophotometer at 562 nm and the metal chelating activity of the sample was compared to that of GSH. The percentage chelating effect (%) was calculated using the following equation:

$$Metal\ chelating\ effect\ (\%) = \frac{Ab - As\ \times 100}{Ab}$$

Where Ab and As, are absorbance of the blank and sample respectively.

5.3.6. Ferric reducing power assay

Ferric reducing power of BPHs and their peptides were measured as described by Girgih *et al.* (2011) with slight adjustments. Peptide sample (250 µL) or GSH was prepared in 0.2 M sodium phosphate buffer (pH 6.6), mixed with 250 µL of buffer and 250 µL of 1% potassium ferricyanide solution dissolved in distilled water. The final peptide or GSH concentration in the assay mixture was 1 mg/mL while the control reaction contained buffer and ferricyanide only. Each resulting mixture was heated at 50 °C and incubated for 20 minutes, followed by addition of 250 µL 10% aqueous trichloroacetic acid. Subsequently, a 250 µL aliquot of the reaction mixture was combined with 50 µL of 0.1% aqueous ferric chloride solution and 200 µL of distilled water was added. The mixture was allowed to stand at room temperature for 10 minutes and then centrifuged at 1,000 × g for 10 minutes. The absorbance of the supernatant was measured at 700 nm.

5.3.7. Statistical Analysis

All experiments were conducted in triplicate. Data were analysed using analysis of variance (ANOVA) and means were compared using Fischer's Least Significant Difference Test ($p< 0.05$).

5.4. Results and Discussion

5.4.1. Surface hydrophobicity (So) of BPHs and their membrane fractions

Hydrophobic properties of peptides of food protein-derived peptides may play important roles in their bioactivities (Pownall *et al.* 2010; Ajibola *et al.* 2011). The maximum DPPH free radical scavenging activities reported for <1 kDa peptide fraction of African yam bean protein hydrolysate was linked to its high hydrophobicity (Ajibola *et al.* 2011). Fig. 5.1-: shows that So was directly proportional to peptide size because the values increased significantly ($p<0.05$) with increase in peptide size from <1 to 5-10 kDa. Given that the non polar amino

acids (hydrophobic groups) are supressed inside the core of the folded structure of native protein molecules, the subsequent exposure of some of these groups after partial hydrolysis may have contributed to increased So (Wu *et al.* 1998; He *et al.* 2013). On the other hand, as enzyme hydrolysis continues, the hydrophobic covers are interrupted and So decreases with increased enzymatic hydrolysis or reduced peptide size. Similar increase in So values with increase in peptide size has been reported for rape seed protein hydrolysate (He *et al.* 2013). Further, the results obtained show that So of BPH obtained for trypsin (1421) is higher than So obtained for alcalase and pepsin (800) hydrolysates. The results suggest that trypsin hydrolysates and its membrane fractions contained higher molecular weight peptides than the other hydrolysates. Similar high hydrophobicity for trypsin hydrolysate has been reported for hemp protein hydrolysate by Tang *et al.* (2009). Trypsin is a highly specific protease that hydrolyses arginine or lysine-containing peptide bonds and will produce bigger-size peptides. In contrast, alcalase and pepsin are more effect random-acting proteases that will produce smaller peptides with lower So.

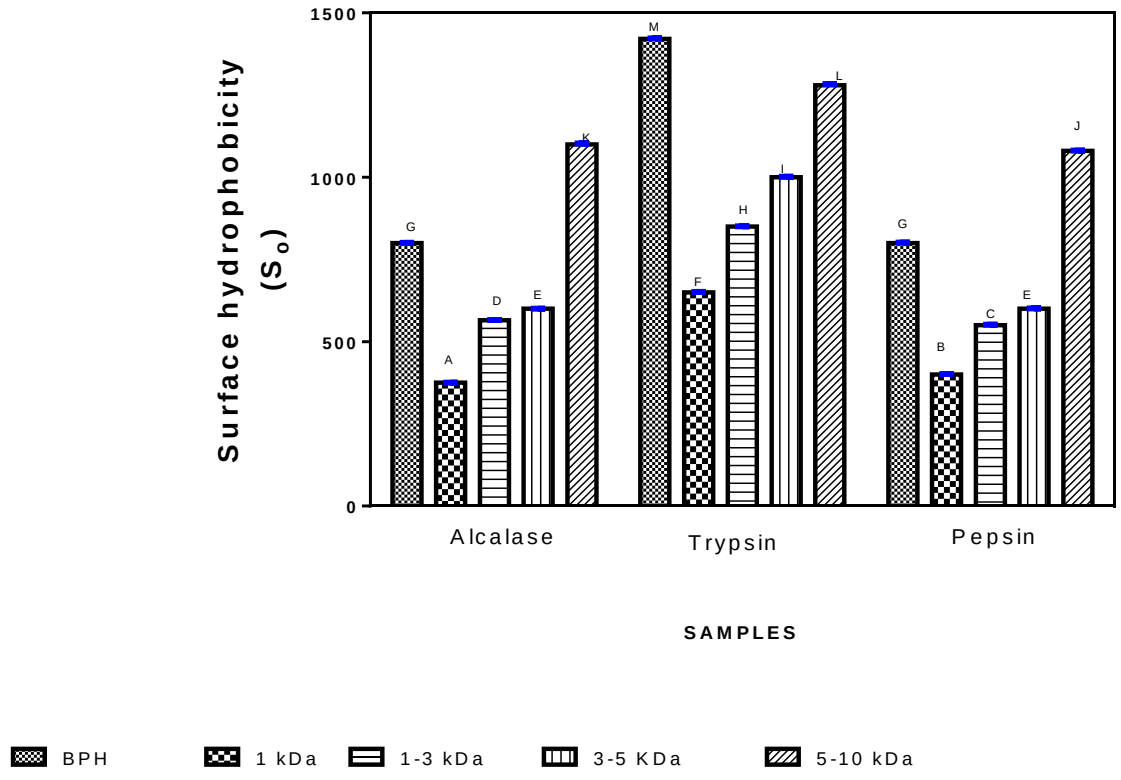


Fig.5.1. Surface Hydrophobicity of enzymatic bambara protein hydrolysates and membrane ultrafiltration fractions (mean± standard deviation, n=3 with different alphabets have mean values that are significantly different ($p<0.05$)

5.4.2. DPPH free radical scavenging activities

DPPH radical is a stable free radical that shows maximal absorbance at 517 nm in methanol and is used to evaluate the antioxidant activity of natural compounds (Pownall *et al.* 2010). The DPPH free radical scavenging activity indicates electron-donating ability of the antioxidant compound, which then converts the radical to a more stable species (Li *et al.* 2008). The ability of BPH and its membrane fractions to scavenge DPPH radical is shown in Fig.5.2. It is interesting to note that pepsin hydrolysate fractions had the highest DDPH scavenging activity (67–72%) in comparison to GSH which was used as standard. All the peptide fractions had better DPPH scavenging activities compared to their hydrolysates. Also, peptide size was inversely related to the DPPH radical scavenging activity. A similar trend was also reported for African yam beans, canola protein hydrolysate and hemp peptide (Ajibola *et al.* 2011; Girgih *et al.* 2011; Alashi *et al.* 2014). The scavenging activity of the BPHs and their fractions revealed that the peptides were able to effectively scavenge the DPPH radical compare to GSH. The DPPH scavenging properties of low molecular weight peptide could make them useful ingredients to avoid oxidative deterioration of foods.

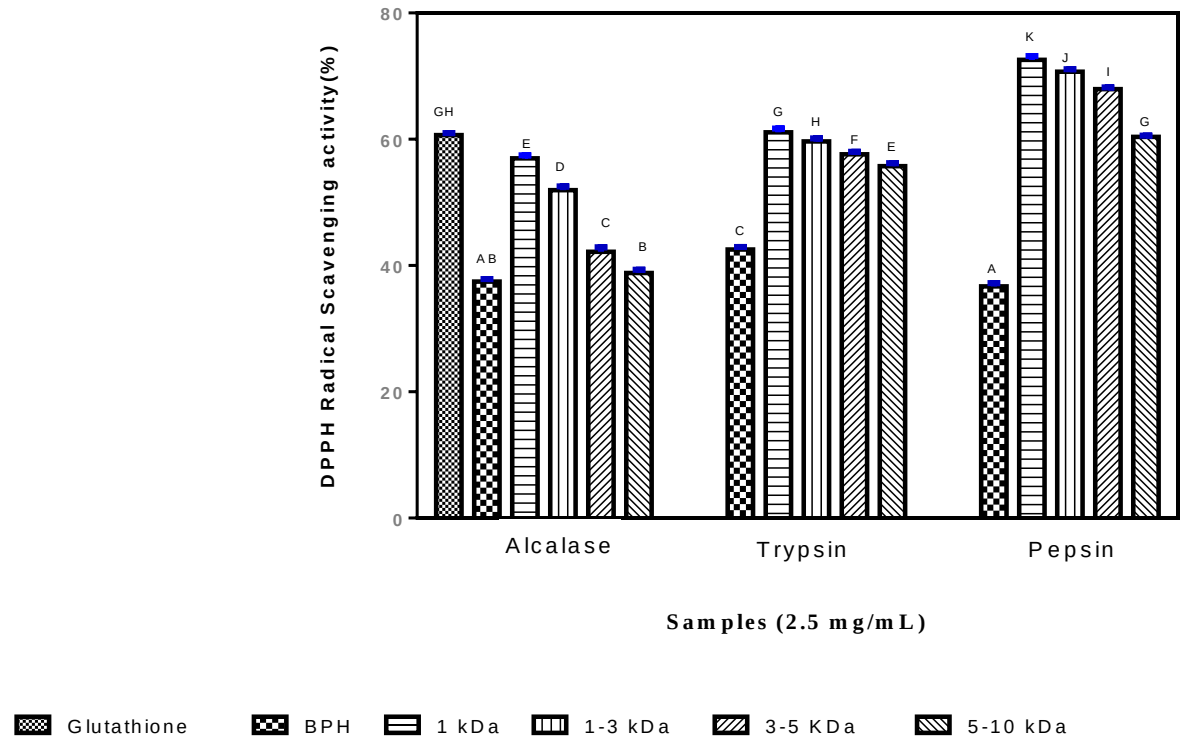


Fig.5.2. DPPH radical scavenging activities of Bambara protein hydrolysates and membrane fractions (mean± standard deviation, n=3 with different alphabets have mean values that are significantly different ($p<0.05$)

5.4.3. Superoxide radical scavenging activities (SRSA)

Numerous biological reactions such as the oxidation of haemoglobin and normal catalytic function of a number of metabolic enzymes generate superoxide radicals (O_2^-), which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of highly reactive species such as hydroxyl radical and therefore the study of the scavenging activity of this radical is important (Li *et al.* 2008). Bambara protein hydrolysates and their membrane-separated fractions showed different SRSA based on the different proteases used at 1 mg/ml final concentration (Fig.5.3). The GSH was significantly ($p<0.05$) a more active superoxide radical scavenger than the BPHs and peptide fractions. Generally, all hydrolysates had lower superoxide activities (< 20%). However, pepsin hydrolysate and its membrane fractions (except <1 kDa) had the highest scavenging activity in comparison to alcalase and trypsin hydrolysates or their corresponding membrane fractions. Similar higher superoxide activity was reported for canola pepsin hydrolysate compared to its alcalase and trypsin hydrolysates (Alashi *et al.* 2014). The results obtained in this study are lower when compared to the values reported for Africa yam beans peptides (Ajibola *et al.* 2011). Bambara protein hydrolysate and peptide fractions may not be as effective as GSH superoxide radical scavenging.

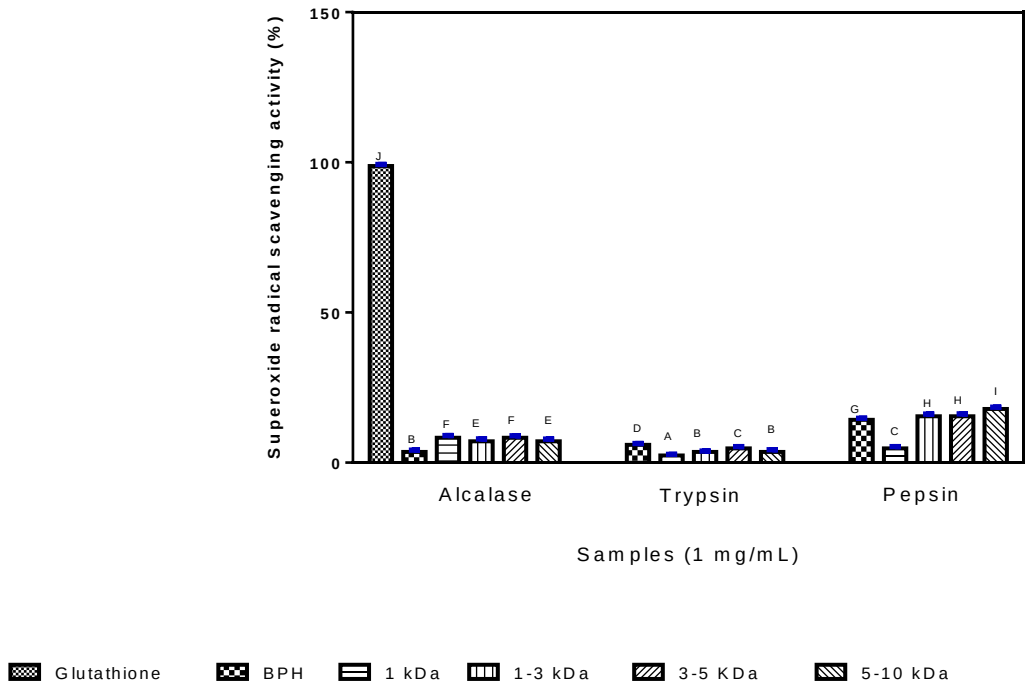


Fig.5.3. Superoxide radical scavenging activities of Bambara protein hydrolysates and membrane fractions (mean± standard deviation, n=3 with different alphabets have mean values that are significantly different ($p<0.05$)

5.4.4. Hydroxyl radical scavenging activities

The reactive oxygen radicals are unstable and react readily with other groups or substances in the body resulting in cell damage hence, human diseases (Zhu *et al.* 2006). Among these reactive oxygen radicals, hydroxyl radical has been found to be the most reactive. The hydroxyl radical brutally damages end-to-end biomolecules such as proteins, DNA, nucleic acid and almost any other biological molecules. These damages may lead to aging as well as development of chronic diseases such as cancer, diabetes and neurodegeneration (Ajibola *et al.* 2011). Therefore, the scavenging of hydroxyl radical is important for protection against various metabolic disorders that are due to hydroxyl radical activities. Bambara protein hydrolysates and its fractions showed different hydroxyl scavenging activity which was dependent on the type of protease used (Fig. 5.4). Trypsin and pepsin hydrolysates had similar hydroxyl radical scavenging, which also compared favourably to GSH (standard). Moreover, the 5-10 kDa pepsin hydrolysate fractions had a slightly higher percentage (88%) of hydroxyl radical scavenging activity than GSH (82%). This high activity of the pepsin hydrolysate may be due to its high surface hydrophobicity as shown in Fig. 5.1. A number of researchers have linked high hydrophobicity to high hydroxyl scavenging activity. For example Pownall *et al.* (2010), described a strong hydroxyl scavenging activity for highly hydrophobic pea protein hydrolysates. In addition, the peptides molecular weight was positively related to hydroxyl radical scavenging. The hydroxyl radical activity obtained in this study is higher than the values reported for African yam bean alcalase hydrolysate but lower than that of chickpea alcalase protein hydrolysates (Li *et al.* 2008; Ajibola *et al.* 2011). The inhibition of hydroxyl radical exhibited by the pepsin hydrolysate and trypsin hydrolysate (82%) is closer to that of a peptide isolated from hoki (81%) (Kim *et al.* 2007). According to the present findings, bambara protein hydrolysate might be useful in providing bioactive peptides with good hydroxyl radical scavenging activity for the formulation of oxidative stress-reducing foods.

