CHARACTERIZATION AND MODIFICATION OF BAMBARA GROUNDNUT GLOBULIN FRACTIONS FOR THE ENHANCEMENT OF FUNCTIONAL PROPERTIES

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Submitted in complete fulfilment for the Degree of Doctor of Food Science and Technology in the Department of Biotechnology and Food Science, Durban University of Technology, Durban, South Africa

Declaration

I, **Opeyemi O. ALABI** hereby declare that this study presents the original work carried out by the author and is submitted to the Department of Biotechnology and Food Science at the Durban University of Technology for the Degree of Doctor of Food Science and Technology. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

10-03-2023

Signature of student **Date**

03/04/2023

Signature of supervisor **Date**

Dedication

This doctoral thesis is dedicated to the Almighty God, the God of all my days.

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Abstract

There is a growing interest in the utilization of leguminous grain proteins for food and industrial applications. Bambara groundnut is a xerophyte pulse grain and a potential source of protein that can replace soybean protein, a trusted and widely used food ingredient in the food industry. However, the use of Bambara groundnut proteins including the subunits (legumin and vicilin) is limited in food applications. The understanding of the composition of Bambara groundnut proteins at the subunit level is vital to unlocking their potential and facilitating utilization. In this study, Bambara groundnut globulin is characterized in terms of the structures, composition, and physicochemical properties at the subunit level, and then modified using atmospheric plasma and enzymatic hydrolysis.

Bambara globulin consisted of about 70% vicilin, whilst legumin protein was found in relatively low quantity. Gel electrophoresis revealed three major protein bands in globulin similar to vicilin with predominant β-sheet structures. The presence of a disulfide bond was also revealed in legumin. Bambara globulin showed major vicilin (7S, Mw: 120 kDa) and minor legumin (11S, Mw: 410 kDa) components. Fluorescence and hydrophobicity data suggested a folded structure for the legumin fraction dominated by the helical secondary structure compared to the vicilin fraction. Bambara proteins contain an appreciable amount of methionine that is even higher than the FAO/WHO recommended value. Bambara vicilin had the highest amount of negatively and positively charged amino acids compared to globulin and legumin. This coincides with its high solubility profile (approximately 82% at pH 3.5). The least gelation concentration (LGC) significantly increased in the order of globulin (8%) < legumin (18%) < vicilin (20%) at pH 7.

Bambara groundnut proteins formed weakly structured gels as indicated by the frequency-dependent behaviours of both the storage (G′) and loss (G″) moduli with a difference of lesser than 1 log cycle. The highest G′ of vicilin gel indicated more firmness of the gel compared to the gel formed by globulin and legumin. The sol-gel transition temperatures increased in the order of globulin (40°C) < legumin (50°C) < vicilin (80°C). The G′ and G″ of globulin showed relatively low dependency on heating time beyond the gel point compared to legumin and vicilin subfractions, suggesting a more rapid establishment of its gel network during gelation. Vicilin gel consisted of a microporous structure with a small lath sheet-like structure compared to globulin and legumin. Emulsifying stability of the proteins significantly differed ($p < 0.05$)

at pH 7. The foaming capacity of the vicilin fraction was significantly ($p < 0.05$) higher than that of the storage protein at pH 3, 7, and 9.

Atmospheric cold plasma-activated water (PAW) and enzymatic modification of Bambara groundnut globulin were further assessed. The cold plasma treatment resulted in the loss of the helical structure of Bambara globulin. The plasma treatment increased the hydrophobicity of Bambara globulin indicating an unfolded structure that was also reflected in the observed redshift in fluorescence intensity. No major changes were observed in gel electrophoresis, protein surface charge, and solubility profiles, except for about a 20% reduction in the glutamic acid content of the amino acid profile. Bambara globulin had reduced emulsifying capacity after treatment with PAW. However, foaming capacities were significantly better and stable at up to 15 mg protein/mL.

Hydrolysates produced from Bambara groundnut globulin and vicilin, respectively using a combination of pepsin and pancreatin were investigated for ACE and renin inhibitory activities. The hydrophobic amino acid residues in both globulin and vicilin hydrolysates are high, improving the entry of their peptides into target organs via hydrophobic associations. Surface hydrophobicity increased significantly ($p<0.05$) with an increase in peptide size from <1 to <3 kDa with that of vicilin hydrolysate and membrane fractions having the highest values. The low molecular weight peptide (<1 kDa) membrane fractions from globulin at 1 mg/mL exhibited significantly higher ($p<0.05$) *invitro* ACE inhibitory activities compared to vicilin hydrolysate and its fractions. However, higher molecular peptide fraction (<3 kDa) favoured renin inhibitory activity at the same concentration.

Vicilin is the major protein fraction of Bambara groundnut globulin. Bambara groundnut globulin was stabilized by disulfide linkages from the legumin, a minor fraction of the storage protein. Bambara globulin and its subfractions formed a weakly structured gel with the dominance of an elastic structure. The dominancy of the β-sheet structure in vicilin protein and the high crosslink density of the vicilin gel could be related to the firmness of the vicilin gel. The variations in the gel points of Bambara globulin and the subfractions were linked to the differences in their amino acid and subunit composition, the thermal unfolding properties of the protein fractions, and the presence of disulfide linkages.

Modification of Bambara groundnut globulin using cold plasma-activated water treatment and enzymatic hydrolysis, respectively increased the hydrophobicity of the protein and influenced

the emulsifying and foaming properties and the *invitro* angiotensin-converting enzyme (ACE) and renin inhibitory activities. Therefore, Bambara groundnut globulin could be a potential functional ingredient in the food system. The low molecular weight peptide (<1 kDa and <3 kDa) membrane fractions from globulin have the potential to serve as functional bioactive peptides against hypertension.

Preface

This thesis is organized into eight chapters and the experimental work is presented in manuscript format. Chapter one gives a general introduction to the thesis. Chapter two provides a critical review of the literature on legume proteins, their nutritional composition, the physicochemical and structural composition of legumes, and the functional properties of legume proteins including emulsifying, foaming properties, gelation, and solubility. Also, a brief review on the modification of Bambara groundnut protein using plasma technology as a potential alternative means of altering the structure of a protein to impact functionality is provided. The subsequent chapters are divided into two sections. Section one (Chapters 3 and 4) reports on the molecular composition and functional properties of Bambara globulin whilst the second section (Chapters 5 and 6) reports on the modification of Bambara groundnut globulin using atmospheric cold plasma technology and enzymatic hydrolysis method. Chapter seven is a general discussion of all the findings. Chapter eight concludes the thesis and suggests recommendations for future studies.

Table of Contents

Declaration i

Dedication ii

Acknowledgments iii

Abstract iv

Preface vii

Table of Contents viii

List of Figures xiv

List of Tables xvi

Abbreviations xvii

Publications and conferences xix

CHAPTER ONE 1

1. Introduction 1

CHAPTER TWO 4

2. Literature Review 4

2.1. Historical background 4
2.2. Nutritional composition of Bambara groundnut 5
2.3. Uses of Bambara groundnut 6
2.4. Pulse protein 8
2.4.1. Composition *8*
2.4.2. Amino acid composition *8*
2.4.3. Types of pulses protein and their subunit composition *9*
2.5. Structural and physicochemical properties of legume protein 13

2.5.1. Surface hydrophobicity *13*
2.5.2. Circular dichroism (CD) spectroscopy *14*
2.5.3. Fluorescence spectroscopy *15*
2.6. Thermal properties **16**
2.7. Functional properties of legume protein **17**
2.7.1. Protein Solubility *18*
2.7.2. Emulsifying properties *19*
2.7.3. Foaming properties *20*
2.7.4. Gel-forming properties of proteins *21*
2.8. Modification of plant proteins **24**
2.8.1. Cold plasma technology *24*
2.8.2. Effect of cold plasma treatment on the functional properties of proteins *25*
2.8.3. Enzymatic modification of proteins to produce bioactive peptides *28*
2.8.4. ACE and renin inhibitory properties of protein *29*
2.9. Conclusion **31**
2.10. Hypothesis **31**
2.11. Aim **32**
2.12. Objectives **32**
CHAPTER THREE **33**