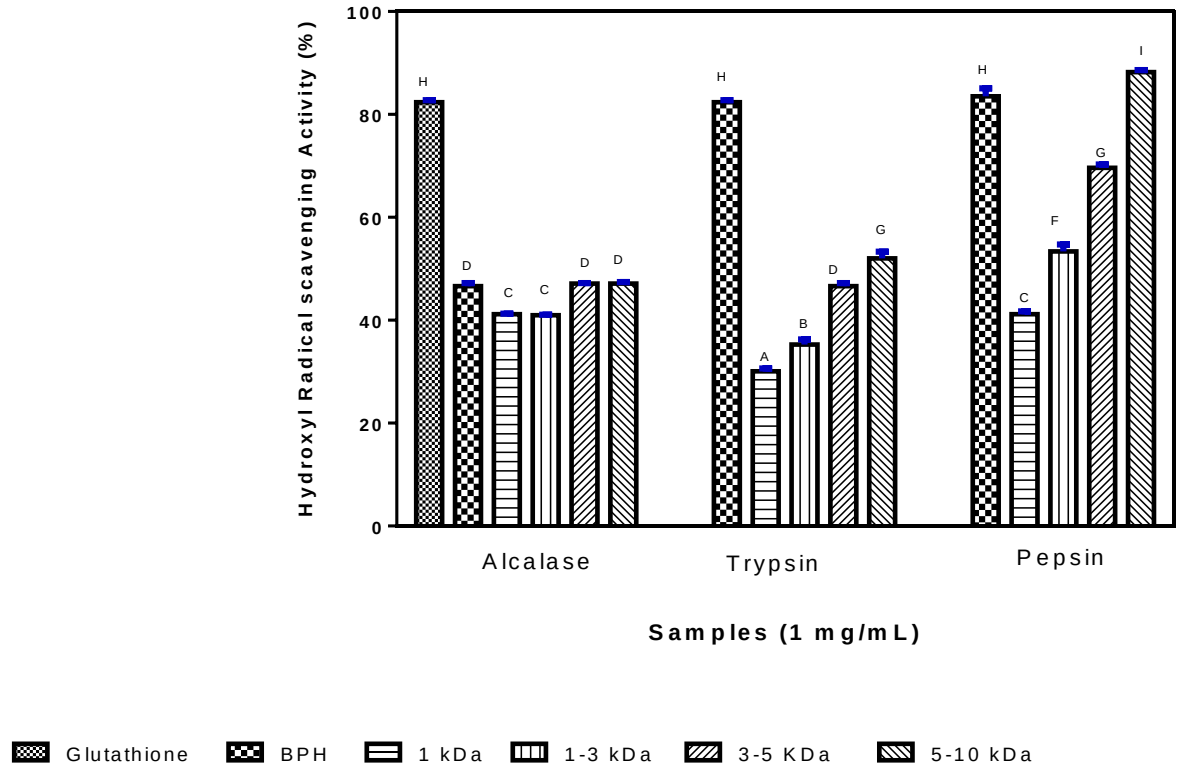


Fig.5.4. Hydroxyl radical scavenging activities of Bambara protein hydrolysates and membrane fractions (mean± standard deviation, n=3 with different alphabets have mean values that are significantly different ($p<0.05$)

5.4.5. Metal chelating activity

Involvement of transition metal ions in many *in vivo* oxidation reactions has been recognized. Ferrous ion ($Fe^{2+)}$ can catalyse the Haber-Weiss reaction and induce superoxide anion to form the more hazardous hydroxyl radical. This hydroxyl radical reacts rapidly with the adjacent biomolecules and can induce severe cellular damage. Ferrous ion is one of the products formed during the Fenton reaction, where hydrogen superoxide produces hydroxyl radicals (Xie *et al.* 2008; Pownall *et al.* 2010). It has been reported that scavenging of hydroxyl radicals by antioxidants was effective mainly through metal ion chelation. Since compounds that interfere with the catalytic activity of metal ions could impair the peroxidative process, measurement of chelating ability is important for evaluating antioxidant potential of a compound (Pownall *et al.* 2010). Fig. 5.5 shows the Fe^{2+} chelating effects of reduced GSH, BPHs and its membrane fractions. The alcalase hydrolysate and its membrane fractions exhibited higher chelating ability than GSH (standard) while pepsin and its membrane fractions did not display any metal chelating activity. Similar results have been observed for lower molecular weight thermolysin pea protein hydrolysate which did not display any metal

chelating activity (Pownall *et al.* 2010). Interestingly, 5-10 kDa trypsin fractions showed the highest percentage of metal chelation (90%), which could be due to the high hydrophobicity of trypsin hydrolysates as shown in Fig. 5.1. In addition, for trypsin hydrolysates, the metal ion chelating activities increased with the molecular weight, which may be due to an additive effect from constituent peptides. However, for alcalase the trend was different because the 1-3 kDa fractions had the highest metal chelation percentage (86%) when compared to other peptide fractions. The results obtained in this study are similar to those obtained for African yam bean and hemp seed (Ajibola *et al.* 2011; Girgih *et al.* 2011). The observed ion chelating properties of the BPHs and their membrane fraction (alcalase and trypsin) may be beneficial towards the protection of cellular components against metal cation – dependent oxidative damage.

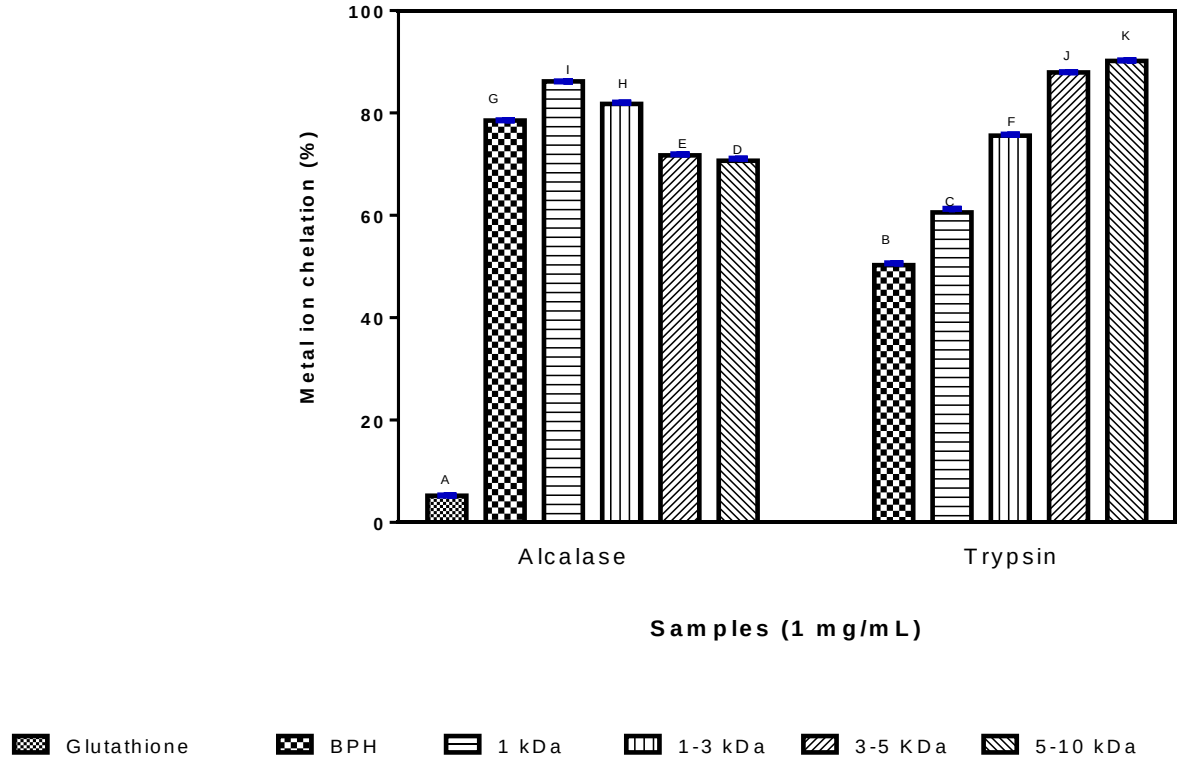


Fig.5.5. Metal chelating effects of Bambara protein hydrolysates and membrane fractions (mean± standard deviation, n=3 with different alphabets have mean values that are significantly different ($p<0.05$)

5.4.6. Ferric reducing power activity (FRAP)

The ability of natural antioxidants to donate electrons or hydrogen can be evaluated by using the ferric reducing antioxidant power (FRAP) assay. Literature indicated that there is a direct correlation between the reducing power of protein hydrolysate fractions and antioxidant activities (Cheng *et al.* 2006; Pownall *et al.* 2010). The reducing power of BPHs and their membrane fractions are shown in Fig. 5.6. An increase in absorbance shows better reducing power of the test sample. The BPHs and their membrane fractions exhibited higher absorbance values of 0.035 - 0.07 in comparison to GSH which had lowest absorbance value of 0.034. This inferred that BPHs and their membrane fractions had highest reducing power in comparison to GSH. Among the BPHs and their fractions, the unfractionated hydrolysates had higher reducing power when compared to their membrane fractions. Trypsin hydrolysate had the highest absorbance (0.077) among the hydrolysates. In addition, the ferric reducing power of BPH fractions increased with increase in molecular size of the peptide. This may be due to the additive effect from constituent peptide. However, the synergistic effects of the fractions may have been instrumental in providing the high activity exhibited by the unfractionated hydrolysate. The results may also be an indication of additive effects of active groups within the long chain peptide contains more reducing groups than in the short chain peptides. This same trend was observed for the So, which increased as the molecular weight increased, Furthermore, the same trend observed for FRAP was observed for the metal chelation of BPHs and their membrane fractions, which had the 5-10 kDa fraction as the most effective. In contrast, Ajibola *et al.* (2011), reported a decrease in reducing activities with increase in peptide size for alcalase hydrolysate of African yam bean. However, results obtained in this study are in line with the reducing power values obtained for hemp seed hydrolysates (Girgih *et al.* 2011).

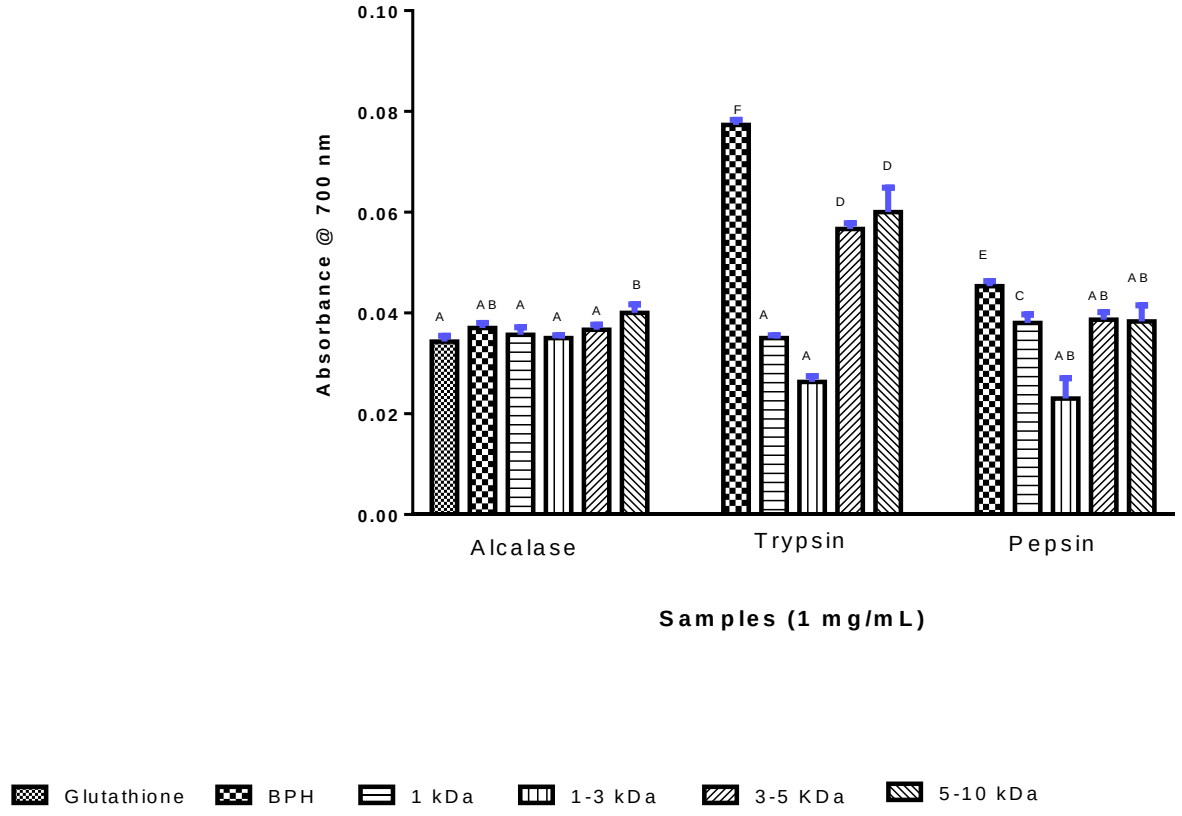


Fig.5.6. Ferric reducing power of Bambara protein hydrolysates and membrane fractions (mean± standard deviation, n=3 with different alphabets have mean values that are significantly different ($p<0.05$)

5.5. Conclusions

Protein hydrolysates derived from bambara groundnut possess antioxidant properties against a variety of physiologically relevant free radicals studied *in vitro*. High surface hydrophobicity and the molecular size of the peptide appear to be important for scavenging of hydroxyl radicals, ferric reducing power and metal chelating. The activity increased with increase in peptide size except for DPPH which had higher activity with smaller molecular size peptide. The activity of <1 kDa for pepsin was higher than that of GSH. Similarly, the peptides had better ferric reducing and metal chelating activities when compared to GSH except for pepsin hydrolysate and its fractions that did not exhibit any metal chelating activity. Bambara protein hydrolysate and its membrane fractions may be suitable ingredients for the formulation of functional foods and nutraceuticals that can be used to prevent or manage oxidative stress.

5.6. Research outputs

a) Journal article

1. **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Oluwatosin A. Ijabadeniyi, Rotimi E. Aluko and Eric O. Amonsou. (2016). Antioxidant activities of bambara groundnut (*Vigna subterranea*) protein hydrolysates and their membrane ultrafiltration fraction. DOI:10.1039/c6f000057

b) Conference Paper

2. **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Oluwatosin A. Ijabadeniyi, Rotimi E. Aluko and Eric O. Amonsou. (2016). Antioxidant activities of bambara groundnut (*Vigna subterranea*) protein hydrolysates and their membrane ultrafiltration fraction. IUFoST-world congress of Food Science and Technology, Dublin, Ireland 21st-25th August, 2016.

CHAPTER SIX

6. Inhibitory properties of bambara protein hydrolysate and its membrane fractions against angiotensin converting enzymes, renin and free radicals

ABSTRACT

Bambara protein hydrolysates (BPHs) were obtained using three different proteases (alcalase, trypsin and pepsin), followed by membrane ultrafiltration to produce peptide fractions with molecular weights (10, 5, 3 and 1 kDa). BPHs had similar and adequate quantities of essential amino acids. Alcalase hydrolysate contained the highest amount of low molecular weight (LMW) peptides. Evaluation of potential antihypertensive properties of the peptides showed that the <1 kDa fractions exhibited the highest ($p<0.05$) inhibitory activity against angiotensin converting enzyme (ACE) for all the enzymes hydrolysates. For renin inhibition, alcalase hydrolysate showed the highest inhibition at 59% when compared to other hydrolysates and their membrane fractions. The antioxidant power of bambara hydrolysates and peptide fractions were evaluated through the inhibition of linoleic acid peroxidation and 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging activity. Among the hydrolysates, alcalase exhibited the highest inhibition of linoleic acid oxidation. Furthermore, all BPHs were able to scavenge ABTS•+ three folds more than the isolate. Therefore, it was concluded that bambara protein hydrolysate and some of the peptide fractions could potentially serve as useful ingredients that can be used to formulate functional foods and nutraceuticals against high blood pressure and oxidative stress.

6.1. Introduction

Peptides with antihypertensive properties have received increasing attention in recent times. There is a high prevalence of high blood pressure (HBP) in most nations. This critical health condition accounts for approximately 45-51% of total global deaths (Malomo *et al.* 2015). Hypertension is one of the primary risk factors for the development of several cardiovascular diseases including coronary heart diseases, heart failure, stroke, peripheral arterial disease and renal failure (Sharp *et al.* 2011). The renin angiotensin system (RAS) plays a vital role in BP regulation with renin and ACE being the main regulators that control the RAS pathway (Daien *et al.* 2012; He *et al.* 2013). Renin is synthesized in the kidneys and then released into

the blood circulatory system where it cleaves the N-terminal region of angiotensinogen to produce a decapeptide, angiotensin (AT) - I (Aluko 2015), which circulates in the blood until its C-terminal dipeptide residue is cleaved by ACE to form an octapeptide AT-II (a potent vasoconstrictor). Angiotensin-II also induces the release of aldosterone thereby increasing plasma sodium concentration and water retention, which leads to HBP. ACE is also known to hydrolyze bradykinin, a potent vasodilator, thus leading to the inability of the blood vessels to relax adequately following contraction (He *et al.* 2013). Consequently, by inhibiting ACE activity, the formation of angiotensin-II and degradation of bradykinin will be reduced leading to a lowering of HBP. Moreover, simultaneous inhibition of both ACE and renin activities could provide a better blockade of the RAS when compared to inhibition of either enzymes alone (Fitzgerald 2011).

Nutritional factors play a significant role in the prevention and treatment of hypertension. Therefore, the ability to use natural plant peptides as a measure to treat or regulate HBP is attractive. ACE-inhibitory peptides have been obtained from plant proteins some of which are rice (Kang and Ahn 2012), soybean (Rho *et al.* 2009) and peanut (Jimsheena and Gowda 2010), which shows that plant proteins are good sources of bioactive peptides. Apart from ACE inhibition, some studies have also proven that food-derived peptides could inhibit the activity of renin and that they also possess free radical scavenging activities (Huang *et al.* 2010; Li and Aluko 2010; Girgih *et al.* 2011). For instance, < 1 kDa peptide of kidney bean protein hydrolysate has been reported to possess both antioxidant and antihypertensive activities (Mundi and Aluko 2014). An improved BP -lowering effects can be obtained by inhibition of both ACE and renin during antihypertensive therapy by food-derived peptides than inhibition of ACE activity alone (Udenigwe and Aluko 2012).

Bambara groundnut is an underutilized and neglected crop of African origin. It is the third most important legume seed after groundnut (*Arachis hypogeal)* and cowpea (*vigna unguiculata*) in Africa (Adegbola and Bamishaiye 2011; Arise *et al.* 2015) and could be considered a potential alternative source of plant protein. Bambara protein is high in lysine (6.1 g/100 g sample) and its amino acid composition can be compared to other commonly consumed legumes such as soybean (Adebowale *et al.* 2011; Kudre *et al.* 2013). These legumes have been used in the development of many products such as in the functional food and nutraceutical industries (Thammarat *et al.* 2015). Both the technological and functional properties of bambara protein have been studied (Kudre *et al.* 2013; Arise *et al.* 2015). Studies on the functional and antioxidative properties of bambara protein has been limited to

a hydrolysate produced using a single digestive protease, with scanty reports on activities of peptides from different proteases (Thammarat *et al.* 2015). The use of different proteases for enzymatic digestion is important in order to determine the specific enzyme or group of enzymes that produces peptides with the best bioactive properties. In addition, information on RAS enzyme-inhibitory activities of bambara protein and the characteristics of their peptides is lacking in literature.

It is well known that, the structure and activity of bioactive peptides can be affected by the enzyme hydrolysis technique. Therefore, it is essential to appraise the effectiveness of proteases in releasing potential antihypertensive peptides from bambara proteins. Thus this study was carried out to determine the ability of several proteases to produce potential antihypertensive bambara protein hydrolysates (BPHs) from bambara protein isolate as measured using *in vitro* inhibition of ACE and renin activities. Since high oxidative stress may also contribute to high blood pressure, the antioxidant activities of the BPHs were determined using ABTS radical scavenging activity and inhibition of linoleic acid oxidation.

6.2. Materials and Methods

6.2.1. Materials

Bambara groundnut seeds were obtained from Josini, KwaZulu-Natal province of South Africa. Alcalase 2.4 L, trypsin, pepsin, ACE, N(3[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS), Captopril, L-glutathione (GSH), and 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), were purchased from Sigma-Aldrich (St. Louis, MO). Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI) while other analytical grade reagents and ultrafiltration membranes (1, 3 .5 and 10 kDa molecular weight cut-offs), were obtained from Fisher Scientific (Oakvile, ON, Canada).

6.2.2. Preparation of bambara seed protein isolate (BPI)

Bambara flour was defatted with n-hexane in the ratio 1:5 (flour: solvent) for 3 h using a magnetic stirrer at speed of 198 rpm. The defatted flour was air- dried in a fume hood overnight. According to the Soxhlet's procedure, the fat content of defatted bambara flour was less than 0.01%. BPI was produced from defatted bambara flour as previously described by Adebowale *et al.* (2011) with slight alterations. Briefly, defatted bambara flour was dispersed in deionized water (1:20, w/v), and the dispersion was adjusted to pH 10.0 with 2 M

NaOH to solubilize the proteins. The resultant dispersion was stirred at 37^{o}C for 2 h followed by centrifugation (7000 x *g* at 4 ^{o}C) for 45 minutes. The residue was discarded and the soluble proteins in the supernatant were subjected to isoelectric precipitation (pH 5.0) with the addition of 2 M HCl. Subsequently, the mixture was centrifuged (7000 x *g* at 4 ^{o}C) for 45 minutes. The resultant precipitate was re-dispersed in deionized water and adjusted to pH 7.0 with 2 M NaOH. The mixture was subsequently freeze- dried to obtain the BPI powder; protein content was determined by the modified Lowry method (Markwell *et al.* 1978) .

6.2.3. Enzymatic hydrolysis of bambara protein isolates and membrane fractionations

BPI was hydrolysed using three food grade enzymes at an enzyme substrate ratio of 1:100 for 4 h to obtain BPHs. The following hydrolysis conditions were used: alcalase (pH 8.0 and 50 $^{\circ}$C), pepsin (pH 2.0 and 37 $^{\circ}$C) and trypsin (pH 8.0 and 37 $^{\circ}$C). The pH was maintained for each hydrolysis process using either 1 M NaOH or 1 M HCl as appropriate with continuous stirring, while the temperature was maintained using a thermostat. After the 4 h digestion period, the enzymes were inactivated by adjusting the reaction mixture to pH 4.0 with 2 M HCl followed by heating and holding at 90 ^{o}C for 15 minutes. The undigested proteins were precipitated by centrifugation at 8000 x *g* for 60 minutes. A portion of the supernatant containing target peptides was freeze dried to obtain BPH, while the remaining portion was passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1, 3, 5, 10 kDa in an Amicon stirred ultrafiltration cell. Supernatant was first passed through the 1 kDa membrane and the retentate passed through 3 kDa. The 3 kDa retentate was passed through a 5 kDa membrane whose retentate was then passed through a 10 kDa membrane. The permeate from each MWCO membrane (<1, 1–3, 3–5, and 5– 10 kDa) was collected, lyophilized, and stored at -20 ^{o}C until needed for further analysis. The protein contents of the freeze-dried bambara protein hydrolysates (BPH) and membrane fractions were also determined using the modified Lowry method as described by Markwell, et al., (Markwell *et al.* 1978).

6.3. Analytical methods

6.3.1. Analysis of molecular weight distribution

Molecular weight distribution of RPH peptides was determined using an AKTA FPLC system (GE Healthcare, Montreal, PQ) equipped with a Superdex Peptide12 10/300 GL column (10 x 300 mm), and UV detector (λ = 214 nm). A 100 µL aliquot 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 at 0.5 mL/minute flow rate. Molecular weight was determined from a plot of log MW versus elution volume of standard proteins (cytochrome C-12 kDa; Aprotinin- 6.5 kDa; vitamin B12- 1.85 kDa; and Glycine- 0.075 kDa).

$$\text{Kav} = \frac{V_e - V_o}{V_c - V_o}$$

Where Kav = partition coefficient

V_e = Elution volume, V_c = column volume and V_o = Void volume

6.3.2. Amino acid composition analysis

The amino acid profiles of bambara protein isolate (BPI) and bambara protein hydrolysates (BPH) samples were determined using the HPLC system after samples were hydrolysed with 6 M HCl at 116^{o}C for 24 h prior to chromatographic analysis, according to the method of Bidlingmeyer *et al.* (1984). This method is based on the principle of reverse phase chromatography with pre-column derivatization following acid digestion. The cysteine and methionine contents were determined after performic acid oxidation (Gehrke *et al.* 1985) while tryptophan content was determined after alkaline hydrolysis (Landry and Delhaye 1992). The digests were separated on a cationic column (4.6 x 150 mm) using a gradient of sodium citrate buffers (pH 3.45 and pH 10.85) at 0.45 mL/minute flow rate.

6.3.3. ACE inhibition assay

The ability of BPH and membrane permeates to inhibit *in vitro* activity of ACE was measured according to the method of Girgih *et al.* (2011) with slight changes, using FAPGG as the substrate. Briefly, 1 mL 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 ACE (1 U/ mL, final activity of 20 mU) and 80 µL sample dissolved in same buffer. The final concentration of samples was 1 mg/mL based

on protein content. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. The buffer was used instead of sample solutions in the blank experiment. ACE activity was expressed as the rate of reaction (ΔA/min) and inhibitory activity was calculated as:

$$\text{ACE inhibition (\%)} = \left(\frac{(\Delta A/\min)_{(blank)} - (\Delta A/\min)_{(sample)}}{(\Delta A/\min)_{(blank)}}\right) * 100$$

Where $\Delta Amin^{-1}_{(sample)}$ and $\Delta Amin^{-1}_{(blank)}$ represent s ACE activities in the presence and absence of the BPH or peptide fractions respectively.

6.3.4. Renin inhibition assay

In vitro inhibition of human recombinant renin activity was investigated using the Renin Inhibitor Screening Assay Kit according to a previously described method (Girgih *et al.* 2011). Prior to the assay, renin buffer was diluted in 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with the assay buffer before use and pre-warmed to 37^{o}C prior to initiating the reaction in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, and Sunnyvale, CA) maintained at 37^{o}C. Before the reaction, (i) 20 µL substrate, 160 µL assay buffer, and 10 µL Milli-Q water were added to the background wells; (ii) 20 µL substrate, 150 µL assay buffer, and 10 µL Milli-Q water were added to the blank wells; and (iii) 20 µL substrate, 150 µL assay buffer, and 10 µL sample were added to the inhibitor wells. The final sample concentration was 1 mg/mL based on protein content. The reaction was initiated by adding 10 µL renin to the blank and sample wells. The microplate was shaken for 10 s to mix, incubated at 37^{o}C for 15 minutes, and the fluorescence intensity (FI) was recorded using an excitation and emission wavelengths of 340 and 490 nm, respectively. The percentage renin inhibition was calculated as follows:

$$\text{Renin inhibition (\%)} = \left(\frac{(\text{FI of blank well} - \text{FI of sample well})}{(\text{FI of blank well})}\right) * 100$$

6.3.5. Inhibition of linoleic acid oxidation

Linoleic acid oxidation was measured using the method described by He *et al.* (2013). Samples (at final concentrations of 1 mg/mL) were dissolved in 1.5 mL of 0.1 M sodium phosphate buffer, pH 7.0. A 1 mL aliquot of 50 mM linoleic acid (dissolved in 95% ethanol) was added to the samples and blank (buffer). The mixtures were incubated at 60^{o}C under dark conditions for 7 days. The degree of colour development was measured as follows at intervals of 24 h. A 100 µL aliquot of the assay mixture above was transferred into a reaction tube to

which 4.7 mL of 75% (v/v) ethanol, 100 µL of 30% (w/v) ammonium thiocyanate and 100 µL of 0.02 M ferric chloride dissolved in1 M HCl was added. After shaking and incubating at room temperature for 3 minutes, 200 µL of each sample was transferred into a clear-bottom 96-well plate and the absorbance measured at 500 nm using a spectrophotometer. An increase in absorbance value implies an increase in the level of linoleic acid oxidation. The percentage inhibition of linoleic acid was calculated using the following equation:

$$\text{Inhibition of linoleic acid (\%)} = \left(1 - \left(\frac{A_s}{(A_b)}\right) \times 100\right)$$

Where A_s and A_b are absorbance of sample and blank respectively.

6.3.6. ABTS radical scavenging activity

This assay is based on the percentage inhibition of the peroxidation of ABTS radical, which is observed as a discoloration of the blue green colour (734 nm). The reaction was carried out according to a previously described method (Arts *et al.* 2004; Alashi *et al.* 2014) with slight modifications. Briefly, $ABTS^{\bullet+}$ was prepared by dissolving 7 mM ABTS and 2.45 mM potassium persulphate in phosphate buffered saline (PBS), pH7.4 and allowing this to stand in the dark for 16 h to generate the ABTS radical cation ($ABTS^{\cdot+}$). For the analysis, the $ABTS^{\bullet+}$ stock was diluted using PBS buffer and equilibrated at 30^{o}C to an absorbance of 0.7 ± 0.02 at 734 nm. Trolox was dissolved in 80% ethanol. The antioxidant capacity was measured by mixing 200 µL of samples with 2 mL of $ABTS^{\bullet+}$ solution and the decline in absorbance was observed for 5 min. Appropriate blanks were run for each sample and the radical scavenging capacity was compared to that of Trolox (6.25–200 µM) and results were expressed as mM Trolox equivalent (TE) per gram of sample on protein equivalent basis. The percentage $ABTS^{\bullet+}$ scavenged was calculated using the following equation:

$$\text{Percentage } ABTS^{\bullet+} \text{ scavenged} = \left(\frac{\text{Ai} - \text{Af}}{\text{Ai}} \times 100\right)$$

The effective concentration that scavenged 50% of the free radicals (EC50, $ABTS^{\bullet+}$) was calculated for each sample by non-linear regression from a plot of percentage $ABTS^{\bullet+}$ scavenged versus sample concentration (1-l0 mg/ml).

6.3.7. Statistical analysis

All assays were conducted in triplicate and analysed by one way analysis of variance (ANOVA). The means were compared using Duncan's multiple range test and significant differences accepted at $p < 0.05$.

6.4. Results and Discussion

6.4.1. Protein content and yield of membrane fractions

BPI had 80.2% protein content (wet weight basis). The protein content and yield (i.e. the amount of protein recovered from the fractionation process for each membrane filtration) of BPHs and fractions shows that the protein content increased with increasing peptide size for all the enzymes (Table 6.1). The yield reflects the amount of peptides that can be obtained from a known quantity of raw materials and is an important parameter for technology adoption and commercialization. This is because a higher yield provides a better cost/benefit ratio than a lower yield, which enhances profitability of protein hydrolysate production. This trend however, was reversed for the protein yield as lower yields were obtained as the peptide molecular weight increased except for alcalase peptide size 1-3 kDa that gave a higher yield (41.8%) when compared to its <1 kDa peptide (21%). This is expected as there was a strong positive correlation between all protein content and the yield for all fractions. All the BPHs had protein content ≥73.5% with pepsin BPH being the lowest while BPH obtained with alcalase had the highest protein content of 93.5%. Furthermore, alcalase BPH had higher yield (79.5%) when compared to trypsin and pepsin BPHs. The result suggests that alcalase is a more effective protease in releasing peptides from bambara proteins. The >68% yields obtained for the hydrolysates indicate that most of the proteins were susceptible to enzymatic hydrolysis and could be converted into peptide products, which would be economically beneficial for industrial purposes. Pepsin BPH had the lowest yield (68.5%), which is probably due to the nature of the enzyme, since pepsin is an endoprotease and is most efficient at cleaving bonds involving the aromatic amino acids phenylalanine, tryptophan and tyrosine. This specificity could have limited the rate of bambara protein hydrolysis by pepsin and hence lowered the hydrolysate yield. The ratio of the percentage protein content to yield was higher for trypsin BPH and its fractions when compared to both alcalase and pepsin BPHs. The percentage yield of protein hydrolysate is not commonly reported in literature; therefore adequate comparison could not be made. In this study, percentage yield obtained for <1 kDa (12.3%) pepsin peptide are higher than those obtained for canola seed (Alashi *et al.* 2014). The result also shows that bambara proteins were better hydrolysed by enzymes when compared to flaxseed protein (Udenigwe *et al.* 2009).

Table 6.1. Protein content (PC) and yield of bambara protein hydrolysates and peptide fractions obtained from ultrafiltration membrane separation

Proteases	Hydrolysates		<1 kDa		1-3 kDa		3- 5 kDa		5-10 kDa	
	PC (%)[1]	Yield(%)[2]	PC (%)[1]	Yield(%)[2]	PC(%)[1]	Yield (%)[2]	PC(%)[1]	Yield(%)[2]	PC(%)[1]	Yield(%)[2]
Alcalase	93.5±0.5^{efg}	79.5±0.5^{j}	89.0±0.7^{de}	21.0±1.0^{g}	91.0±0.6^{de}	41.8±0.2^{h}	91.5±0.5^{def}	14.0±0.1^{f}	96.0±1.0^{fg}	11.9±0.2^{de}
Trypsin	80.5±0.5^{c}	76.0±1.0^{i}	75.5±1.5^{b}	7.1±0.2^{c}	76.0±2.0^{bc}	4.7±0.1^{ab}	78.0±2.0^{bc}	5.6±0.2^{b}	93.0±1.5^{def}	4.1±0.1^{a}
Pepsin	73.5±0.7^{b}	68.5±0.5^{h}	67.5±0.5^{a}	12.3±0.1^{e}	88.0±0.8^{d}	13.6±0.2^{f}	88.5±1.0^{d}	11.9±0.1^{de}	97.5±0.5^{g}	10.7±0.1^{d}

[1] Weight of protein in a hydrolysate expressed as a ratio of the weight of protein in the starting material (Bambara protein isolate).

1 Weight of protein in a fraction expressed as a ratio of the weight of protein in the respective hydrolysate

6.4.2. Molecular size distribution of bambara protein hydrolysates

The size exclusion chromatogram of BPHs shows differences between the molecular weight distribution of BPI and its protease hydrolysates (Fig.6.1). The specificity of pepsin endoprotease activity is revealed as shown by the high peak intensity in the chromatogram (C and D) of MW ranging between 19.6 and 6 kDa. In contrast, these two peak intensities were absent in the chromatogram of Alcalase and trypsin hydrolysates. Trypsin proteolysis is even more specific because it is restricted to peptide bonds formed by arginine or lysine only, hence the larger peptide size of the BPH when compared to pepsin and alcalase BPHs. However, bambara alcalase hydrolysates had a larger range of low molecular weight (LMW) peptides as observed (peak E), while bambara trypsin hydrolysates had a peak (B) similar to that of the isolate (peak A) with a significant overlap between both peaks A and B. The results reflect the higher proteolytic activity and non-specificity of alcalase (acts randomly), which is consistent with the higher BPH peptide yield as shown in Table 6.1. It was reported that low MW peptides possess a stronger effect on ACE inhibitory activity of protein hydrolysates in comparison to high MW peptides because they are easily absorbed and reach the active sites to inhibit ACE activity without further digestion (Terashima *et al.* 2011). Therefore, bambara Alcalase hydrolysates may have high potential for use as ingredients to formulate antihypertensive products.