3. Composition and some functional properties of Bambara vicilin fraction **33**

3.1. Introduction **34**
3.2. Materials and methods **35**
3.2.1. Chemicals *35*
3.2.2. Preparation of defatted flour *35*
2.2.3. Extraction of Storage protein and vicilin *35*
3.2.4. Protein content and yield *36*
3.2.5. Amino acid composition analysis *36*
3.2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) *36*
3.2.7. Surface hydrophobicity determination *36*
3.2.8. Intrinsic fluorescence emission *37*
3.2.9. Differential scanning calorimetry (DSC) *37*
3.2.10. Protein solubility *37*
3.2.11. Emulsifying activity and stability *38*
3.2.12. Foaming capacity and stability *38*

3.2.13. Statistical analysis *39*
3.3. Results and discussion **39**
3.3.1. Protein content and yield *39*
3.3.2. Amino acid profile of Bambara storage protein and vicilin *39*
3.3.3. Gel electrophoresis profile of Bambara vicilin *40*
3.3.4. Fluorescence emission spectra of Bambara vicilin *40*
3.3.5. Hydrophobicity (S_O) property of Bambara vicilin *44*
3.3.6. Differential scanning calorimetry (DSC) *44*
3.3.7. Solubility profile of Bambara vicilin *44*
3.3.8. Emulsifying properties of Bambara vicilin *47*
3.3.9. Foaming properties of Bambara vicilin *47*
3.4. Conclusions **50**
3.5. Research outputs **50**
CHAPTER FOUR **51**

4. Extraction, characterization and rheological properties of vicilin and legumin proteins derived from Bambara globulin **51**

4.1. Introduction **52**
4.2. Materials and methods **53**
4.2.1. Materials *53*
4.2.2. Preparation of defatted flour *53*
4.2.3. Extraction of Bambara globulin, legumin, and vicilin *53*
4.2.4. Protein content and yields *54*
4.2.5. Amino acid analyses *54*
4.2.6. Gel electrophoresis *55*
4.2.7. High-performance size-exclusion chromatography *55*
4.2.8. Surface hydrophobicity (S_o) *55*
4.2.9. Intrinsic fluorescence emission *55*
4.2.10. Circular dichroism (CD) *56*
4.2.11. Differential scanning calorimetry *56*
4.2.12. Particle size and zeta potential (ζ) *56*
4.2.13. Protein solubility *56*
4.2.14. Least gelation concentration *57*
4.2.15. Steady-shear viscosity of Bambara groundnut proteins dispersion *57*

4.2.16. Preparation of Bambara protein fractions gels *57*
4.2.17. Rheological properties of Bambara protein fraction gels *58*
4.2.18. Scanning electron microscopy (SEM) *58*
4.2.19. Gelation of Bambara dispersion *58*
4.2.20. Statistical analysis *58*
4.3. Results and discussion **58**
4.3.1. Protein content and yield *58*
4.3.2. Amino acid profile *59*
4.3.3. SDS-PAGE of Bambara globulin, legumin, and vicilin *61*
4.3.4. Size-exclusion chromatography *61*
4.3.5. Surface Hydrophobicity of Bambara globulin and its fractions *62*
4.3.6. Fluorescence emission spectra of Bambara globulin *62*
4.3.7. CD Spectra *66*
4.3.8. Differential scanning calorimetry *68*
4.3.9. Surface charges (ζ) and particle size distribution *68*
4.3.10. Solubility profile of Bambara globulins and fractions *69*
4.3.11. Critical protein concentration for gel formation *73*
4.3.12. Steady-shear viscosity of Bambara groundnut proteins dispersion *73*
4.3.13. Mechanical spectra of Bambara protein gels *74*
4.3.14. The microstructure of Bambara protein gels *79*
4.3.15. Gelation profile of Bambara dispersion *79*
4.4. Conclusions **83**
4.5. Research outputs **83**
CHAPTER FIVE **84**

5. Modification of Bambara globulin with plasma-activated water. **84**

5.1. Introduction **85**
5.2. Materials and methods **87**
5.2.1. Preparation of defatted flour *87*
5.2.2. Extraction of Bambara Globulin *87*
5.2.3. Preparation of plasma-activated water and treatment of Bambara globulin with plasma 87
5.2.4. Protein content *88*
5.2.5. Amino acid composition analysis *88*
5.2.6. Gel electrophoresis *88*

5.2.7. Intrinsic fluorescence *88*
5.2.8. Surface hydrophobicity *89*
5.2.9. Fourier transform infrared spectroscopy (FTIR) *89*
5.2.10. Particle size and zeta potential (ζ) *89*
5.2.11. Protein solubility *89*
5.2.12. Emulsifying formation and oil droplet size measurement *89*
5.2.13. Foaming capacity (FC) and stability (FS) *90*
5.2.13. Statistical analysis *90*
5.3. Result and discussion **91**
5.3.1. Amino acid composition *91*
5.3.2. Gel electrophoresis profile of plasma-treated Bambara globulin *91*
5.3.3. Fluorescence emission spectra of plasma-treated Bambara globulin. *93*
5.3.4. Hydrophobicity (S_O) property of plasma-treated Bambara globulin *93*
5.3.5. Fourier transform infrared spectroscopy *96*
5.3.6. Surface charges (ζ-potential) and particle size distribution *96*
5.3.7. Solubility profile of Plasma Bambara globulin *97*
5.3.8. Emulsifying properties *101*
5.4. Conclusion **106**
5.5. Research outputs **106**
CHAPTER SIX **107**

6. Inhibition of renin and angiotensin-I-converting enzyme by Bambara vicilin hydrolysate fractions **107**

6.1. Introduction **108**
6.2. Materials and methods **110**
6.2.1. Preparation of defatted flour *110*
6.2.2. Extraction of Bambara globulin and vicilin *110*
6.2.3. Protein content and yields *111*
6.2.4. Preparation of hydrolysate from Bambara globulin and vicilin with their peptide fractions *111*
6.2.5. Amino acid analyses *111*
6.2.6. Degree of Hydrolysis (DH) *112*
6.2.7. Surface hydrophobicity (So) *112*
6.2.8. ACE inhibition assay *113*

6.2.9. Renin inhibition assay *113*
6.2.10. Statistical analysis *114*
6.3. Result and discussion **114**
6.3.1. Protein content *114*
6.3.2. Amino acid *116*
6.3.3. Degree of Hydrolysis (DH) *118*
6.3.4. Surface Hydrophobicity *118*
6.3.5. ACE Inhibitory properties of Bambara proteins hydrolysates and membrane fractions *118*
6.3.6. Renin Inhibitory properties of Bambara proteins hydrolysates and membrane fractions *123*
6.4. Conclusion **123**
CHAPTER SEVEN **125**

7. General Discussion **125**

CHAPTER EIGHT **130**

8. Conclusion and recommendations **130**

8.1. Conclusion **130**
8.2. Recommendation **131**
References **132**

Appendices **161**

List of Figures

Figure 2. 1: Varieties of Bambara groundnut (*Vigna subterranean*) differentiated based on the colour of the seed coat. 11
Figure 2. 2: SDS-PAGE patterns of various legume seed protein isolates under non–reducing and reducing conditions. 12
Figure 2. 3: Heat-induced gelation process 23
Figure 2. 4: Cleavage of peptide bond by diamiade and α-amidation pathways 27
Figure 2. 5: (A) Cleavage by oxidation of glutamyi residues and (B) clevage by oxidation of Aspartyl residues 27
Figure 2. 6: Oxidation of aromatic amino acid residues 28

Figure 3. 1: SDS-PAGE of Bambara proteins. 42
Figure 3. 2: Fluorescence spectra of Bambara storage protein and its vicilin fraction 43
Figure 3. 3: Surface hydrophobicity of Bambara storage protein and vicilin fraction 45
Figure 3. 4: Heat flow pattern of Bambara storage protein and its vicilin fraction. 46
Figure 3. 5: Solubility profile of Bambara storage protein and its vicilin fraction as a function of pH 46

Figure 4. 1: SDS-PAGE pattern of Bambara globulin, legumin, and vicilin. 63
Figure 4. 2: Size exclusion chromatography elution profile of Bambara protein fractions....64
Figure 4. 3: Physicochemical properties of Bambara groundnut protein fractions measured at pH 7....65
Figure 4. 4: Circular Dichroism spectra of Bambara groundnut protein fractions measured at pH 7....67
Figure 4. 5: Thermal properties of Bambara globulin, legumin, and vicilin. 70
Figure 4. 6: Zeta potential and solubility profile of Bambara groundnut protein fractions....71
Figure 4. 7: Steady-shear viscosity of Bambara globulin, legumin, and vicilin 75
Figure 4. 8: Frequency sweep data of gels prepared from Bambara groundnut protein fractions at their critical protein concentration (LGC) 76
Figure 4. 9: Frequency sweep data of gels prepared from Bambara groundnut protein fractions at a protein concentration of 22.5% 77
Figure 4. 10: Scanning electron microscopy images of gels from Bambara protein fractions. 81
Figure 4. 11: Temperature dependence of G′ and G″ moduli of Bambara proteins at 22.5% concentration during heating ramp. 82

Figure 5. 1: SDS-PAGE of plasma-treated Bambara globulin. 94
Figure 5. 2: Physicochemical properties of plasma-treated Bambara globulin 95
Figure 5. 3: (A) Zeta potential and (B) Solubility profile of plasma-treated Bambara globulin 99
Figure 5. 4: Emulsifying properties of plasma-treated Bambara globulin at varying concentrations. 103

Figure 5. 5: Oil droplet size distribution properties of emulsion formed at pH 7 with plasma-treated Bambara globulin. Protein concentrations: 104
Figure 5. 6: Foaming properties of plasma-treated Bambara globulin at varying concentrations. 105

Figure 6. 1: Enzymatic hydrolysis curve on Bambara globulin and vicilin isolate by proteases. 120
Figure 6. 2: Surface hydrophobicity of Bambara globulin and vicilin isolate with their membrane fractions. 121
Figure 6. 3: Inhibition of angiotensin-converting enzyme (ACE) by enzymatic Bambara protein hydrolysates and membrane ultrafiltration fractions at 0.5 mg/mL and 1.0 mg/mL, respectively. 122
Figure 6. 4: Inhibition of renin by enzymatic Bambara protein hydrolysate and membrane ultrafiltration fractions at 0.5 mg/mL and 1.0 mg/mL, respectively. 124

List of Tables

Table 2. 1: Chemical composition of Bambara groundnut seeds 7

Table 3. 1: Amino acid composition (g/100 g protein) of Bambara storage protein and its vicilin fraction 41
Table 3. 2: Emulsifying properties (%) of Bambara storage protein and vicilin fraction....... 48
Table 3. 3: Foaming properties (%) of Bambara storage protein and vicilin fraction 49

Table 4. 1: Amino acid composition (g/100 g protein) of globulin, legumin, and vicilin from Bambara groundnut 60
Table 4. 2: Particle size distribution of Bambara globulin and its legumin and vicilin fractions 72
Table 4. 3: Power law parameters for gels from Bambara globulin and its subunits at 22.5% protein concentration 78

Table 5. 1: Amino acid compositions (g/100g) of plasma-treated Bambara globulin 92
Table 5. 2: Secondary structure of plasma-treated Bambara globulin 98
Table 5. 3: Particle size distribution of plasma-treated Bambara globulin 100

Table 6. 1: The protein content of hydrolysates from Bambara globulin and vicilin and their membrane fractions. 115
Table 6. 2: Amino acid composition (g/100 g protein) of hydrolysates from Bambara globulin and vicilin 117

Abbreviations

ACE: Angiotensin-converting enzyme

ANOVA: Analysis of variance

APS: Ammonium persulfate

BP: Blood pressure

BPH: Bambara protein hydrolysate

VH: Vicilin hydrolysate

CD: Circular Dichroism

DSC: Differential scanning calorimetry

FAO: Food and Agriculture Organization of the united nation

FI: Fluorescence intensity

FTIR: Fourier Transform Infra-Red

G″: Loss modulus

G′: Storage modulus

G*: Complex modulus

HCl: Hydrochloric acid

ΔH: Protein denaturation enthalpy

HPLC: High-Performance Liquid Chromatography

kDa: kilo-Dalton

LGC: Least gelation concentration

ME: Mercaptoethanol

MW: Molecular Weight

kDa: kilo-Dalton

PAW: Plasma-activated water

PAGE: polyacrylamide gel electrophoresis

SD: Standard deviation

SDS: Sodium dodecyl sulfate

SEM: Scanning Electron Microscopy

So: Surface hydrophobicity

TCA: Tricholoroacetic acid

Td: Peak Temperature of Transition

TEMED: Tetramethylethylene-Diamine

WHO: World Health Organisation

So: Surface hydrophobicity

TCA: Tricholoroacetic acid

Td: Peak Temperature of Transition

TEMED: Tetramethylethylene-Diamine

Publications and conferences

Publications

- Alabi O.O., Ali, N., Nwachukwu, I.D., Aluko, R.E., and Amonson, E.O. 2020 Composition and some functional properties of Bambara vicilin fraction. *LWT-Food Science and Technology,* 125: 109256
- Alabi, O.O., Annor, G., and Amonsou, E.O. 2023. Effect of cold plasma-activated water on physicochemical, and functional properties of Bambara groundnut globulin. *Food Structure*, 36:100321

Submitted manuscripts

- Opeyemi Alabi, Eric O. Amonsou (2022). Impact of structure-composition on gel microstructures and rheological properties of vicilin and legumin-like proteins derived from Bambara globulin. Submitted to Food Research International Journal

Conferences

- Alabi, O.O., Annor, G., and Amonsou, E.O. Impact of plasma-activated water treatment on the physicochemical and functional properties of Bambara globulin. Presented at the 2022 AOCS Annual Meeting and Expo: Advancing the science and technology of oils, fats, proteins, surfactants and related material, enriching the lives of people everywhere, 3rd-14th May, Atlanta, USA. (Oral presentation)
- Alabi, O.O. and Amonsou, E.O. Impact of structure-composition on gel microstructures and rheological properties of vicilin and legumin-like proteins derived from Bambara globulin. Presented at the 2021 AOCS Annual Meeting and Expo: Advancing the science and technology of oils, fats, proteins, surfactants and related material, enriching the lives of people everywhere, 3rd-14th May, Chicago, USA. (Oral presentation)
- Alabi, O.O. and Amonsou, E.O. Comparative study of legumin (11S) and vicilin (7S) of Bambara protein, 23rd SAAFoST Biennial International Congress and Exhibition, Birchwood hotel and conference centre, Johannesburg, 1-4 September 2019. (Oral presentation)

- Alabi, O.O. and Amonsou, E.O. Rheological properties of selected food biopolymers. 1st World of Rheology workshop, Anton Paar Building, Midrand, 28-29 August 2019. (Oral presentation)
- Ali, N., Alabi, O.O., Udechukwu, M., Nwachukwu, I.D., Aluko, R.E., Amonsou, E.O. Fractionation and functionality of Bambara groundnut vicilin (7S) Protein. Presented at the 2018, 32nd EFFOST International Conference: Developing innovative food structures and functionality through process and reformulation to satisfy consumers need and expectations, 6th-8th November, Nantes, France (Poster presentation).

CHAPTER ONE

1. Introduction

Bambara groundnut (*Vigna subterranea*) is an underutilized pulse grain of African origin (Mazahib *et al.* 2013; Oyeyinka *et al.* 2017). It is the third most consumed legume in semi-arid Africa after peanut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) (Adebowale, Schwarzenbolz and Henle 2011; Ogundele, Amanda and Emmambux 2017). The Bambara groundnut is drought tolerant with good resistance to pests and diseases (Bamshaiye, Adegbola and Bamshaiye 2011; Thammarat *et al.* 2015). Bambara, just like any other legume, contributes to soil fertility through nitrogen fixation (Cleasby, Massawe and Symonds 2016; Mubaiwa *et al.* 2017), and is usually intercropped with crops like maize, cowpea, sorghum, melon, and tuberous crops to improve productivity (Bamshaiye, Adegbola and Bamshaiye 2011; Mubaiwa *et al.* 2017).