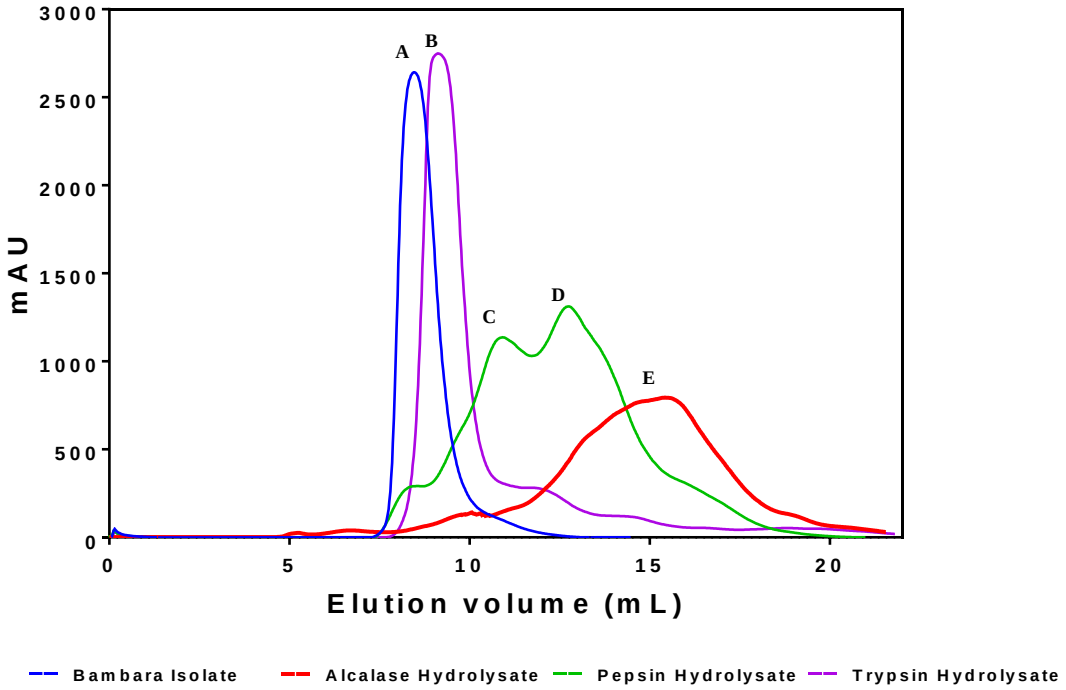


Fig.6.1. Gel-permeation chromatograms of bambara protein isolate and hydrolysates after passage through a Superdex Peptide 10/300 GL column

6.4.3. Amino acid composition of BPI and BPHs

The amino acid composition of BPI and BPHs is shown in Table 6.2. The similarities in the amino acid compositions, especially some of the hydrophobic amino acids when compared to BPI suggest that the protein hydrolysis process did not have a negative effect on the amino acid composition of the hydrolysates. Hydrolysates obtained from alcalase and trypsin contained higher concentration of hydrophobic amino acid (HAA) when compared to pepsin hydrolysate (Table 6.2). HAA have been reported to act as antioxidants by increasing the solubility of peptides in lipids which facilitates better interaction with free radicals (Rajapakse *et al.* 2005). Similar values of HAA has been reported for rapeseed protein hydrolysates (Pan *et al.* 2011) and canola hydrolysates (Alashi *et al.* 2014). Generally, BPI and BPHs contained low levels of methionine and cysteine, which is typical of legume proteins that are usually deficient in sulphur-containing amino acids (Moure *et al.* 2006). Furthermore, the amino acid compositions of all the hydrolysates revealed that they have high levels of Glu, Asp, Arg, and Leu. Previous reports have indicated that acidic amino acids such as Glu and Asp have strong antioxidant effects due to the presence of excess electrons that can be donated during interaction with free radicals (Udenigwe and Aluko 2012; Alashi *et al.* 2014). Further, threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine and lysine values were also found to be higher than the FAO/WHO recommendations for animal protein (Table 6.2). The result shows that bambara proteins are of high nutritional quality and may be used as a protein source in human diet.

Table 6.2. Amino acid composition of bambara protein isolates and hydrolysates (g/100g sample)

Amino acid	Isolate	Trypsin	Pepsin	Alcalase	FAO/WHO 1991
ASP	9.0	8.1	7.4	7.5	
THR	5.2	5.1	4.1	5.1	3.4
SER	4.7	4.2	3.7	4.1	
GLU	14.4	12.4	11.8	12.3	
PRO	4.1	3.7	2.3	4.3	
GLY	2.4	2.2	1.9	2.1	
ALA	2.7	2.4	1.5	2.3	
CYS	0.3	0.3	0.2	0.3	
VAL	4.1	3.9	3.6	3.9	3.5
MET	0.7	0.5	0.3	0.6	
ILE	3.5	3.6	3.3	3.8	2.8
LEU	7.0	6.8	6.7	7.5	6.6
TYR	2.7	2.4	1.8	2.7	1.1
PHE	5.1	4.5	4.1	4.9	6.3
HIS	2.7	2.3	2.1	2.4	1.9
LYS	6.1	5.9	5.8	6.1	5.8
ARG	5.8	5.1	4.6	5.1	
TRP	0.5	0.9	0.5	0.6	
HAA	30.7	29.0	24.3	30.1	
PCAA	14.6	13.3	12.5	13.6	
NCAA	23.4	20.5	19.2	19.9	
AAA	8.3	7.8	6.4	8.2	

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).

6.4.4. ACE-inhibitory activities of BPHs and membrane fractions

The ACE- inhibitory activities of BPHs and membrane fractions showed the same trends as observed in Fig. 6.2. The activity of peptide fractions for all the hydrolysates was clearly molecular weight -dependent. The LMW peptides (<1 kDa) showed a higher percentage of ACE- inhibitory activity than the high molecular weight (HMW) peptides (5-10 kDa). Generally, all peptides fractions and BPHs showed higher percentage (above 57%) of ACE-inhibitory activity. The highest ACE inhibition at 93.9% was achieved by alcalase peptide fraction <1 kDa while trypsin peptide fraction 5-10 kDa showed minimum inhibition at 59.6%. Among the hydrolysates, alcalase hydrolysate showed the highest inhibition at 70.1%. Hydrophobic and bulky amino acids have been reported as structural requirement for ACE-inhibitory peptides (Udenigwe *et al.* 2012). Thus the high hydrophobic and aromatic amino acids of alcalase hydrolysate could have resulted to the high ACE- inhibitory activity. Similar results were reported for rapeseed (He *et al.* 2013) and peanut protein isolate and its alcalase hydrolysate at different degrees of hydrolysis (Jamdar *et al.* 2010). In addition, this result also suggests that LMW peptides were more active at inhibiting ACE activity when compared to

HMW peptides. The results are consistent with data from studies carried out by Zhu *et al.* (2010), which showed that the best ACE- inhibitory activity was attributed to LMW fractions. Similar ACE-inhibitory activity behaviour of peptides was also reported for cowpea hydrolysates, kidney bean peptide fractions and alcalase derived peptides from azufrado beans (Segura Campos *et al.* 2010; Valdez-Ortiz *et al.* 2012; Mundi and Aluko 2014). The alcalase BPH ACE inhibition obtained in this study (93.9%) is greater than 80% obtained for alcalase kidney bean hydrolysate (Mundi and Aluko 2014) and slightly higher than 89.4% reported for alcalase rapeseed protein hydrolysate (Mäkinen *et al.* 2012).

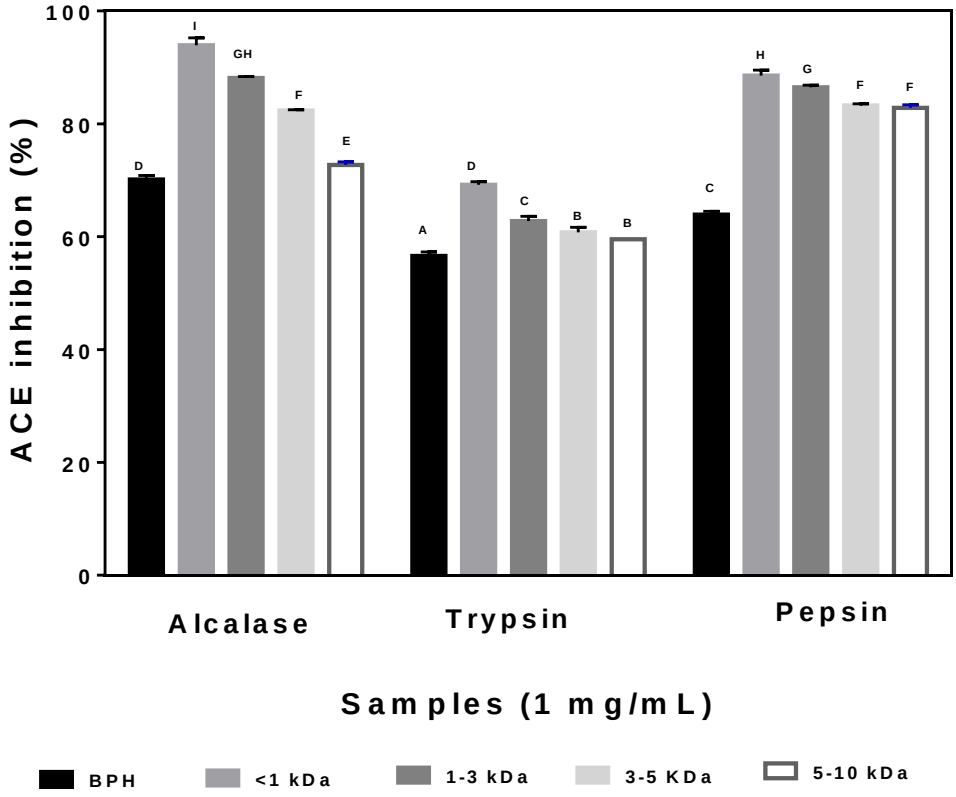


Fig. 6.2. Inhibition of angiotensin converting enzyme (ACE) by enzymatic bambara protein hydrolysate and membrane ultrafiltration fractions at a concentration of 1 mg/mL. Error bars (mean ± standard deviation, n = 3) with different alphabets have mean values that are significantly different ($p < 0.05$)

6.4.5. Renin-inhibitory activities of BPHs and membrane fractions

The type of enzyme used significantly affected the renin inhibitory potential of bambara protein hydrolysates (Fig.6.3). No renin inhibitory activity was observed for pepsin and trypsin hydrolysates. This result agrees with previous studies that showed that renin activity was dependent on the type of proteolytic treatment of the substrate protein (Udenigwe *et al.* 2009). Renin inhibition was significantly ($P<0.005$) higher for alcalase hydrolysate. The

ultrafiltration peptide fractions did not show a similar trend to the unfractionated hydrolysate (Fig. 6.3). Alcalase fraction did not show any level of renin inhibition. This same trend was observed for *palmaria palmata* protein hydrolysate from alcalase in which there was a loss in renin inhibition activities of the hydrolysates (Harnedy and FitzGerald 2013). Among the peptides, trypsin 5-10 kDa, pepsin <1 kDa and 5-10 kDa were the fractions that are able to inhibit renin. Trypsin 5-10 kDa and pepsin <1 kDa showed higher renin inhibition when compared to other peptide fractions. The renin inhibition obtained for alcalase hydrolysate in this study (59%) is higher than 44.5% reported for alcalase flaxseed protein at an even higher concentration of 7.5 mg/mL (Udenigwe *et al.* 2012). In addition, the value is also higher than 23% reported for alcalase *palmaria palmata* protein hydrolysate (Harnedy and FitzGerald 2013).

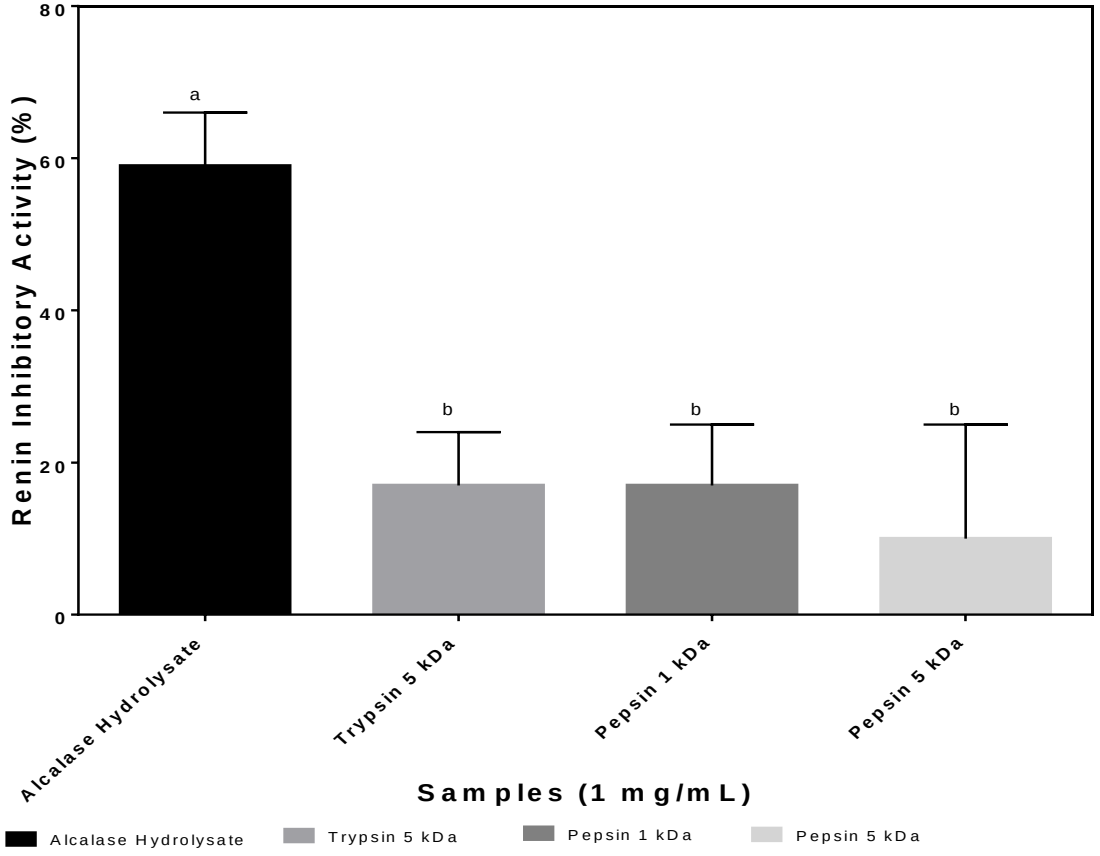


Fig. 6.3: Inhibition of renin by enzymatic bambara protein hydrolysate and membrane ultrafiltration fractions at a concentration of 1 mg/mL. Error bars (mean ± standard deviation, n = 3) with different alphabets have mean values that are significantly different ($p < 0.05$

6.4.6. Inhibition of linoleic acid peroxidation

The lipid peroxidation inhibition activities of GSH, BPI, BPHs and their membrane fractions were evaluated at 1 mg/mL using a linoleic acid system .The results obtained after 7 days of incubation indicated that addition of peptide inhibitors was effective in decreasing linoleic acid oxidation up till day 5 of the incubation for most of the samples except for alcalase peptide 1-3 kDa that was effective for the whole 7 days (Fig.6.4). The samples generally exhibited a significantly higher percentage inhibition ($p < 0.05$) on day 2 when compared to day 5. This may be attributed to the gradual loss in their ability to adequately reduce the linoleic peroxyl radical with continuous incubation, due to the slow formation of peroxidation of linoleic acid in the system. However, BPHs showed better linoleic acid oxidation inhibition when compared to peanut protein hydrolysates, which only showed inhibition activity at higher concentrations (Chen *et al.* 2007). The unhydrolysed BPI showed the least effective inhibition (53.3%) after day 5 of incubation while alcalase hydrolysate showed highest inhibition (82.7%) after 5 days of incubation. These results are comparable to those of previously reported studies from hempseed (Girgih *et al.* 2011) and rapeseed protein hydrolysates (He *et al.* 2013), which also showed effective inhibition of linoleic acid oxidation . In addition, our results showed that the BPHs had superior inhibition activity of linoleic acid oxidation when compared to Alcalase-digests of wheat gluten that lost inhibitory activity after 3 days at a higher concentration of 4 mg/mL (Zhu *et al.* 2010) . In lipids such as free and ester forms of polyunsaturated fatty acids, lipid peroxidation occurred through radical mediated abstraction of hydrogen atoms from methylene. This starts a sequence of reactions that produces aldehydes, ketones with odours and other potential toxic substances. As a result of this, inhibition of lipid peroxidation is an important indicator for measuring antioxidant activity of peptides.