Bambara groundnut contains about 19% - 20.5% of protein which is comparable to pulses such as cowpea and pea (Arise, Amonsou and Ijabadeniyi 2015; Chinma *et al.* 2015). Bambara protein is rich in essential amino acids such as lysine (6.6%) and particularly methionine (1.3%) (Chinma *et al.* 2015) which is limiting in leguminous grain. Bambara groundnut is cultivated mainly by local farmers for sustenance reasons in Limpopo, Mpumalanga and KwaZulu–Natal provinces of South Africa (Mabhaudhi and Modi 2013). Even with these agronomic and nutritional potentials of Bambara groundnut, the grain is largely naturalized (Yao *et al.* 2015) which may be attributed to a lack of market value (Mayes *et al.* 2011; Yao *et al.* 2015). Recent studies have shown that Bambara groundnut could be used in the preparation of value-added products like vegetable milk and yogurt (Falade *et al.* 2014), meat patties (Alakalia, Irtwange and Mzera 2010), bread (Chinma *et al.* 2015; Abdulrahman *et al.* 2017), and extruded snacks to meet the growing demand for healthier products, and to reduce food insecurity.

Knowledge of the molecular and structural composition of the protein is essential in explaining the functionality of protein and its various application as ingredients in the food system. Globulins, the salt-soluble protein, is the major storage protein in pulses (Boye, Zare and Pletch 2010). Globulin is classified into 7S fraction (vicilin) and 11S fraction (legumin) based on their sedimentation coefficients (S20.w) (Mertens *et al.* 2012; Stone *et al.* 2015b). Vicilin, with a molecular weight of 150-250 kDa, accounted for about 65%-80% of the total protein in legumes (Stone *et al.* 2015b). Vicilin is composed of heterogeneous polypeptides held together by hydrophobic interaction rather than a covalent disulfide bond in legumin (Lam *et al.* 2016).

Vicilin has been reported to have a more hydrophilic surface than legumin in pea (Stone *et al.* 2015a) owing to its higher level of acidic and basic amino acids (Lam *et al.* 2016). Vicilin consists of varying subunits which may be glycosylated; three subunits in pea (~47-50 kDa) (Mession *et al.* 2015; Stone *et al.* 2015b), kidney beans (55, 45-41 kDa) (Shevkani *et al.* 2015), chickpea (19.2, 34.6-51 kDa) (Chang *et al.* 2011), and two subunits in cowpea (Vasconcelos *et al.* 2010) and red beans (Mundi and Aluko 2012).

Recent studies tentatively identify vicilin (7S) as the major storage protein fraction of Bambara groundnut (Adebowale, Schwarzenbolz and Henle 2011; Arise *et al.* 2017). The vicilin identification was based on molecular weight distribution compared to other pulses. The vicilin in Bambara groundnut globulin was never characterized and the legumin fraction was never isolated. There is, therefore, a need for a more detailed evaluation of the composition and structure of Bambara groundnut globulin and how these impact functionalities of protein in food systems.

Furthermore, the poor functional properties of legume proteins limiting their utilization as a food ingredient may be enhanced through the modification process. Modification processes such as physical, chemical, and enzymatic hydrolysis had been employed to effect changes in the composition and structural attributes of Bambara groundnut proteins (Lawal, Adebowale and Adebowale, 2007; Mune mune, 2015). These methods were reported to enhance the functional properties of Bambara groundnut protein, but the processes involved were complex, expensive, and time-consuming. Also, the danger of chemical residual effects from chemical modification.

Recently, cold plasma technology, a green, environmentally friendly, cost-effective, energy and water-saving technology (Šimončicová *et al.* 2019), have been of interest for protein modification because it has no undesirable effects (Misra, *et al.* 2015; Thirumdas *et al.* 2018). Cold plasma (CP) is a partially ionized gas consisting of reactive species containing reactive oxygen species (ROS), or reactive nitrogen species (RNS) generated by ionizing gases with electricity (Coutinho *et al.* 2018; Ekezie, Cheng and Sun, 2019; Luo *et al.* 2020). Previous studies have reported that plasma reactive species from dielectric barrier discharge (DBD) interacted with proteins in various ways, causing structural modification, which influences their functionality in food systems. (Bußler *et al.* 2015; Segat *et al.* 2015; Dong *et al.* 2017). However, optimization of the reactive species, produced in DBD plasma, to induce changes in protein foods is still challenging as the surface accumulation of these excessive reactive species

may cause protein oxidation (Wojtyla *et al.* 2016; Qian *et al.* 2021). Therefore, protein isolate can be subjected to plasma-activated water (PAW) treatment to allow for uniform and sufficient interactions between reactive species and protein molecules (Bermudez-Aguirre, 2020). Thus, preventing excessive oxidation, and impacting the functional properties of Bambara groundnut globulin by structural modification.

The growing global burden of hypertension threatening an estimated one billion individuals (approximately 14.9% of the global population) has made it a serious public health issue in both developed and developing countries (Bavishi, Bangalore and Messerli 2016; Fu *et al.* 2017). Hypertension can be controlled through the renin-angiotensin system (RAS) that helps to regulate the key enzymes, renin and angiotensin I-converting enzyme (ACE), which are responsible for causing blood pressure (BP) elevation (Crowley and Coffman 2012; Girgih *et al.* 2016; Fu *et al.* 2017). The simultaneous inhibition of ACE and renin activities will exert a synergistic effect on the efficient treatment of hypertension, compared to the individual enzyme inhibitors that are currently used (Harel *et al.* 2012; Connelly *et al.* 2013; Fu *et al.* 2017).

Moreover, there is a need for the development and production of plant food-derived peptides to replace or complement synthetic ACE and renin inhibitors due to the high costs and negative side effects such as erectile dysfunction, dry cough, angioedema, taste disturbance, decrease in white blood cells, fatigue, skin rash, and diarrhoea after prolonged use (Chen *et al.* 2013; Aluko 2015; Tao *et al.* 2017) associated with them (Udenigwe and Aluko 2012). Although the previous study suggested hydrolysates of Bambara groundnut storage protein from alcalase, pepsin, and trypsin, respectively to have dual potential antioxidant and antihypertensive properties (Arise *et al.* 2016), there is a paucity of information on the plant protein-derived peptide from the Bambara protein fraction (vicilin) compared to the storage protein (globulin), and the effect of successive hydrolysis on the peptides.

CHAPTER TWO

2. Literature Review

2.1. Historical background

Bambara groundnut (*Vigna subterranea*) is an underutilized pulse of African origin cultivated by subsistence farmers (Mubaiwa *et al.* 2017; Oyeyinka *et al.* 2017). It is a member of the family of Fabaceae and the sub-family of Faboidea (Mazahib *et al.* 2013). There are two botanical varieties of Bambara namely *Vigna subterranean var. spontanea* which includes wild varieties and *Vigna subterranean var subterranean* which includes cultivated varieties (Cleasby, Massawe and Symonds 2016). Bambara is an intermediate, herbaceous annual legume with a compact full-grown taproot with many profuse geotropic short lateral roots which are about 20 cm long (Murevanhema and Jideani 2013). The symbiotic relationship between Bambara groundnut and bacteria that form root nodules, a characteristic of legume grains, increases the level of nitrogen in the soil thereby improving the soil fertility (Mubaiwa *et al.* 2017). The flank stems of Bambara develop from the root and the leaves are produced on it. The flower of the branching types is usually self-pollinated while that of the spreading types is cross-pollinated by ants. The leaves are trifoliate (± 5 cm long) and are attached to the stem by the petiole. The petioles are about 15 cm long, stiff, and grooved with a green or purple color base. Leaves and flower buds arise alternately at each node. The podding habit of the Bambara seed is like 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, Bennett and Mponda 2012; Yao *et al.* 2015). The Bambara groundnut is a fast-growing plant, which requires warm temperatures and does not tolerate freezing temperatures during the growing season. The optimum temperature for germination of the Bambara groundnut is 30°C to 35°C and sprouting takes 5 to 21 days. Bambara groundnut grows well at an average temperature of 20°C to 28°C and has a growth period of 110 to 150 days (Bamshaiye, Adegbola and Bamshaiye 2011). Better than most crops, Bambara groundnut is highly pliant and tolerates harsh climatic conditions (Mazahib *et al.* 2013).

Furthermore, Bambara groundnut can be grown on poor, sandy to sandy loam and well-drained soil, which makes it easier to harvest. Bambara grows better with a higher yield on poor, low-fertility soils, and soil with a pH of 5.0 to 6.5. Bambara groundnut required an annual rainfall of 500 to 1200 mm during the growing season. Bambara is not susceptible to the menace of total crop failure, especially in low and erratic rainfall (Bamshaiye, Adegbola and Bamshaiye

2011; Hillocks, Bennett and Mponda 2012). Although the Bambara plant tolerates heavy rainfall, too much rainfall at harvest may result in yield losses. In this era of global warming and food security threat in Africa, Bambara groundnut will be an important crop of hope for improving food security because of its capacity to tolerate harsh climatic conditions under a low-input agricultural system where many other crops fail (Cleasby, Massawe and Symonds 2016). Bambara groundnut is majorly cultivated in Limpopo, Mpumalanga, Northwest, Gauteng, and Kwazulu-Natal provinces of South Africa. The seed is hard, smooth, usually round, and varies in size. The colour of Bambara groundnut varies from white, cream, dark brown, red, or black and may be speckled or patterned with a combination of colours.

2.2. Nutritional composition of Bambara groundnut

The chemical composition of Bambara groundnut (Table 2.1) revealed that the grain made a nutritious and complete food because of the protein (18.9-22.9 g/100g), carbohydrate (61.7-65.0 g/100g), and fat content (2.0- 6.5 g/100g) (Mahazib *et al.* 2013; Mubaiwa *et al.* 2017). Bambara groundnut has been reported to be nutritionally richer than peanuts in essential amino acids such as isoleucine, leucine, lysine, phenylalanine, threonine, valine, and particularly methionine, which is a limiting amino acid in most legumes, (Chinma *et al.* 2016; Halimi *et al.* 2019; Mbuma *et al.* 2022). This makes Bambara groundnut a potential complement for foods lacking in these essential amino acids. All the other essential and non-essential amino acids in Bambara groundnut meet FAO requirements (Mubaiwa *et al.* 2017). Bambara groundnut has been reported to be higher in protein quality because the amino acid score of the most limiting amino acid is 80% compared to 74% for soybean, 65% for groundnut, and 64% for cowpea (Mubaiwa *et al.* 2017). Bambara groundnut is a potential replacement for animal protein in areas where animal protein is expensive, and where unfavourable environmental conditions make the cultivation of other legumes economically risky (Murevanhema and Jideani, 2013; Yao *et al.* 2015). It contains slowly digestible starch (SDS) that promotes slow and moderate postprandial glucose and insulin responses and has low glycaemic values like other legumes (Chinma *et al.* 2016). The predominant fatty acids in Bambara groundnut are linoleic, palmitic, and linolenic acids (Baptista *et al.* 2017; Halimi *et al.* 2019; Mbuma *et al.* 2022).

Furthermore, Murevanhema and Jideani (2013) reported that Bambara groundnut contains a substantial quantity of vitamin A, thiamine, riboflavin, niacin, carotene, and trace quantities of ascorbic acid. Bambara groundnut was reported to contain micronutrients such as zinc and iron (Murevanhema and Jideani 2013), calcium and potassium but is poor in phosphorus and

magnesium (Bamshaiye, Adegbola and Bamshaiye 2011). The red seeds have been proposed to be beneficial in areas where iron deficiency is prevalent because of the report that it contains almost twice as much iron as the cream seeds (Bamshaiye, Adegbola and Bamshaiye 2011). Bambara groundnut was reported to possess anti-oxidative properties (Ademiluyi and Oboh 2011; Arise *et al.* 2016; Chinma *et al.* 2016).

2.3. Uses of Bambara groundnut

Bambara groundnuts are essentially grown for human consumption. Mostly, the immature and mature Bambara groundnut, either boiled with salt and pepper, fried or grilled, is consumed as a snack (Mahazib *et al.* 2013). Bambara groundnuts are also roasted and crushed to make soup with or without condiments (Murevanhema and Jideani 2013). Bambara groundnut can be used as a condiment (Akanni *et al.* 2018) for cooking, and flour (Abdualrahman *et al.* 2019). Milk produced from Bambara groundnut is often used as weaning milk in many African countries (Bamshaiye, Adegbola and Bamshaiye 2011; Falade *et al.* 2015). Bambara milk was the most accepted in terms of colour when compared to other legume-based milk such as soybean and cowpea (Murevanhema and Jideani 2013). Recent studies had demonstrated the inclusion of Bambara groundnut in the development of various value-added food products such as biscuits and cake (Chinma *et al.* 2022), vegetable milk and yogurt (Falade *et al.* 2015; Murevanhema and Jideani 2013), bread (Abdualrahman *et al.* 2019; Chinma *et al.* 2016), meat patties (Alakali, Irtwange and Mzer 2010).

Bambara groundnut had been successfully used to feed chicks and the leaves are suitable for animal grazing owing to its high nitrogen and phosphorus content (Jideani, and Jideani 2021a). Other than the nutritional uses, Bambara groundnut has medicinal benefits as well. The Luo tribe in Kenya drinks water from boiled Bambara and maize grain mixture to cure diarrhoea (Okafor *et al.* 2022). Bambara leaves extract is applied to abscesses and infected wounds and its sap is applied to the eye for treating epilepsy. Bambara roots are sometimes taken as an aphrodisiac, and pulverized Bambara grains mixed with water are used for the treatment of cataracts in Senegal. The Igbos in Nigeria use the plant to treat venereal diseases (Hillocks, Bennett and Mponda 2012). It was reported that chewing and swallowing raw Bambara groundnut is being used to curb nausea and vomiting in South African pregnant women (Jideani and Diedericks 2014; Jideani, and Jideani 2021b).

Table 2. 1: Chemical composition of Bambara groundnut seeds

Parameter	Minimum	Average	Maximum
Proximate composition (g/100g)			
Moisture	5.8	8.6	11.5
Crude protein	17.1	18.9	22.9
Crude lipid	2.9	6.3	9.2
Crude fiber	1.4	4.0	7.2
Ash	2.9	3.7	5.1
Carbohydrate	55.8	61.7	68.0
Mineral composition (mg/100g)			
Potassium	470	1234	2200
Phosphorus	33.3	31.7	61.3
Calcium	37	56	70
Sodium	0.9	5.3	20.2
Magnesium	57.1	184.0	335
Zinc	0.6	1.1	1.2
Manganese	1.0	1.8	5.5
Iron	1.2	3.1	7.0
Copper	0.2	0.6	1.3
Vitamin composition (mg/100g)			
Thiamine	0.22	0.47	0.62
β-carotene	N/A	0.01	N/A
Riboflavin	0.14	0.15	1.12
Niacin	0.60	1.80	2.40
Ascorbic acid	N/A	Trace	N/A

Source: Bamshaiye, Adegbola and Bamshaiye (2011); Murevanhema and Jideani (2013); Chinma *et al.* (2016); Mubaiwa *et al.* (2017)

*N/A: Not available

2.4. Pulse protein

2.4.1. Composition

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 the germination of the seed to provide necessary free amino acids and nitrogen to the growing plant during the initial stages of germination. Bambara groundnut has been reported to be a good source of protein. The value reported for the protein content of Bambara groundnut (20.6-25.0%) (Chinma *et al.* 2016) was similar to those reported for other pulses such as cowpea (Gerrano *et al.* 2019), kidney bean (Shevkani *et al.* 2015), chickpea (Anitha, Govindaraj and Kane-Potaka 2020), and mung bean (Dahiya *et al.* 2015). The variation in the chemical composition of legumes protein may be attributed to differences in genotypes, environmental conditions, and agricultural practices (Singh 2017; Shevkani *et al.* (2019).