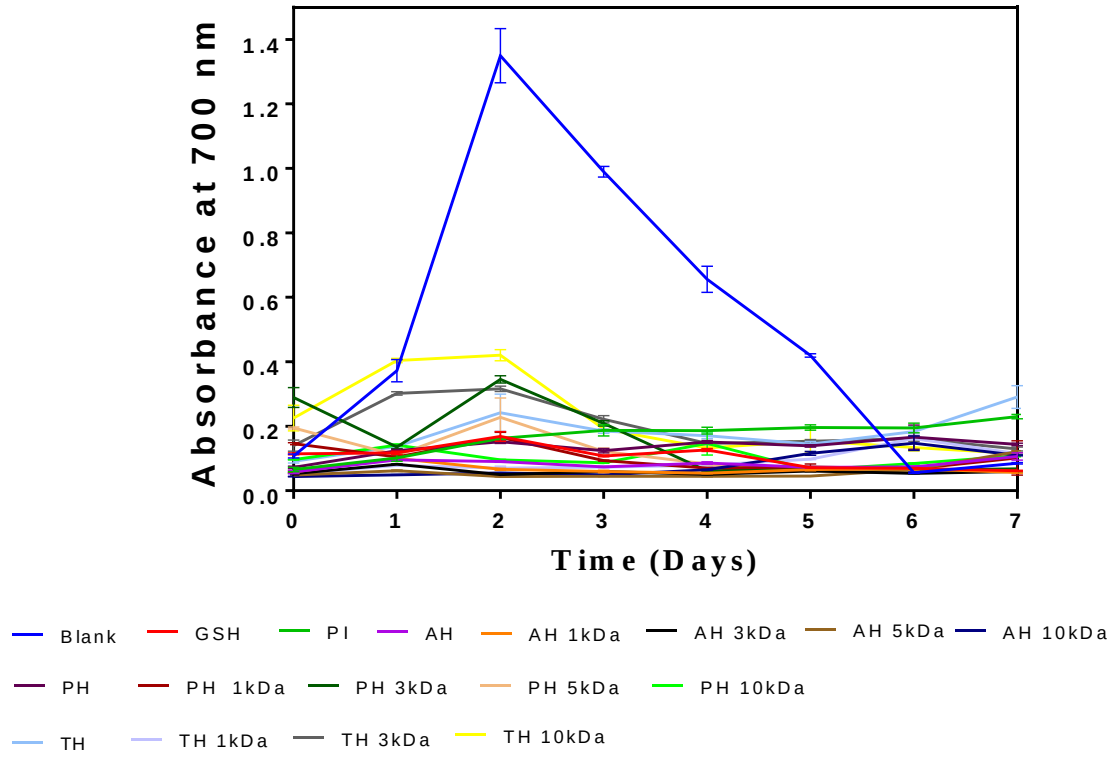


Fig. 6.4:.Inhibition of linoleic acid oxidation of bambara protein isolate (BPI), hydrolysates(BPHs) and membrane ultrafiltration peptide fractions measured over 7 days at 500 nm.

6.4.7. ABTS radical scavenging activity

Fig.6.5 shows that the $ABTS^{\cdot\bullet+}$ scavenging ability (EC_{50}) for peptide was significantly ($p < 0.05$) better (<25 µg/mL) when compared to that of BPI (84 µg/mL). This is because scavenging potency is inversely related to peptide EC_{50} value. However, glutathione reduced (GSH) had the lowest ($p < 0.05$) EC_{50} value of 1.8 µg/mL, which indicates highest radical scavenging potency among all the tested samples. Therefore, the BPHs have superior $ABTS^{\bullet+}$ scavenging ability when compared to the unhydrolysed BPI. The higher activity observed for the hydrolysates when compared to the isolates showed that antioxidant peptides were released from BPI during enzyme hydrolysis. This is an indication that the peptide was able to donate hydrogen atoms for $ABTS^{\bullet+}$ reduction. Generally, among the hydrolysates, trypsin hydrolysate was found to be better $ABTS^{\bullet+}$ scavengers with a low value of 22 µg/mL when compared to alcalase and pepsin hydrolysates. In addition, trypsin <1 kDa and 1-3 kDa fractions with value of 19.8 µg/mL was the most effective ($p<0.05$) $ABTS^{\bullet+}$ scavenger. The values obtained for the alcalase hydrolysate in this study is similar to that reported for Alcalase hydrolysed amaranth protein (22.4 µg/mL) and cocoa seed protein (Preza *et al.* 2010; Tironi and Añón 2010) but higher than that reported for canola alcalase hydrolysates

(Alashi *et al.* 2014). In general, there is no significant difference between the BPHs and peptide fractions.

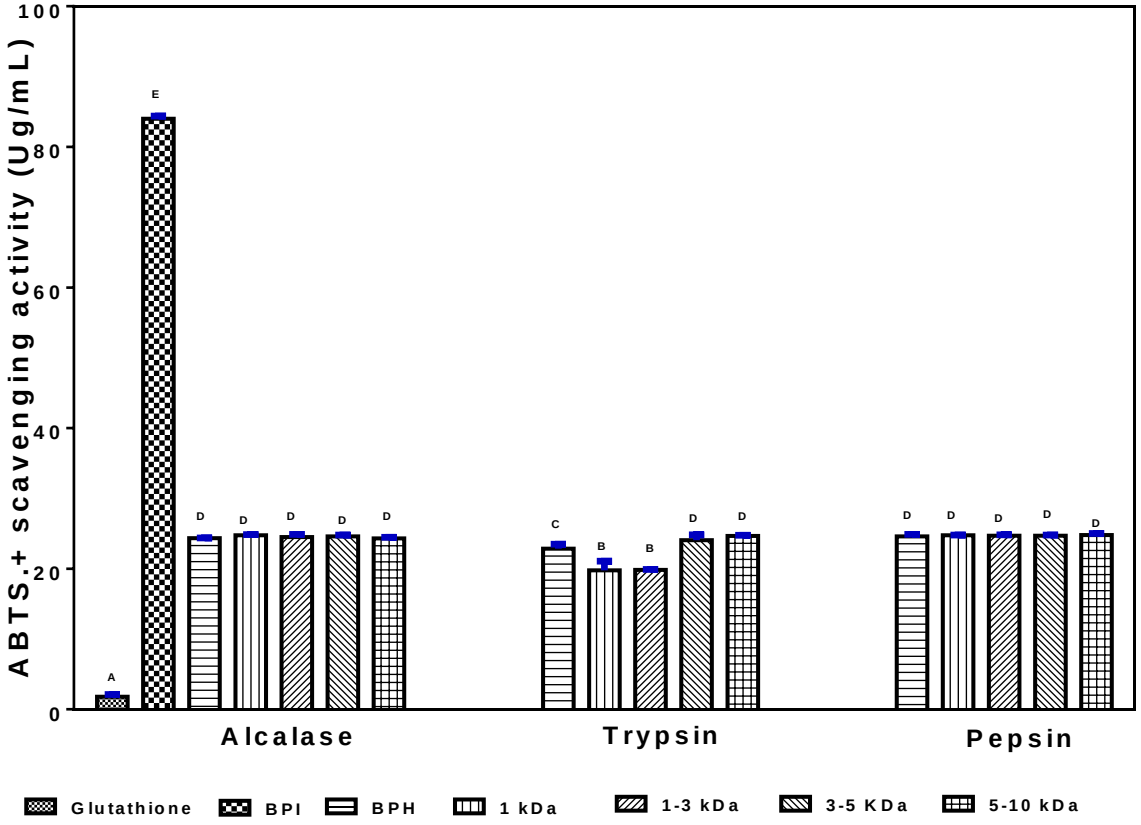


Fig.6.5. The effective concentration that scavenged 50% (EC50) values for ABTS$^{\bullet+}$ Scavenging activity values of bambara protein isolate (BPI), hydrolysates (BPHs) and membrane ultrafiltration peptide fractions

6.5. Conclusions

The <1 and 1-3 kDa fractions exhibited significantly higher (p<0.05) inhibition, as well as the ability to inhibit peroxidation of linoleic acid and scavenge ABTS$^{\bullet+}$. Therefore, the fractions with <1 and 1-3 kDa peptides showed a higher potential as antihypertensive and antioxidant peptides. However, it is also well known that smaller size peptides exhibit better bioactivity because of the higher possibility for increased rate of intestinal absorption (without structural degradation) and entry into cells when compared with the larger sized peptides. Therefore, *in vivo* tests will be required to confirm the observed enzyme inhibition and antioxidant properties of the BPHs and peptide fractions.

6.6. Research outputs

a) Journal article

1. **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Sunday A. Malomo, Rotimi E. Aluko and Eric O. Amonsou. Inhibitory properties of bambara protein hydrolysate and its membrane fractions against angiotensin converting enzymes, renin and free radicals Submitted to LWT-journal of Food Science and Technology.

b) Conference paper

2. **Abimbola K. Arise**, Adeola M. Alashi, Ifeanyi D. Nwachukwu, Sunday A. Malomo, Rotimi E. Aluko and Eric O. Amonsou. Inhibitory properties of bambara protein hydrolysate and its membrane fractions against angiotensin converting enzymes, renin and free radicals. Food Safety and Security 2016 Autumn Scientific Conference (FSaS), Johannesburg. South Africa, 16-18th May, 2016.

CHAPTER SEVEN

7. SUMMARY AND CONCLUSIONS

The first aspect of this study reveals the effect of extraction methods on functional properties of South African bambara groundnut protein landraces. The findings revealed that the method of extraction has influence on the yield, protein content and functional properties of bambara protein rather than the landrace. Acid precipitation produces bambara concentrates with high protein content and yield compared to concentrates prepared by salt solubilisation. Protein concentrates prepared through salt solubilisation method exhibited better functional properties. It also reveals that manipulation of pH could be used to increase foaming ability, which is dependent on protein solubility. This implies that protein extraction by salt solubilisation may be the most appropriate method for the enhanced functionality and utilisation of bambara groundnuts' protein concentrates.

The second study yields information on the composition and structure of bambara protein isolates landraces. It is interesting to note that all the landraces examined in this research had a similar composition and structural properties except for the protein spots that differ. Intrinsic fluorescence and CD data from this study revealed that bambara proteins existed in different conformational states within different pH environments. The study suggested that the effect of pH on protein functionality is dependent on protein concentration and also confirmed the high dependency of protein functionality on structural conformation as being affected by modulation in pH changes. CD data revealed that bambara proteins existed in different conformational states when evaluated at various pH values but with well- defined structure at pH 3.0. Molecular structure revealed two subunits of vicilin as the major storage proteins in bambara protein. In addition, DSC revealed two endothermic peak for bambara protein which results in high thermal stability. This suggests that the protein could be used as an ingredient in food systems in which heat treatment (but not protein denaturation) is a required process. Furthermore, bambara protein has potential as an ingredient for the production of food foams and emulsions due to their high foaming and emulsion capacity. The proteomic map of bambara was established for the first time and this also confirms vicilin as the major storage protein in bambara as the map is dominated with acidic spot which is characteristic of vicilin. Also different number of spots are obtained for each landraces.

Functional peptides are now an important category within the nutraceuticals food sector. Bioactive peptides could help in providing a solution to most of the life threatening diseases. The third and fourth phase of this study examined the structure-activity relationships of bambara protein hydrolysates and constituent amino acids as they relate to antioxidative and antihypertensive properties. The third phase described the production of enzymatic protein (Trypsin, Pepsin and Alcalase) hydrolysates from bambara protein and membrane separation of the hydrolysate by the use of specific molecular weight cut-offs. Protein hydrolysates derived from bambara protein possess antioxidant properties against a variety of physiologically relevant free radicals studied in vitro. High surface hydrophobicity and the molecular size of the peptide appear to be important for scavenging of hydroxyl radicals, ferric reducing power and metal chelation. The activity increased with an increase in peptide size except for DPPH which had higher activity with a smaller molecular size peptide. The activity of <1 kDa for pepsin hydrolysate was higher than that of GSH. Similarly, the peptides had better ferric reducing and metal chelating activities when compared to GSH except for the pepsin hydrolysate and its fractions that did not exhibit any metal chelating activity. The bambara protein hydrolysate and its membrane fractions may be suitable ingredients for the formulation of functional foods and nutraceuticals that can be used to prevent or manage oxidative stress.

To the best of our knowledge, the fourth study is the first to report the *in vitro* ACE and renin inhibition activities of bambara protein derived-peptides obtained from enzymatic hydrolysis of bambara proteins.This study showed that the low molecular weight peptides (MW <1 and 1-3 kDa) exhibited significantly higher (p<0.05) ACE- inhibition, as well as the ability to inhibit the peroxidation of linoleic acid and scavenge ABTS•+. Therefore, it is concluded that the fractions with <1 and 1-3 kDa peptides showed a high potential as antihypertensive and antioxidant peptides that can be used to prevent oxidation and as functional foods and nutraceutical ingredients.

Future work should include use of the isolated or purified bambara proteins in actual food formulations such as stabilization of food emulsions (salad dressings and mayonnaise, frozen desserts) and replacement of animal fat in meats to manufacture low-fat products. For the protein hydrolysates, it will be necessary for future work to identify and purify active peptides, including elucidation of amino acid sequence especially of the <1 and 1-3 kDa . Finally, *in vivo* activities of the protein hydrolysates and peptides need to be determined using

appropriate animal disease models such as the spontaneously hypertensive rats that can enable determination of blood pressure-lowering effects.

REFERENCES

Abioye, V., Olanipekun, B. and Omotosho, O. (2015). Effect of Varieties on the Proximate, Nutritional and Anti-nutritional Composition of Nine Variants of African Yam Bean Seeds (*Sphenostylis Stenocarpa*). *Donnish Journal of Food Science and Technology*, 1 (2): 17 -21.

Abugoch, L. E., Romero, N., Tapia, C. A., Silva, J. and Rivera, M. (2008). Study of some physicochemical and functional properties of quinoa (*Chenopodium quinoa* Willd) protein isolates. *Journal of Agricultural and Food Chemistry*, 56 (12): 4745-4750.

Adebiyi, A. P. and Aluko, R. E. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (*Pisum sativum* L.) seed protein isolate. *Food Chemistry*, 128 (4): 902-908.

Adebowale, K. and Lawal, O. (2003). Foaming, gelation and electrophoretic characteristics of mucuna bean (*Mucuna pruriens*) protein concentrates. *Food Chemistry*, 83 (2): 237-246.

Adebowale, Y. A., Adeyemi, I. A., Oshodi, A. A. and Niranjan, K. (2007). Isolation, fractionation and characterisation of proteins from mucuna bean. *Food Chemistry*, 104 (1): 287-299.

Adebowale, Y. A., Schwarzenbolz, U. and Henle, T. (2011). Protein isolates from bambara groundnut (*voandzeia subterranean L.*): chemical characterization and functional properties. *International Journal of Food Properties*, 14 (4): 758-775.

Adegbola, O. B. J. and Bamishaiye, E. (2011). Bambara groundnut: an Under-Utilized Nut in Africa. *Advances in Agricultural Biotechnology*, 1: 60-72.

Adegunwa, M., Adebowale, A., Bakare, H. and Kalejaiye, K. (2013). Effects of Treatments on the Antinutritional Factors and Functional Properties of Bambara Groundnut (*Voandzeia subterranea*) Flour. *Journal of Food Processing and Preservation*, 38 (4): 1875.

Ajibola, C. F., Fashakin, J. B., Fagbemi, T. N. and Aluko, R. E. (2011). Effect of peptide size on antioxidant properties of African yam bean seed (*Sphenostylis stenocarpa*) protein hydrolysate fractions. *International Journal of Molecular Sciences*, 12 (10): 6685-6702.

Ajibola, C. F., Fashakin, J. B., Fagbemi, T. N. and Aluko, R. E. (2013). Renin and angiotensin converting enzyme inhibition with antioxidant properties of African yam bean protein hydrolysate and reverse-phase HPLC-separated peptide fractions. *Food Research International*, 52 (2): 437-444.

Akintayo, E., Oshodi, A. and Esuoso, K. (1999). Effects of NaCl, ionic strength and pH on the foaming and gelation of pigeon pea (*Cajanus cajan*) protein concentrates. *Food Chemistry*, 66 (1): 51-56.

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., Girgih, A. and Aluko, R. E. (2014). Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chemistry*, 146: 500-506.

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., Malomo, S. A., Girgih, A. T. and Aluko, R. E. (2014). Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats. *Food Research International*, 55: 281-287.

Ali, N. A., Ahmed, S. H., Mohamed, E. A., Ahmed, I. A. M. and Babiker, E. E. (2010). Changes in functional properties by transglutaminase cross linking as a function of pH of legumes protein isolate. *Innovative Romanian Food Biotechnology*, 7: 12-20.