2.4.2. Amino acid composition

The main amino acids of legumes storage protein are glutamic and aspartic acid which may include glutamine and asparagine, respectively (Shevkani *et al.* 2019). Aspartic and glutamic amino acids account for 25-40% of the total amino acids in leguminous seeds (Kudre *et al.* 2013; Pastor-Cavada *et al.* 2014; Arise *et al.* 2017). A higher amount of arginine has been reported in Faba bean (Pastor-Cavada *et al.* 2014) and cowpea (Elhardallou *et al.* 2015). Arise *et al.* (2017) reported a higher arginine content of approximately 7.31 g/100g for the Bambara storage protein which was close to the 8.1 g/100g reported by Adebowale *et al.* (2011). The solubility profile of protein had been reported to be influenced by the synergetic effect of both positively charged amino acids such as arginine and negatively charged amino acids such as glutamic acid (Shukla and Trout 2011; Li *et al.* 2018). Plant proteins rich in glutamic acid and arginine is of great interest because arginine helps in preventing heart diseases, while glutamine supports the immune system and improves athletic performance (Wu *et al.* 2021). Arginine, is also, involved in the synthesis of protein and nitric oxide (NO) as well as amino acids such as proline and glutamate in humans (Wu *et al.* 2021). The lysine content of Bambara (6.3 g/100 g protein) is similar to that of mung bean (Kudre *et al.* 2013). Research findings had shown that indigenous pulse grains such as Bambara groundnut, mung bean (Kudre *et al.* 2013), and faba bean (Pastor-Cavada *et al.* 2014) contained an adequate amount of lysine comparable to that of soya bean based on the report of FAO/WHO (2007). Thus, these indigenous legumes offer some potentially significant nutritional attributes which make legumes a good protein

supplement to cereals that are known to be deficient in lysine. Proline can impart a rigid structure to proteins by participating in folding and unfolding processes. The cyclic sidechain of proline introduces a kink in protein structure that prevent the formation of protein beta-pleated conformation (Aider, Djenane and Ounis 2012; Damodaran, Parkin and Fennema, 2008). The formation of beta-pleated conformation in protein prevents aggregation in protein and thus increases protein solubility. The hydrophobic amino acids influence the thermal stability, conformation (Arise *et al.* 2017), and foaming properties of the plant proteins (Aryee, Agyei and Udenigwe 2018). The functionality of pulse proteins had been linked to their amino acid composition (Aryee, Agyei and Udenigwe 2018; Shevkani *et al.* 2019). Therefore, the findings from the amino acid content of Bambara groundnut globulin in terms of the subunits (vicilin (7S) and legumin (11S)) that has not been investigated would give an insight into how amino acids impact the functionality of legume proteins and their fractions.

2.4.3. Types of pulses protein and their subunit composition

Pulses proteins including albumins (water soluble), globulins (salt soluble), prolamins (alcohol soluble), and glutens (alkali/acid soluble) based on their solubility in solvent (Osborne 1924). The major storage protein fractions found in pulses are albumin (10-20%) and globulin (70-80%) and in total protein (Boye, Zare and Pletch 2010; Lu *et al.* 2020). The ratio of albumins to globulins is usually between 1:3 and 1:6.3 depending on the type of pulses (beans, lentils, black gram, and chickpea) (Shevkani *et al.* 2019). Albumins primarily contain metabolic, enzymatic, and non-enzymatic proteins (Chang *et al.* 2022). Pulse albumins, a low molecular weight (MW: 5–80 kDa) protein contain a high amount of cysteine and methionine content than pulse globulins (Boye, Zare and Pletch 2010; Shevkani *et al.* 2019). The utilization of pulse storage protein as food ingredients in the food system is largely influenced by the functionality of globulin (Junejo *et al.* 2021).

Globulins including legumin (11S) and vicilin (7S) are the major storage protein in most leguminous grains based on their sedimentation coefficient (s) (Agarwal, 2017; Klupšaitė and Juodeikienė 2015). The ratio of legumin to vicilin varies considerably with factors including the methods used in the preparation of protein materials, processing parameters like pH and temperature, and agronomic factors (cultivar type and species) (Barac *et al.* 2010; Karaca, Low and Nickerson 2011; Shen and Tang 2014). These factors can also impact significant changes in the molecular and structural composition of protein, thus affecting its functionality (Sun and Arntfield 2012). Boye, Zare and Pletch (2010) reported ratios of 10.5:1, 1:6–9, 1:9, 1–3:1, and

4–6:1 for legumin to vicilin ratios in lentil, French bean, cowpea, pea, and chickpea, respectively. Legumin is a hexameric protein with a molecular weight of about 320–400 kDa. The polypeptides are linked together by disulfide bonds (Mertens *et al.* 2012; Mession *et al.* 2015). The sizes of these polypeptides range from 38-40 kDa for the acidic and 19-22 kDa for the basic polypeptides. Vicilin is a trimer of monomers of 50–70 kDa held together by non-covalent hydrophobic interactions. It shows an overall MW of 150–250 kDa (Mertens *et al.* 2012). This globulin is not disulfide-bonded because it lacks cysteine residue in its structure. Vicilin can be cleaved at one or two sites (called the α-β and β-γ processing sites) as specified by the coding sequence of the vicilin genes. Cleavage at the α-β site generates fragments of 19 and 30 kDa, whereas that at the β-γ site generates fragments of 12.5 or 16 and 33 kDa. Cleavage at both sites generates fragments of 12.5, 13.5, and 16 or 19 kDa.

Differences in terms of the number and molecular weight of constituent polypeptide subunits have been studied through one-dimensional SDS-PAGE and high-performance liquid chromatography (HPLC). The globulin fraction of kidney bean protein is composed of two major bands with a molecular weight of 43 and 45 kDa including three minor polypeptides with less than a molecular weight of 30 kDa (Mundi and Aluko 2012). A molecular weight of 196 kDa was reported for Bambara vicilin isolated using the Osborne extraction method (Diedericks *et al.* 2019). SDS-PAGE analysis showed three major bands at 55, 62 and 141 kDa for Bambara protein isolate under both reducing and non-reducing conditions (Arise *et al.* 2017). Whilst three major bands at 40, 65, and 72 kDa under both non-reducing and reducing conditions were revealed for Bambara protein as described by Mune Mune and Sogi (2016). However, Kudre *et al.* (2013), reported two major bands for Bambara protein isolate at 52 and 62 kDa under both reducing and non-reducing conditions. The different number of bands reported by the authors might be due to different extraction methods employed in the preparation of the protein isolates (Mune Mune and Sogi 2016). Therefore, there is a need to further explore the gel electrophoresis of Bambara protein to confirm the actual bands that it possesses as this will help in determining its functionality in the food system (Tang 2017). The protein band distributions of some legumes are shown in Fig. 2.2. The protein isolates from mung bean, black bean, and Bambara was compared using the SDS-PAGE. The major protein band of Bambara had molecular weights of 58 and 66 kDa. The polypeptide bands with MW 58 and 66 KDa were observed under both conditions of reducing and non-reducing (Kudre *et al.* 2013). These bands were considered 7S vicilin proteins which are widely present in seed proteins and devoid of disulfide bonds between some units. Vicilin (7S) was then reported as

the major storage protein fraction in Bambara groundnut protein. However, vicilin (7S) was not further subjected to other experimental analyses by these authors to ascertain it as the major storage protein in Bambara. Subunits that were stabilized by disulfide bonds were reported for mung bean as new bands emerged under reducing conditions (Kudre *et al.* 2013). This shows that the protein compositions may vary in size, type, and bond involved in stabilizing the structure of protein depending on different isolates. Vicilin has been established as the major storage protein in kidney beans, cowpea, mung bean, and red beans accounting for about 88% of total globulins (Meng and Ma 2001; Tang *et al.* 2009; Tang and Sun 2010; Shevkani *et al.* 2015). While the storage proteins in Feld pea and Fava bean and Faba bean comprise both vicilins and legumins (Kimura *et al.* 2008; Shevkani and Singh 2014; Shevkani *et al.* 2015).

The differences in terms of the molecular weights of polypeptide subunits of pulse proteins influence their functional properties such as gelation capacity, emulsion, and foaming properties (Merten *et al.* 2012; Shevkani *et al.* 2019).

Figure 2. 1: Varieties of Bambara groundnut (*Vigna subterranean*) differentiated based on the colour of the seed coat.

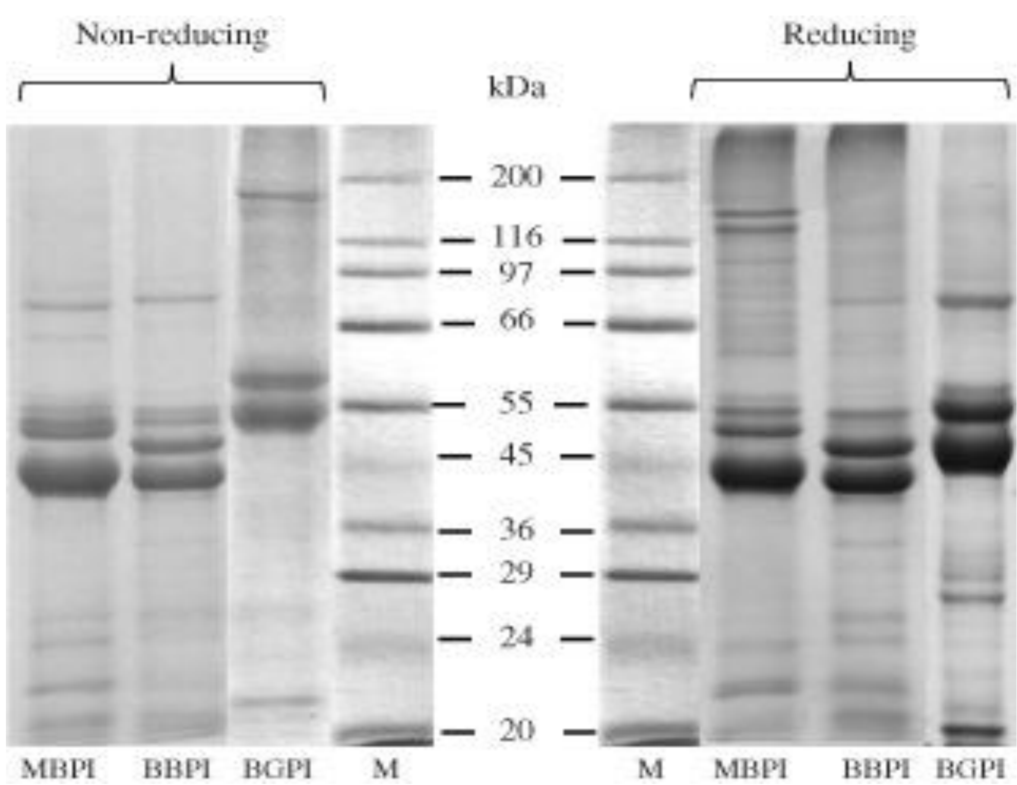


Figure 2. 2: SDS-PAGE patterns of various legume seed protein isolates under non–reducing and reducing conditions.

M, marker; MBPI, Mung bean protein isolate; BBPI, Black bean protein isolate; BGPI, Bambara groundnut protein isolate.

Source: Kudre *et al.* (2013)

2.5. Structural and physicochemical properties of legume protein

Conformational changes occurring at the molecular level which is due to changes in the protein environment (Kelly, Jess and Price 2005) are measured using spectroscopic methods such as surface hydrophobicity, circular dichroism, and fluorescence spectroscopy. Globular proteins in their native form have folded structures. This native structure can be altered by changes in the environment or by subjecting them to extremes of pH, high temperatures, detergents, and organic solvents. This alteration changes the structural and physicochemical properties of the native proteins to open or unfolded proteins. It is therefore important to investigate the structure and physicochemical properties of proteins to understand the folding of proteins into biologically active states and the stabilization of these states. The spectroscopic studies are performed when the proteins are in solution. It provides information on the rate of change in the structure of proteins which determines their biological functions.

2.5.1. Surface hydrophobicity

Surface hydrophobicity, measured using (anilino naphthalene sulphate) ANS-as fluorescence probe, is the characteristics of protein relating to the extent to which hydrophobic amino acids are exposed at the surface of protein molecules and is affected by protein unfolding and denaturation (Tang and sun 2010; Timilsena *et al.* 2016). It is a structure-related function, dependent on the size and shape of the protein molecule, amino-acid composition, and sequence, as well as any intramolecular or intermolecular cross-links (Jiang *et al.* 2015). The surface hydrophobicity of a protein can be used as an indicator of the ability of molecules to interact with each other (Jiang *et al.* 2015). Hydrophobicity plays a major role in the structural and functional properties such as solubility, gelation, emulsification, and foaming of food proteins (Carbonaro, Maselli and Nucara 2015; Karami and Akbari-Adergani 2019). Hydrophobic interactions of native protein structures are solution-dependent because the hydrophobic side chain amino acid residues which are buried in the interior of the protein structure need to be exposed to the surface to measure hydrophobicity (Wolfenden *et al.* 2015). Therefore, 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 are essential. The solubility of proteins is determined by the distribution of polar and hydrophobic groups of amino acid side chains since these two groups always have opposite interactions in solution (Qing *et al.* 2022).

Tang and Sun (2010) reported that the vicilin protein with higher content of the polypeptide exhibited more exposure to hydrophobic clusters on its molecular surface. Zhang, Gao and Yang (2009) reported that the hydrophobicity of the 7S globulin fraction of protein extracted from cowpea is higher than those from French beans. This was attributed to the fact that proteins with higher charged amino acids might exhibit more compact conformation. Surface hydrophobicity values of 148.6, 225.3, and 259.3 were reported for kidney bean, red bean, and mung bean vicilin, respectively (Tang and Sun 2011), which were lower than that of the corresponding vicilin-rich protein from the same legume sources. Makeri *et al.* (2017) reported 234.79 and 252.44 for winged bean and soy protein hydrophobicity, respectively. The hydrophobicity value obtained for globulin is higher than 162.10 reported for peanut protein isolate but lower than 746.4 reported for soybean isolate (Ma *et al.,* 2018). Surface properties of 7S globulin protein revealed larger hydrophobic areas than myoglobin and serum albumin (Nucara *et al.* 2013).

2.5.2. Circular dichroism (CD) spectroscopy

The functional and biological activities of proteins are controlled by the protein structural conformation that is sustained by their carbon-bonded sulfhydryl, thioethers, and carbonyl functional groups (Marsh and Teichmann 2015). These activities are subjected to the amino acid sequence (primary structure), α-helices and β-sheets stabilized largely by hydrogen bonds (secondary structure), 3-D organization of secondary structures via disulfide bonds (tertiary structure), and assembly of multiple folded or coiled protein subunits (quaternary structure) of the protein (Sang and Liu 2022). Any alteration in the secondary, tertiary, and quaternary structures of the protein could lead to denaturation. Denaturation occurs when changes in temperature, pH, interfacial area, and presence of organic compounds disrupted the hydrogen, ionic, or hydrophobic bonds that are in proteins (Fatma *et al.* 2021).