Alli, I., Gibbs, B. F., Okoniewska, M. K., Konishi, Y. and Dumas, F. (1993). Identification and characterization of phaseolin polypeptides in a crystalline protein isolated from white kidney beans (*Phaseolus vulgaris*). *Journal of Agricultural and Food Chemistry*, 41 (11): 1830-1834.

Aluko, R. and Yada, R. (1995). Structure-function relationships of cowpea (*Vigna unguiculata*) globulin isolate: influence of pH and NaCl on physicochemical and functional properties. *Food Chemistry*, 53 (3): 259-265.

Aluko, R. E. (2015). Antihypertensive Peptides from Food Proteins. *Annual Review of Food Science and Technology*, 6: 235-262.

Aluko, R. E. (2015). Structure and function of plant protein-derived antihypertensive peptides. *Current Opinion in Food Science*, 4: 44-50.

Aluko, R. E. and McIntosh, T. (2001). Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. *Journal of the Science of Food and Agriculture*, 81 (4): 391-396.

Amonsou, E. O. 2010. Characterisation of marama bean protein. PhD Thesis (Unpubished). University of Pretoria.

Amonsou, E. O., Siwela, M. and Dlamini, N. (2014). Chemical composition and microstructure of Bauhinia grains. *Journal of Food Science and Technology*, 51 (9): 2263-2269.

Amonsou, E. O., Taylor, J., Beukes, M. and Minnaar, A. (2012). Composition of marama bean protein. *Food Chemistry*, 130 (3): 638-643.

Anwer, K., Rahman, S., Sonani, R., Khan, F. I., Islam, A., Madamwar, D., Ahmad, F. and Hassan, M. I. (2016). Probing pH sensitivity of αC-phycoerythrin and its natural truncant: A comparative study. *International Journal of Biological Macromolecules*,

AOAC. 2000. *Official methods of analysis (17th ed.)*. Rockville Maryland: Association of Official Analytical Chemists.

Arise, A. K., Amonsou, E. O. and Ijabadeniyi, O. A. (2015). Influence of extraction methods on functional properties of protein concentrates prepared from South African bambara groundnut landraces. *International Journal of Food Science and Technology*, 50: 1095-1101.

Arts, M. J., Dallinga, J. S., Voss, H.-P., Haenen, G. R. and Bast, A. (2004). A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chemistry*, 88 (4): 567-570.

Avanza, M., Acevedo, B., Chaves, M. and Anon, M. (2013). Nutritional and anti-nutritional components of four cowpea varieties under thermal treatments: Principal component analysis. *LWT-Food Science and Technology*, 51 (1): 148-157.

Barać, M. and Stanojević, S. (2005). The effect of microwave roasting on soybean protein composition and components with trypsin inhibitor activity. *Acta Alimentaria*, 34 (1): 23-31.

Barac, M. B., Pesic, M. B., Stanojevic, S. P., Kostic, A. Z. and Bivolarevic, V. (2015). Comparative study of the functional properties of three legume seed isolates: adzuki, pea and soy bean. *Journal of Food Science and Technology*, 52 (5): 2779-2787.

Basu, S., Roberts, J., AZAM-ALI, S. and Mayes, S. (2007). Development of microsatellite markers for bambara groundnut (*Vigna subterranea L. Verdc*.)—an underutilized African legume crop species. *Molecular Ecology Notes*, 7 (6): 1326-1328.

Belova, L. (2000). Angiotensin II-generating enzymes. *Biochemistry (Moscow)*, 65 (12): 1337-1345.

Benjakul, S., Visessanguan, W. and Thummaratwasik, P. (2000). Isolation and characterization of trypsin inhibitors from some Thai legume seeds. *Journal of Food Biochemistry*, 24 (2): 107-127.

Bidlingmeyer, B., Cohen, S. and Tarvin, T. (1984). The PICO-TAG method for amino acid determination. *Journal of Chromatography*, 33: 93-104.

Boateng, M. A., Addo, J. K., Okyere, H., Adu-Dapaah, H., Berchie, J. N. and ATetteh. (2013). Physicochemical and functional properties of proteinates of two bambara groundnut (*vigna substerran)* landraces. *African Journal of Food Science and Technology*, 4 (4): 64-70.

Bora, P., Brekke, C. J. and Powers, J. R. (1994). Heat induced gelation of pea (Pisum sativum) mixed globulins, vicilin and legumin. *Journal of Food Science*, 59 (3): 594-596.

Boye, J., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E. and Rajamohamed, S. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, 43 (2): 537-546.

Boye, J., Zare, F. and Pletch, A. (2010). Pulse proteins: processing, characterization, functional properties and applications in food and feed. *Food Research International*, 43 (2): 414-431.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72 (1): 248-254.

Brough, S., Azam-Ali, S. and Taylor, A. (1993). The potential of bambara groundnut (Vigna subterranea) in vegetable milk production and basic protein functionality systems. *Food Chemistry*, 47 (3): 277-283.

Carrasco-Castilla, J., Hernández-Álvarez, A. J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, J., Vioque, J. and Dávila-Ortiz, G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. *Food Chemistry*, 135 (3): 1789-1795.

Castel, V., Andrich, O., Netto, F. M., Santiago, L. G. and Carrara, C. R. (2012). Comparison between isoelectric precipitation and ultrafiltration processes to obtain *Amaranth mantegazzianus* protein concentrates at pilot plant scale. *Journal of Food Engineering*, 112 (4): 288-295.

Chen, G. t., Zhao, L., Zhao, L. y., Cong, T. and Bao, S. f. (2007). In vitro study on antioxidant activities of peanut protein hydrolysate. *Journal of the Science of Food and Agriculture*, 87 (2): 357-362.

Chen, H.-M., Muramoto, K. and Yamauchi, F. (1995). Structural analysis of antioxidative peptides from Soybean. beta.-Conglycinin. *Journal of Agricultural and Food chemistry*, 43 (3): 574-578.

Chen, H.-M., Muramoto, K., Yamauchi, F., Fujimoto, K. and 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, Y.-h., Wang, Z. and Xu, S.-y. (2006). Antioxidant properties of wheat germ protein hydrolysates evaluated in vitro. *Journal of Central South University of Technology*, 13 (2): 160-165.

Chinedu, S. N. and Nwinyi, C. O. (2012). Proximate analysis of Sphenostylis stenocarpa and Voadzeia subterranean consumed in South Eastern Nigeria. *Journal of Agricultural Extension and Rural Development*, 4 (3): 57-62.

Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M., Lopes, F. C., Segalin, J., Risso, P. H. and Brandelli, A. (2011). Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. *Journal of the Science of Food and Agriculture*, 91 (12): 2247-2254.

Cui, C., Zhao, M., Yuan, B., Zhang, Y. and Ren, J. (2013). Effect of pH and pepsin limited hydrolysis on the structure and functional properties of soybean protein hydrolysates. *Journal of Food Science*, 78 (12): C1871-C1877.

Dahiya, P., Linnemann, A., Nout, M., van Boekel, M. and Grewal, R. (2013). Nutrient composition of selected newly bred and established mung bean varieties. *LWT-Food Science and Technology*, 54 (1): 249-256.

Daien, V., Duny, Y., Ribstein, J., Du Cailar, G., Mimran, A., Villain, M., Daures, J. P. and 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.

Damodaran, S. and Parkin, K. L. 2008. *Fennema's food chemistry*. CRC press Boca Raton, FL.

De la Caba, K., Pena, C., Ciannamea, E., Stefani, P., Mondragon, I. and Ruseckaite, R. (2012). Characterization of soybean protein concentrate—stearic acid/palmitic acid blend edible films. *Journal of Applied Polymer Science*, 124 (3): 1796-1807.

Deng, Q., Wang, L., Wei, F., Xie, B., Huang, F., Huang, W., Shi, J., Huang, Q., Tian, B. and Xue, S. (2011). Functional properties of protein isolates, globulin and albumin extracted from *Ginkgo biloba* seeds. *Food Chemistry*, 124 (4): 1458-1465.

Durak, A., Baraniak, B., Jakubczyk, A. and Świeca, M. (2013). Biologically active peptides obtained by enzymatic hydrolysis of Adzuki bean seeds. *Food Chemistry*, 141 (3): 2177-2183.

Dziuba, J., Szerszunowicz, I., Nałęcz, D. and Dziuba, M. (2014). Proteomic Analysis of Albumin and Globulin fractions of pea (*Pisum sativum L.*) Seeds. *Acta Scientiarium Polonorum Technology Aliment*, 13 (2): 181-190.

El Nasri, N. A. and El Tinay, A. (2007). Functional properties of fenugreek (*Trigonella foenum graecum*) protein concentrate. *Food Chemistry*, 103 (2): 582-589.

Elhardallou, S. B., Khalid, I. I., Gobouri, A. A. and Abdel-Hafez, S. H. (2015). Amino Acid Composition of Cowpea (*Vigna ungiculata L.* Walp) Flour and Its Protein Isolates. *Food and Nutrition Sciences*, 6 (9): 790.

Eltayeb, A. R. S., Ali, A. O., Abou-Arab, A. A. and Abu-Salem, F. M. (2011). Chemical composition and functional properties of flour and protein isolate extracted from Bambara groundnut (*Vigna subterranean*). *African Journal of Food Science*, 5 (2): 82-90.

Falade, K. O., Ogundele, O. M., Ogunshe, A. O., Fayemi, O. E. and Ocloo, F. C. (2014). Physico-chemical, sensory and microbiological characteristics of plain yoghurt from bambara groundnut (*Vigna subterranea*) and soybeans (*Glycine max*). *Journal of Food Science and Technology*: 1-8.

FAO/WHO. 1989. *Protein quality evaluation. Report of the joint FAO/WHO expert consultation. Food and nutrition No.51*. Rome: Food and Agriculture Organizations and the World Health Organization.

Feyzi, S., Varidi, M., Zare, F. and Varidi, M. J. (2015). Fenugreek (*Trigonella foenum graecum*) seed protein isolate: extraction optimization, amino acid composition, thermo and functional properties. *Journal of the Science of Food and Agriculture*,

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

Forman-Kay, J. D. and Mittag, T. (2013). From sequence and forces to structure, function, and evolution of intrinsically disordered proteins. *Structure*, 21 (9): 1492-1499.

Gehrke, C. W., Wall Sr, L., Absheer, J., Kaiser, F. and Zumwalt, R. (1985). Sample preparation for chromatography of amino acids: acid hydrolysis of proteins. *Journal of the Association of Offical Analytical Chemists (USA)*, 68: 811-821.

Girgih, A. T., Udenigwe, C. C. and 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.

Goli, A., Begemann, F. and Ng, N. 1997. Characterization and evaluation of IITA's Bambara groundnut collection. In: Proceedings of *Proceedings of the workshop on conservation and improvement of bambara groundnut*. 101-118.

Gomes, L. S., Senna, R., Sandim, V., Silva-Neto, M. r. A., Perales, J. E., Zingali, R. B., Soares, M. r. R. and Fialho, E. (2014). Four conventional soybean [*Glycine max* (L.) Merrill]

seeds exhibit different protein profiles as revealed by proteomic analysis. *Journal of Agricultural and Food Chemistry*, 62 (6): 1283-1293.

Graham, D. and Phillips, M. (1976). The conformation of proteins at the air-water interface and their role in stabilizing foams. *Foams*: 237-255.

Harnedy, P. A. and FitzGerald, R. J. (2013). In vitro assessment of the cardioprotective, anti-diabetic and antioxidant potential of Palmaria palmata protein hydrolysates. *Journal of Applied Phycology*, 25 (6): 1793-1803.

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

He, R., Malomo, S. A., Alashi, A., Girgih, A. T., Ju, X. and Aluko, R. E. (2013). Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides. *Journal of Functional Foods*, 5 (2): 781-789.

Hernández-Álvarez, A. J., Carrasco-Castilla, J., Dávila-Ortiz, G., Alaiz, M., Girón-Calle, J., Vioque-Peña, J., Jacinto-Hernández, C. and Jiménez-Martínez, C. (2013). Angiotensin-converting enzyme-inhibitory activity in protein hydrolysates from normal and anthracnose disease-damaged Phaseolus vulgaris seeds. *Journal of the Science of Food and Agriculture*, 93 (4): 961-966.

Hillocks, R., Bennett, C. and Mponda, O. (2012). Bambara nut: A review of utilisation, market potential and crop improvement. *African Crop Science Journal*, 20 (1): 1-16.

Hirano, H., Kagawa, H. and Okubo, K. (1992). Characterization of proteins released from legume seeds in hot water. *Phytochemistry*, 31 (3): 731-735.

Hojilla-Evangelista, M. P., Selling, G. W., Berhow, M. A. and Evangelista, R. L. (2014). Preparation, composition and functional properties of pennycress (*Thlaspi arvense L.*) seed protein isolates. *Industrial Crops and Products*, 55: 173-179.

Huang, W.-Y., Majumder, K. and Wu, J. (2010). Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals. *Food Chemistry*, 123 (3): 635-641.

Ijarotimi, O. S. and Esho, T. R. (2009). Comparison of nutritional composition and anti-nutrient status of fermented, germinated and roasted bambara groundnut seeds (*vigna subterranea*). *British Food Journal*, 111 (4): 376-386.

Jamdar, S., Rajalakshmi, V., Pednekar, M., Juan, F., Yardi, V. and Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chemistry*, 121 (1): 178-184.

Jideani, V. A. and Diedericks, C. F. 2014. *Nutritional, Therapeutic, and Prophylactic Properties of Vigna subterranea and Moringa oleifera*. INTECH.pp. 178 - 207. Available: http://dx.doi.org/10.5772/57338 (Accessed 19 June 2016).

Jimsheena, V. and Gowda, L. R. (2010). Arachin derived peptides as selective angiotensin I-converting enzyme (ACE) inhibitors: structure–activity relationship. *Peptides*, 31 (6): 1165-1176.

Joshi, M., Adhikari, B., Aldred, P., Panozzo, J. and Kasapis, S. (2011). Physicochemical and functional properties of lentil protein isolates prepared by different drying methods. *Food Chemistry*, 129 (4): 1513-1522.

Kang, M. G. and Ahn, B. H. (2012). Characterization of new antihypertensive angiotensin I-converting enzyme inhibitory peptides from Korean traditional rice wine. *Journal of Microbiology and Biotechnology*, 22 (3): 339-342.

Karaca, A. C., Low, N. and Nickerson, M. (2011). Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. *Food Research International*, 44 (9): 2991-2998.

Kelly, S. M., Jess, T. J. and Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1751 (2): 119-139.

Kim, S.-Y., Je, J.-Y. and Kim, S.-K. (2007). Purification and characterization of antioxidant peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry*, 18 (1): 31-38.

Kudre, T. G. and Benjakul, S. (2014). Physicochemical and functional properties of beany flavour-free bambara groundnut protein isolate. *Journal of the Science of Food and Agriculture*, 94 (6): 1238-1247.

Kudre, T. G., Benjakul, S. and Kishimura, H. (2013). Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. *Journal of the Science of Food and Agriculture*, 93 (10): 2429-2436.

Kumar, A., Prasad, N. and Sinha, S. K. (2015). Nutritional and antinutritional attributes of faba bean (Vicia faba L.) germplasms growing in Bihar, India. *Physiology and Molecular Biology of Plants*, 21 (1): 159-162.

Kwon, D. Y., Daily III, J. W., Kim, H. J. and Park, S. (2010). Antidiabetic effects of fermented soybean products on type 2 diabetes. *Nutrition Research*, 30 (1): 1-13.

Landry, J. and 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.

Latif, S., Pfannstiel, J., Makkar, H. and Becker, K. (2013). Amino acid composition, antinutrients and allergens in the peanut protein fraction obtained by an aqueous enzymatic process. *Food Chemistry*, 136 (1): 213-217.

Lawal, O., Adebowale, K. and Adebowale, Y. (2007). Functional properties of native and chemically modified protein concentrates from bambarra groundnut. *Food Research International*, 40 (8): 1003-1011.

Li, H. and Aluko, R. E. (2006). Structural modulation of calmodulin and calmodulin-dependent protein kinase II by pea protein hydrolysates. *International Journal of Food Sciences and Nutrition*, 57 (3-4): 178-189.

Li, H. and Aluko, R. E. (2010). Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. *Journal of Agricultural and Food Chemistry*, 58 (21): 11471-11476.

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

Liu, B., Peng, J., Zhang, S., Zou, B. and Zhong, G. (2013). Chemical composition, in vitro starch digestibility and amino acid analysis of a underexplored kidney bean (*Phaseolus vulgaris*. L.) grown in Chongqing, China. *International Journal of Food Science & Technology*, 48 (3): 527-532.

López-Barrios, L., Gutiérrez-Uribe, J. A. and Serna-Saldívar, S. O. (2014). Bioactive Peptides and Hydrolysates from Pulses and Their Potential Use as Functional Ingredients. *Journal of Food Science*, 79 (3): R273-R283.