Circular Dichroism (CD) is a technique for studying the interactions of soluble proteins. It is used to determine the dynamics, folding pathways, and the rate of structural changes that occurred within the protein structure (Zhao, Wang, and Zhao 2023). The principle of this technique is based on the interaction of plane polarised light with an asymmetric molecule (which either contains a chiral center or has a three-dimensional structure that provides a chiral environment) (Haque *et al.* 2022). CD signals in the far ultraviolet (UV) wavelength region between 250 nm and 190 nm report on the secondary structure of the protein (α-helix, β-sheet, β-turns and unordered) because of the amide chromophores of the peptide bonds. The Far UV

CD help to observe the process of folding and unfolding in protein. The environmental-dependent CD spectra of protein aromatic residues in the near UV (250 nm to 320 nm) wavelength range give information on the tertiary secondary structure of a protein (Haque *et al.* 2022). The α-helical proteins are detected when the protein has a negative band(s) at 222 nm and 208 nm and a positive band at 190 nm whilst the β-sheet structure shows a negative band at 215 nm and a positive band at 195 nm. The random coil has a negative peak at 200 nm (Zhong and Xiong 2020). Near UV region in the CD spectrum (250–320 nm) provide essential information related to aromatic amino acids residues (Phenylalanine, Tyrosine, and Tryptophan) of proteins in an asymmetric environment. This has been commonly used to assess and monitor the tertiary structure of a protein, and periodically used as well for quaternary structures of proteins during processing (Wang *et al.* 2017). The bands arising from Tyr and Trp residues are found at 275–282 nm and 290–305 nm in the near-UV CD spectrum, respectively whilst a sharp fine structure peak appears for Phe residues at 262 and 268 nm (Martin and Schilstra 2008).

The β-sheet structure had been reported to be dominant in most plant proteins compared to the helical and unordered secondary structure (Tan *et al.* 2019). The far-UV CD analysis revealed that the β-Sheet secondary structure is the dominant secondary structure in pea vicilin at 20°C (O'Kane *et al.* 2004). Chen *et al.* (2022) reported the dominant secondary structure in common vetch protein isolate as the α-helix structure. The structural integrity of storage protein is altered during extraction, fractionation into subunits, and characterization. Therefore, it is essential to assess the conformational changes in such proteins using the circular dichroism (CD) technique.

2.5.3. Fluorescence spectroscopy

Fluorescence is the release of light energy when fluorophores are excited with a high-energy light source (Carstea *et al.* 2016). Fluorescence spectroscopy is used to monitor conformational changes in proteins (Shaikh and O'Donnell 2017). It is used to characterize molecular interactions and chemical reactions. Fluorescence occurs from the aromatic amino acids including tyrosine, phenylalanine, and tryptophan since they are intrinsically fluorescent. The intrinsic fluorescence spectrum is determined mainly by the polarity of the environment of the tryptophan and tyrosine residues, with tryptophan being the most dominant residue. This is because the absorbance at the wavelength of excitation for tryptophan, and its quantum yield of emission are much more than that of tyrosine and phenylalanine to give a good fluorescence

signal (Malomo and Aluko 2015). Fluorescence spectroscopy provides another way of exploring functional folding, stability, and ligand interactions of purified recombinant proteins. Tryptophan fluorescence is very sensitive to protein conformational changes and provides valuable information about changes in protein tertiary structure (Royer 2006).

Fluorescence intensity is influenced by absorbance, optical path length, solute concentration, and quantum yield. Tryptophan (Trp) and Tyrosine (Tyr) are excited at a wavelength of 280 nm will be excited. Residues of Trp and Tyr (chromophores) are involved in the monitoring of protein folding as their fluorescence properties such as quantum yields are responsive to their environment to indicate changes when the protein folds or unfolds. Tryptophan and Tyrosine residues are buried within the core of the natively folded protein (hydrophobic environment) whilst they become exposed to solvent in an unfolded and partially folded state (hydrophilic environment). The fluorescence emission maximum (ƛmax) suffers a red shift when chromophores are exposed to solvent, and the quantum yield of fluorescence decreases resulting in low fluorescence intensity. This also occurs when the chromophores interact with quenching agents either in a solvent or in the protein itself. For example, the interaction of tryptophan with other amino acids such as phenylalanine and tyrosine within the peptide chain might have reduced the amount of absorbed light energy and enhanced fluorescence quenching (Royer 2006). A compact tertiary conformation was reported for kidney bean vicilin because it had a low fluorescence emission maximum of 331.8 nm and a high quantum yield of fluorescence of 2201 (Tang and Sun 2010).

2.6. Thermal properties

The thermal property of protein is related to its heat-induced aggregation and gelation behaviors (Wu *et al.* 2020). It can be studied from the endothermic peaks of the differential scanning calorimetry (DSC) profiles. The disruption of hydrogen bonds involved in the formation and maintenance of tertiary and quaternary structure/conformations of the proteins is reflected by the denaturation temperature (Td) (Shevkani *et al.* 2015; Wan, Liu and Guo 2018). A higher Td value demonstrated a more compact tertiary conformation of the polypeptides in the protein (Tang and Sun 2011; Wan, Liu and Guo 2018). This is an indication of higher thermal stability (Wang *et al.* 2014; Mession *et al.* 2015). The enthalpy (ΔH) value reflected the extent of protein structural unfolding, especially the unfolding extent of tertiary conformation (Tang and Sun 2011). The temperature of denaturation (Td) and enthalpy (ΔH)

are two commonly used parameters in describing the thermal property of pulse proteins (Kaushik *et al.* 2016; Arise *et al.* 2017).

Factors such as amino acid composition, differences in protein structure, and environmental factors have been reported to influence the thermal stability of pulse protein (Kudre *et al.* 2013; Shevkani *et al.* 2019). In addition, the interactions of proteins with residual salts from extraction have been reported to influence thermal stability (Shevkani *et al.* 2015; Kaushik *et al.* 2016). Adebowale, Schwarzenbolz and Henle (2011) reported lower denaturation temperature (Td) for Bambara isolates extracted using isoelectric precipitate compared to the micellised Bambara isolates. This was attributed to the differences in the method of isolation employed as there might be structural rearrangements caused by the extraction method. Wu *et al.* (2020) reported one single endothermic peak for 11S soy protein with an onset of 84.23℃ compared to the 7S soy protein with two endothermic transitions. Flaxseed protein isolate was observed to exhibit a peak denaturation temperature of 105℃ demonstrating 11S globulin as the predominant fraction (Kaushik *et al.* 2016). Denaturation temperatures (Td) of 91.4℃, 85.4℃, and 80.8℃ were observed for vicilin extracted from kidney beans, red beans, and mung beans, respectively. Ionic strength affects the denaturation temperature of the 7S protein fraction (Kimura *et al.* 2008). Denaturation temperature (Td) and enthalpy (ΔH) of 68.5℃ and 7.6 J/g protein, respectively were reported for pea vicilin (Mession *et al.* 2015). The thermal denaturation profile of Mung bean, Black bean, and Bambara protein isolates by DSC showed two major endothermic peaks that were attributed to the thermal denaturation of the 7S protein at lower temperatures and the 11S protein at the higher temperature (Kudre *et al.* 2013). It is vital to investigate the thermal property of the Bambara groundnut globulin including their subunits such as legumin and vicilin, respectively since the thermal property is related to heat-induced aggregation and gelation behaviors of proteins.

2.7. Functional properties of legume protein

Functional properties are the essential properties of a protein, other than nutritional properties that affect the behaviour of proteins in food systems during processing, preparation, manufacturing, and storage (Aryee, Agyei and Udenigwe 2018). These functional properties of a protein are influenced by both intrinsic factors such as physicochemical properties, protein structure, conformation, amino acid composition, hydrophobicity, and hydrophilicity, and extrinsic factors such as pH, temperature, time, the influence of and interaction with other food components (Aryee, Agyei and Udenigwe 2018; Shevkani *et al.* 2019). These functional

properties include solubility, water, and oil absorption, emulsifying, foaming, gelling properties, and rheological properties (Barac *et al.* 2015; Lam *et al.* 2018). Some pulses grain such as Bambara groundnut is exploited as food ingredients in the development of value-added food products such as biscuits and yoghurt. Various studies have exploited the functionalities of legumin