Mabhaudhi, T. and Modi, A. T. (2013). Growth, phenological and yield responses of a bambara groundnut (*Vigna subterranea* (L.) Verdc.) landrace to imposed water stress under field conditions. *South African Journal of Plant and Soil*, 30 (2): 69-79.

Mahala, A. G. and Mohammed, A. A. A. (2010). Nutritive evaluation of bambara groundnut (*Vigna subterranean*) pods, seeds and Hulls as Animal Feeds. *Journal of Applied Science and Research*, 6 (5): 383-386.

Mäkinen, S., Johannson, T., Gerd, E. V., Pihlava, J. M. and Pihlanto, A. (2012). Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. *Journal of Functional Foods*, 4 (3): 575-583.

Malomo, S. (2015). Structure-function properties of hemp seed proteins and protein-derived acetylcholinesterase-inhibitory peptides. PhD Thesis. University of Manitoba.

Malomo, S. A. and Aluko, R. E. (2015). A comparative study of the structural and functional properties of isolated hemp seed (*Cannabis sativa* L.) albumin and globulin fractions. *Food Hydrocolloids*, 43: 743-752.

Malomo, S. A., He, R. and Aluko, R. E. (2014). Structural and Functional Properties of Hemp Seed Protein Products. *Journal of Food Science*, 79 (8): C1512-C1521.

Malomo, S. A., Onuh, J. O., Girgih, A. T. and Aluko, R. E. (2015). Structural and Antihypertensive Properties of Enzymatic Hemp Seed Protein Hydrolysates. *Nutrients*, 7 (9): 7616-7632.

Mao, X. and Hua, Y. (2012). Composition, structure and functional properties of protein concentrates and isolates produced from walnut (*Juglans regia* L.). *International Journal of Molecular Sciences*, 13 (2): 1561-1581.

Marambe, H. K., Shand, P. J. and Wanasundara, J. P. (2013). In vitro digestibility of flaxseed (*Linum usitatissimum* L.) protein: effect of seed mucilage, oil and thermal processing. *International Journal of Food Science & Technology*, 48 (3): 628-635.

Markwell, M. A. K., Haas, S. M., Bieber, L. and Tolbert, N. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry*, 87 (1): 206-210.

Marques, M. R., Fontanari, G. G., Pimenta, D. C., Soares-Freitas, R. M. and Arêas, J. A. G. (2015). Proteolytic hydrolysis of cowpea proteins is able to release peptides with hypocholesterolemic activity. *Food Research International*,

Marsh, J. A. and Teichmann, S. A. (2014). Parallel dynamics and evolution: protein conformational fluctuations and assembly reflect evolutionary changes in sequence and structure. *BioEssays*, 36 (2): 209-218.

Mazahib, A., Nuha, M., Salawa, I. and Babiker, E. (2013). Some nutritional attributes of bambara groundnut as influenced by domestic processing. *International Food Research Journal*, 20 (3): 1165-1171.

Medina-Godoy, S., Ambriz-Pérez, D. L., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., Reyes-Moreno, C. and Valdez-Ortiz, A. (2012). Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard-to-cook chickpeas. *Journal of the Science of Food and Agriculture*, 92 (9): 1974-1981.

Mine, Y., Li-Chan, E. C. and Jiang, B. (2010). Biologically active food proteins and peptides in health: an overview. *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals*: 3-11.

Moure, A., Domínguez, H. and Parajó, J. C. (2006). Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochemistry*, 41 (2): 447-456.

Moyano, F. J., Saénz de Rodrigáñez, M. A., Díaz, M. and Tacon, A. G. (2015). Application of in vitro digestibility methods in aquaculture: constraints and perspectives. *Reviews in Aquaculture*, 7 (4): 223-242.

Mujoo, R., Trinh, D. T. and Ng, P. K. (2003). Characterization of storage proteins in different soybean varieties and their relationship to tofu yield and texture. *Food Chemistry*, 82 (2): 265-273.

Mundi, S. 2012. Structural and functional characterization of red kidney bean (Phaseolus vulgaris) proteins and enzymatic protein hydrolysates. University of Manitoba.

Mundi, S. and Aluko, R. (2012). Physicochemical and functional properties of kidney bean albumin and globulin protein fractions. *Food Research International*, 48 (1): 299-306.

Mundi, S. and Aluko, R. E. (2013). Effects of NaCl and pH on the structural conformations of kidney bean vicilin. *Food Chemistry*, 139 (1): 624-630.

Mundi, S. and 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): 1008.

Mune, M., Minka, S. R., Mbome, I. L. and Etoa, F. (2011). Nutritional potential of Bambara bean protein concentrate. *Pakistan Journal of Nutrition*, 10 (2): 112-119.

Mune Mune, M. A. and Sogi, D. S. (2015). Functional Properties of Protein Concentrates of Cowpea and Bambara Bean Involving Different Drying Techniques. *Journal of Food Processing and Preservation*, 39 (6): 2304-2313.

Murevanhema, Y. and Jideani, V. (2014). Production and Characterization of Milk Produced from Bambara Groundnut (*Vigna Subterranea)* Varieties. *Journal of Food Processing and Preservation*,

Murevanhema, Y. Y. and Jideani, V. A. (2013). Potential of Bambara Groundnut (*Vigna subterranea* (L.) Verdc) Milk as a Probiotic Beverage—A Review. *Critical reviews in food science and nutrition*, 53 (9): 954-967.

Mwasaru, M. A., Muhammad, K., Bakar, J. and Man, Y. B. C. (1999). Effects of isolation technique and conditions on the extractability, physicochemical and functional properties of pigeonpea (*Cajanus cajan*) and cowpea (*Vigna unguiculata*) protein isolates. I. Physicochemical properties. *Food Chemistry*, 67 (4): 435-443.

Nakahara, T., Sugimoto, K., Sano, A., Yamaguchi, H., Katayama, H. and Uchida, R. (2011). Antihypertensive Mechanism of a Peptide-Enriched Soy Sauce-Like Seasoning: The Active

Constituents and Its Suppressive Effect on Renin–Angiotensin–Aldosterone System. *Journal of Food Science*, 76 (8): H201-H206.

Natarajan, S., Xu, C., Caperna, T. J. and Garrett, W. M. (2005). Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. *Analytical Biochemistry*, 342 (2): 214-220.

Natarajan, S. S., Pastor-Corrales, M., Khan, F. H. and Garrett, W. M. (2013). Proteomic Analysis of Common Bean (Phaseolus vulgaris L.) by Two-Dimensional Gel Electrophoresis and Mass Spectrometry. *Journal of Basic and Applied Sciences*, 9: 424-437.

Natarajan, S. S., Xu, C., Bae, H., Caperna, T. J. and Garrett, W. M. (2006). Characterization of storage proteins in wild (*Glycine soja*) and cultivated (*Glycine max*) soybean seeds using proteomic analysis. *Journal of Agricultural and Food Chemistry*, 54 (8): 3114-3120.

Nishinari, K., Fang, Y., Guo, S. and Phillips, G. (2014). Soy proteins: a review on composition, aggregation and emulsification. *Food Hydrocolloids*, 39: 301-318.

Nosenko, T., Kot, T. and Kichshenko, V. (2014). Rape seeds as a source of feed and food proteins. *Polish Journal of Food and Nutrition Sciences*, 64 (2): 109-114.

Odeigah, P. and Osanyinpeju, A. (1998). Evaluating the genetic biodiversity of Bambara groundnut accessions from Nigeria using SDS-polyacrylamide gel electrophoresis. *Genetic Resources and Crop Evolution*, 45 (5): 451-458.

Okafor, J., Okafor, G., Leelavathi, K., Bhagya, S. and Elemo, G. (2015). Effect of Roasted Bambara Groundnut (Voandzeia subterranea) Fortification on Quality and Acceptability of Biscuits. *Pakistan Journal of Nutrition*, 14 (10): 653-657.

Okezie, B. O. and Bello, A. (1988). Physicochemical and functional properties of winged bean flour and isolate compared with soy isolate. *Journal of Food Science*, 53 (2): 450-454.

Olalekan, A. J. and Bosede, B. F. (2010). Comparative study on chemical composition and functional properties of three Nigerian legumes (Jack beans, Pigmeon pea and Cowpea). *Journal of Emerging Trends in Engineering and Applied Sciences*, 1: 89-95.

Omoni, A. O. and Aluko, R. E. (2006). Effect of cationic flaxseed protein hydrolysate fractions on the in vitro structure and activity of calmodulin-dependent endothelial nitric oxide synthase. *Molecular Nutrition and Food Research*, 50 (10): 958-966.

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

Osborne, T. B. (1924). The vegetable proteins. (2nd edition). New York: Longman

Pain, R. (2003). Determining the CD spectrum of a protein. *Current Protocols in Food Analytical Chemistry*, B 3.5.1-B3.5.25.

Pan, M., Jiang, T. S. and Pan, J. L. (2011). Antioxidant activities of rapeseed protein hydrolysates. *Food and Bioprocess Technology*, 4 (7): 1144-1152.

Paredes-López, O., Ordorica-Falomir, C. and Olivares-Vázquez, M. (1991). Chickpea protein isolates: physicochemical, functional and nutritional characterization. *Journal of Food Science*, 56 (3): 726-729.

Park, S. J., Kim, T. W. and Baik, B. K. (2010). Relationship between proportion and composition of albumins, and in vitro protein digestibility of raw and cooked pea seeds (*Pisum sativum* L.). *Journal of the Science of Food and Agriculture*, 90 (10): 1719-1725.

Pastor-Cavada, E., Juan, R., Pastor, J. E., Alaiz, M. and Vioque, J. (2014). Protein and amino acid composition of select wild legume species of tribe Fabeae. *Food Chemistry*, 163: 97-102.

Paul, M. and Somkuti, G. A. (2010). Hydrolytic breakdown of lactoferricin by lactic acid bacteria. *Journal of Industrial Microbiology and Biotechnology*, 37 (2): 173-178.

Pedroche, J., Yust, M., Lqari, H., Girón-Calle, J., Alaiz, M., Vioque, J. and Millán, F. (2004). Brassica carinata protein isolates: chemical composition, protein characterization and improvement of functional properties by protein hydrolysis. *Food Chemistry*, 88 (3): 337-346.

Peyrano, F., Speroni, F. and Avanza, M. (2015). Physicochemical and functional properties of cowpea protein isolates treated with temperature or high hydrostatic pressure. *Innovative Food Science and Emerging Technologies*, 33: 38-46.

Pownall, T. L., Udenigwe, C. C. and 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.

Preza, A. M., Jaramillo, M. E., Puebla, A. M., Mateos, J. C., Hernández, R. and Lugo, E. (2010). Antitumor activity against murine lymphoma L5178Y model of proteins from cacao (*Theobroma cacao L*.) seeds in relation with in vitro antioxidant activity. *BMC Complementary and Alternative Medicine*, 10 (1): 61.

Ptaszek, P. (2013). The non-linear rheological properties of fresh wet foams based on egg white proteins and selected hydrocolloids. *Food Research International*, 54 (1): 478-486.

Qayyum, M., Butt, M., Anjum, F. and Nawaz, H. (2012). Composition analysis of some selected legumes for protein isolates recovery. *The Journal of Animal and Plant Sciences*, 22: 1156-1162.

Ragab, D. M., Babiker, E. E. and Eltinay, A. H. (2004). Fractionation, solubility and functional properties of cowpea (*Vigna unguiculata*) proteins as affected by pH and/or salt concentration. *Food Chemistry*, 84 (2): 207-212.

Rajapakse, N., Mendis, E., Jung, W.-K., Je, J.-Y. and Kim, S.-K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International*, 38 (2): 175-182.

Rangel, A., Domont, G. B., Pedrosa, C. and Ferreira, S. T. (2003). Functional properties of purified vicilins from cowpea (Vigna unguiculata) and pea (Pisum sativum) and cowpea protein isolate. *Journal of Agricultural and Food Chemistry*, 51 (19): 5792-5797.

Rebholz, C., Reynolds, K., Wofford, M., Chen, J., Kelly, T., Mei, H., Whelton, P. and He, J. (2012). Effect of soybean protein on novel cardiovascular disease risk factors: a randomized controlled trial. *European Journal of Clinical Nutrition*, 67 (1): 58-63.

Rho, S. J., Lee, J.-S., Chung, Y. I., Kim, Y.-W. and Lee, H. G. (2009). Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract. *Process Biochemistry*, 44 (4): 490-493.

Rubio, L. A., Pérez, A., Ruiz, R., Guzman, M., Aranda-Olmedo, I. and Clemente, A. (2014). Characterization of pea (*Pisum sativum*) seed protein fractions. *Journal of the Science of Food and Agriculture*, 94 (2): 280-287.

Rui, X., Boye, J. I., Ribereau, S., Simpson, B. K. and Prasher, S. O. (2011). Comparative study of the composition and thermal properties of protein isolates prepared from nine Phaseolus vulgaris legume varieties. *Food Research International*, 44 (8): 2497-2504.

Ruiz-ruiz, j., dávila-ortíz, g., chel-guerrero, l. and betancur-ancona, d. (2013). Angiotensin i-converting enzyme inhibitory and antioxidant peptide fractions from hard-to-cook bean enzymatic hydrolysates. *Journal of Food Biochemistry*, 37 (1): 26-35.

Sanjeewa, W. T., Wanasundara, J. P., Pietrasik, Z. and Shand, P. J. (2010). Characterization of chickpea (Cicer arietinum L.) flours and application in low-fat pork bologna as a model system. *Food Research International*, 43 (2): 617-626.

Sari, Y. W., Bruins, M. E. and Sanders, J. P. (2013). Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Industrial Crops and Products*, 43: 78-83.

Sarmadi, B. H. and Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides*, 31 (10): 1949-1956.

Sathe, S. (2002). Dry bean protein functionality. *Critical Reviews in Biotechnology*, 22 (2): 175-223.

Segura Campos, M. R., Chel Guerrero, L. A. and Betancur Ancona, D. A. (2010). Angiotensin-I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted by ultrafiltration of cowpea *Vigna unguiculata* hydrolysates. *Journal of the Science of Food and Agriculture*, 90 (14): 2512-2518.

Sharp, S. I., Aarsland, D., Day, S., Sønnesyn, H. and Ballard, C. (2011). Hypertension is a potential risk factor for vascular dementia: systematic review. *International Journal of Geriatric Psychiatry*, 26 (7): 661-669.

Shegro, A., van Rensburg, W. and Adebola, P. (2013). Assessment of genetic variability in bambara groundnut (*Vigna subterrenea* L. Verdc.) using morphological quantitative traits. *Academia Journal of Agricultural Research*, 1 (3): 045-051.

Shevkani, K., Kaur, A., Kumar, S. and Singh, N. (2015). Cowpea protein isolates: Functional properties and application in gluten-free rice muffins. *LWT-Food Science and Technology*, 63 : 927-933.

Shevkani, K., Singh, N., Kaur, A. and Rana, J. C. (2015). Structural and functional characterization of kidney bean and field pea protein isolates: a comparative study. *Food Hydrocolloids*, 43: 679-689.

Shevkani, K., Singh, N., Rana, J. C. and Kaur, A. (2014). Relationship between physicochemical and functional properties of amaranth (Amaranthus hypochondriacus) protein isolates. *International Journal of Food Science and Technology*, 49 (2): 541-550.

Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D. and Nickerson, M. T. (2015). Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. *Food Research International*, 76: 31-38.

Szabo, A. G. (2000). Fluorescence principles and measurement. *Spectrophotometry & Spectrofluorimetry, Oxford University Press, Oxford*: 33.

Tan, E.-S., Ying-Yuan, N. and Gan, C.-Y. (2014). A comparative study of physicochemical characteristics and functionalities of pinto bean protein isolate (PBPI) against the soybean protein isolate (SPI) after the extraction optimisation. *Food Chemistry*, 152: 447-455.

Tan, S. H., Mailer, R. J., Blanchard, C. L. and Agboola, S. O. (2011). Canola proteins for human consumption: extraction, profile, and functional properties. *Journal of Food Science*, 76 (1): R16-R28.

Tang, C.-H. (2015). Emulsifying Properties of Soy Proteins: A Critical Review with Emphasis on the Role of Conformational Flexibility. *Critical reviews in food science and nutrition*, (just-accepted): 10.1080/10408398.2015.1067594

Tang, C.-H., Wang, X.-S. and 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.

Tchiagam, L., Bell, J. M., Nassourou, A. M., Njintang, N. Y. and Youmbi, E. (2013). Genetic analysis of seed proteins contents in cowpea (*Vigna unguiculata*. L. Walp.). *African Journal of Biotechnology*, 10 (16): 3077-3086.

Teixeira, D. C., Farias, D. F., Carvalho, A. F. U., Arantes, M. R., Oliveira, J. T. A., Sousa, D. O. B., Pereira, M. L., Oliveira, H. D., Andrade-Neto, M. and Vasconcelos, I. M. (2013). Chemical Composition, Nutritive Value, and Toxicological Evaluation of Bauhinia cheilantha

Seeds: A Legume from Semiarid Regions Widely Used in Folk Medicine. *BioMed Research International*, 2013: 1-7.

Terashima, M., Oe, M., Ogura, K. and Matsumura, S. (2011). Inhibition strength of short peptides derived from an ACE inhibitory peptide. *Journal of Agricultural and Food Chemistry*, 59 (20): 11234-11237.

Thammarat, K., Leena, N., Punnanee, S. and Soottawat, B. (2015). Functional and Antioxidative properties of Bambara groundnut (*Voandzeia subterranea*) protein hydrolysates. *International Food Research Journal*, 22 (4): 1584 - 1595.

Tironi, V. A. and Añón, M. C. (2010). Amaranth proteins as a source of antioxidant peptides: Effect of proteolysis. *Food Research International*, 43 (1): 315-322.

Udenigwe, C. C., Adebiyi, A. P., Doyen, A., Li, H., Bazinet, L. and 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. and Aluko, R. E. (2012). Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. *Journal of Food Science*, 77 (1): R11-R24.

Udenigwe, C. C., Lin, Y.-S., Hou, W.-C. and 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.

Valdez-Ortiz, A., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R. and Medina-Godoy, S. (2012). Protein hydrolysates obtained from Azufrado (*sulphur yellow*) beans (*Phaseolus vulgaris*): Nutritional, ACE-inhibitory and antioxidative characterization. *LWT-Food Science and Technology*, 46 (1): 91-96.

Vose, J. (1980). Production and functionality of starches and protein isolates from legume seeds (field peas and horsebeans). *Cereal Chemistry (USA)*,

Wani, I. A., Sogi, D. S., Shivhare, U. S. and Gill, B. S. (2015). Physico-chemical and functional properties of native and hydrolyzed kidney bean (*Phaseolus vulgaris* L.) protein isolates. *Food Research International*, 76 (1): 11-18.

Wani, I. A., Sogi, D. S., Wani, A. A. and Gill, B. S. (2013). Physico-chemical and functional properties of flours from Indian kidney bean (*Phaseolus vulgaris* L.) cultivars. *LWT-Food Science and Technology*, 53 (1): 278-284.

Wu, W., Hettiarachchy, N. and Qi, M. (1998). Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. *Journal of the American Oil Chemists' Society*, 75 (7): 845-850.

Xie, Z., Huang, J., Xu, X. and Jin, Z. (2008). Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. *Food Chemistry*, 111 (2): 370-376.

Yang, J. T., Wu, C.-S. C. and Martinez, H. M. (1986). [11] Calculation of protein conformation from circular dichroism. *Methods in Enzymology*, 130: 208-269.

Yao, D. N., Kouassi, K. N., Erba, D., Scazzina, F., Pellegrini, N. and Casiraghi, M. C. (2015). Nutritive Evaluation of the Bambara Groundnut Ci12 Landrace [*Vigna subterranea* (L.) Verdc.(Fabaceae)] Produced in Côte d'Ivoire. *International Journal of Molecular Sciences*, 16 (9): 21428-21441.

Yin, S. W., Tang, C. H., Yang, X. Q., Wen, Q. B. and Qi, J. R. (2011). Surface charge and conformational properties of phaseolin, the major globulin in red kidney bean (Phaseolus vulgaris L): effect of pH. *International Journal of Food Science and Technology*, 46 (8): 1628-1635.

Zayas, J. F. 1997. *Introduction: Functionality of proteins in food*. Springer. pp. 1 - 5.

Zhang, T., Li, Y., Miao, M. and Jiang, B. (2011). Purification and characterisation of a new antioxidant peptide from chickpea (*Cicer arietium* L.) protein hydrolysates. *Food Chemistry*, 128 (1): 28-33.

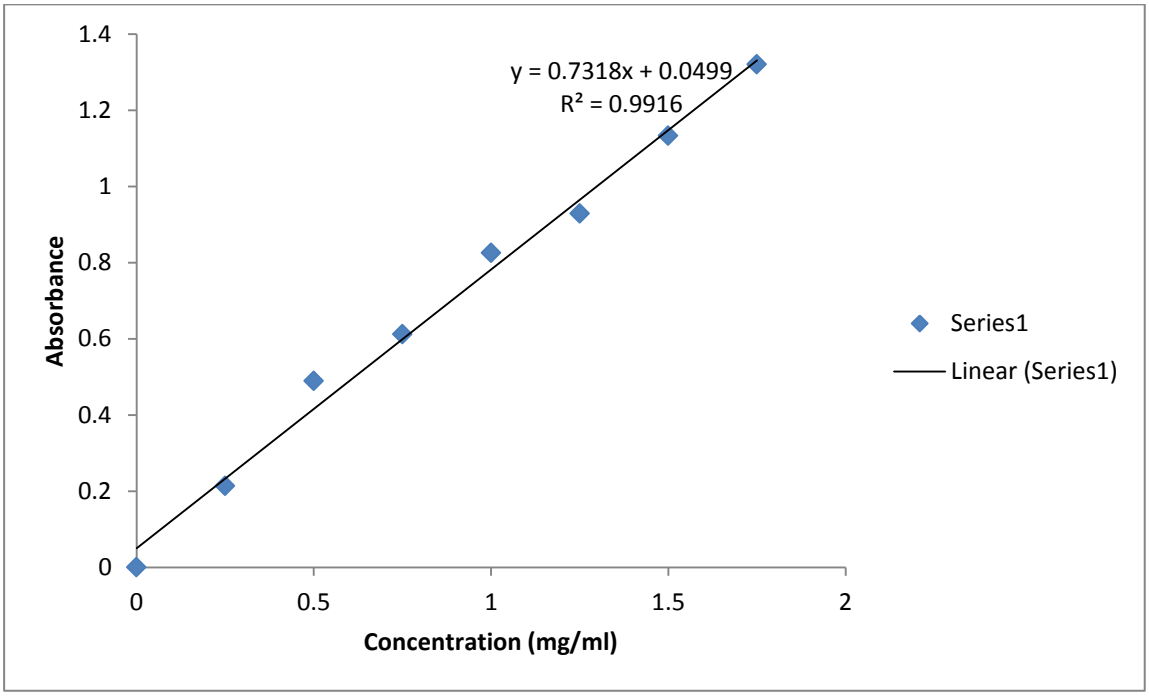
Zhao, G., Liu, Y., Zhao, M., Ren, J. and Yang, B. (2011). Enzymatic hydrolysis and their effects on conformational and functional properties of peanut protein isolate. *Food Chemistry*, 127 (4): 1438-1443.

Zhu, K., Zhou, H. and Qian, H. (2006). Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. *Process Biochemistry*, 41 (6): 1296-1302.

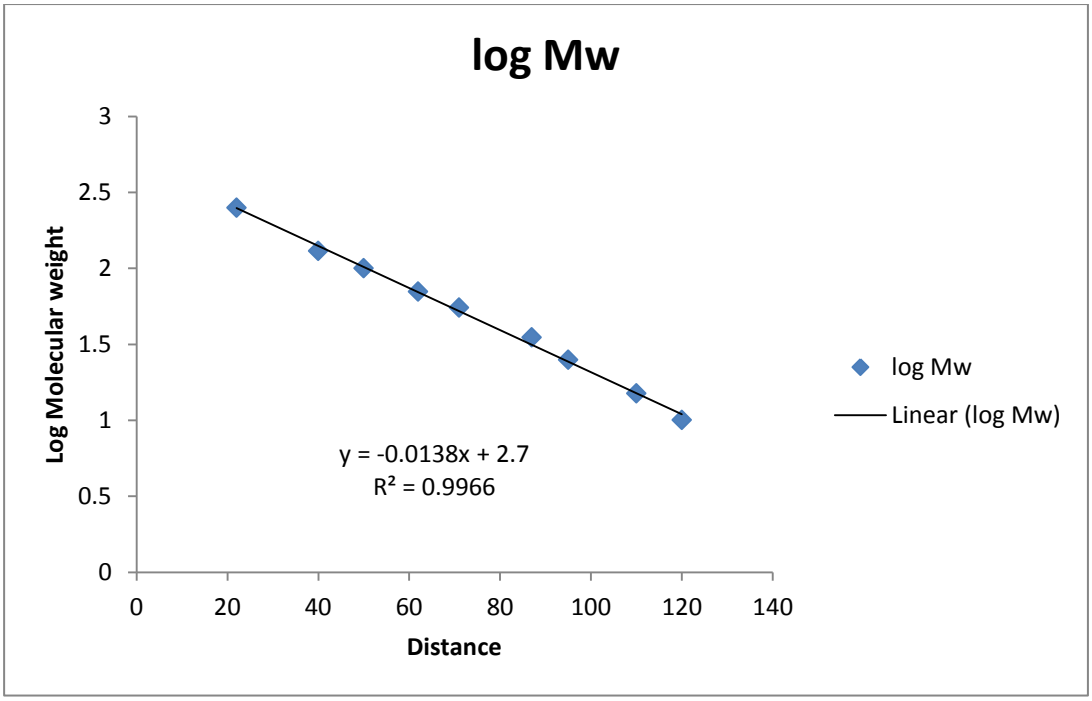
Zhu, Z., Qiu, N. and Yi, J. (2010). Production and characterization of angiotensin converting enzyme (ACE) inhibitory peptides from apricot (Prunus armeniaca L.) kernel protein hydrolysate. *European Food Research and Technology*, 231 (1): 13-19.

APPENDIX

APPENDIX ONE: Graph to determine protein concentration by Braford (Chapter one)



APPENDIX TWO: Standard curve generated by plotting the log of the molecular weight of protein standards vs. the relative mobility (Chapter two)



APPENDIX THREE : Publications

International Journal of Food Science and Technology 2015

Original article

Influence of extraction methods on functional properties of protein concentrates prepared from South African bambara groundnut landraces

Abimbola K. Arise,[1,2]* Eric O. Amonsou[1] & Oluwatosin A. Ijabadeniyi[1]

1 Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, PO Box 1334, Durban 4001, South Africa

2 Department of Home Economics and Food Science, Faculty of Agriculture, University of Ilorin, P.M.B. 1515, Ilorin, Nigeria

(Received 25 September 2014; Accepted in revised form 7 December 2014)

Summary Functional properties of protein concentrates prepared from three bambara groundnut landraces using acid precipitation and salt solubilisation methods were evaluated. The protein content of bambara grains (26–27%) was similar for the three landraces. The acid precipitation gave a much higher yield of protein concentrates (52%), which were also high in protein (79%) compared to the salt solubilisation method (yield: 25%, protein content: 57%). Functional properties of proteins were more influenced by the methods of preparation rather than the landraces. Protein concentrate prepared by salt solubilisation method showed higher emulsifying (63–66%), foaming (53–57%), water (1.4–2.0 mg mL^{-1}) and oil absorption properties (2.2–2.6 mg mL^{-1}) than the acid-precipitated concentrates (53–57%, 63–66%, 2.0–2.7 mg mL^{-1}, 1.4–1.7 mg mL^{-1}). The foaming capacity and stability of all the protein concentrates decreased with increasing pH from 3 to 8. Salt solubilisation may be the most appropriate method for the enhanced functionality and utilisation of bambara groundnuts' protein concentrates.

Keywords Acid precipitation, bambara groundnut, functional properties, landrace, protein concentrate, salt solubilisation.

Introduction

Bambara groundnut (*Vigna subterranea* L. *Verdc*) is a neglected legume of the African origin (Adegbola & Bamishaiye, 2011). It is the third most important after groundnut (*Arachis hypogeal*) and cowpea (*Vigna unguiculata*) in Africa (Adegbola & Bamishaiye, 2011). Bambara is indigenous to South Africa and is grown mainly in Limpopo, Mpumalanga and KwaZulu-Natal provinces of South Africa (Mabhaudhi & Modi, 2013). The protein content of bambara grain may vary between 15% and 25% (Adegbola & Bamishaiye, 2011; Hillocks *et al.*, 2012; Murevanhema & Jideani, 2013). This is similar to cowpea and slightly lower when compared to that of soya bean (Adegbola & Bamishaiye, 2011). Furthermore, bambara groundnut is highly drought tolerant and produces better yield under harsh agronomic condition, which gives it an advantage over other legume grains such as groundnut and soybean (Mazahib *et al.*, 2013). Despite these attributes, the agro-ecological, genetic potential as well as the nutritional importance of bambara groundnuts has not been fully researched (Boateng *et al.*, 2013). The crop is still cultivated from local landraces in South Africa.

Plant protein concentrates may be utilised in foods for the improvement of both nutritional and functional quality of the food products. The yield, composition and functionality of proteins may vary depending on the grain varieties and method of extraction (Zayas, 1997). The functional properties of soya bean protein and concentrates have been extensively researched (Kwon *et al.*, 2010; De la Caba *et al.*, 2012; Rehholz *et al.*, 2012). For example, the protein yield of soya bean concentrate (16.2%) prepared by micellisation method was found to be substantially low, three to four times that obtained by isoelectric precipitation. However, soya bean protein concentrate obtained by isoelectric precipitation showed lower foaming capacity compared to soya bean concentrate extracted using the micellisation method (Adebowale *et al.*, 2011). This suggests that methods of extraction may have influence on the functionality of proteins and, consequently, their application in foods. A comparative study on

*Correspondent: E-mail: abimbolatmi@gmail.com

doi:10.1111/ijfs.12746

Food & Function

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c6fo00057f

Antioxidant activities of bambara groundnut (*Vigna subterranea*) protein hydrolysates and their membrane ultrafiltration fractions

Abimbola K. Arise,[a,b] Adeola M. Alashi,[b] Ifeanyi D. Nwachukwu,[b] Oluwatosin A. Ijabadeniyi,[a] Rotimi E. Aluko[b] and Eric O. Amonsou*[a]

In this study, the bambara protein isolate (BPI) was digested with three proteases (alcalase, trypsin and pepsin), to produce bambara protein hydrolysates (BPHs). These hydrolysates were passed through ultrafiltration membranes to obtain peptide fractions of different sizes (<1, 1–3, 3–5 and 5–10 kDa). The hydrolysates and their peptide fractions were investigated for antioxidant activities. The membrane fractions showed that peptides with sizes <3 kDa had significantly ($p < 0.05$) reduced surface hydrophobicity when compared with peptides >3 kDa. This is in agreement with the result obtained for the ferric reducing power, metal chelating and hydroxyl radical scavenging activities where higher molecular weight peptides exhibited better activity ($p < 0.05$) when compared to low molecular weight peptide fractions. However, for all the hydrolysates, the low molecular weight peptides were more effective diphenyl-1-picrylhydrazyl (DPPH) radical scavengers but not superoxide radicals when compared to the bigger peptides. In comparison with glutathione (GSH), BPHs and their membrane fractions had better ($p < 0.05$) reducing power and ability to chelate metal ions except for the pepsin hydrolysate and its membrane fractions that did not show any metal chelating activity. However, the 5–10 kDa pepsin hydrolysate peptide fractions had greater (88%) hydroxyl scavenging activity than GSH, alcalase and trypsin hydrolysates (82%). These findings show the potential use of BPHs and their peptide fraction as antioxidants in reducing food spoilage or management of oxidative stress-related metabolic disorders.

Received 13th January 2016,
Accepted 23rd April 2016
DOI: 10.1039/c6fo00057f
www.rsc.org/foodfunction

Introduction

Value addition to underutilized crops has become popular in recent times to maximize their potential use for human nutrition and health.[1] Bambara groundnut (*Vigna subterranea*), a scarcely studied crop of African origin, is the third most important legume seed after groundnut (*Arachis hypogea*) and cowpea (*vigna unguiculata*) in Africa.[2,3] The protein content of bambara may vary between 15 and 27%,[2–5] which is similar to that of cowpea[3,6,7] and slightly lower than the values reported for soya bean.[4,5,7] Bambara protein contains a high lysine content (6.5–6.8%) and a reasonable amount of methionine (1.8 g per 100 g) which is normally limiting in legumes.[5,8,9] Other important attributes of bambara include tolerance to drought and poor soil conditions, resilience in the face of extreme weather conditions such as hot temperatures and heavy rainfall and resistance to pests and diseases.[10] Despite these attributes, the use of bambara groundnut remains restricted to domestic food consumption.[3,4] However, with further research, bambara could be used for the manufacture of value-added products and its utilization may assist to solve the problem of food insecurity and poverty in developing countries.

In recent years, research has focused on the generation of bioactive peptides from food protein sources.[11,12] Bioactive peptides contain 2–20 amino acids per peptide as inactive sequences within large proteins. These peptides are released when the parent protein is hydrolysed by digestive enzymes (*in vitro* and *in vivo*), microbial enzymes or during food processing.[13] Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides without adversely affecting the nutritive value.[10,14] Peptides can be used in the formulation of functional foods and nutraceuticals to prevent damage related to oxidative stress in human disease conditions. Also, natural antioxidants are desirable because they can be used at higher concentrations without the toxic side effects associated with the use of their synthetic equivalents.[13,15] They also exhibit enhanced nutritional and func-

[a]Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, PO Box 1334, Durban 4001, South Africa. E-mail: eamonsou@dut.ac.za; Tel: +27785066339
[b]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada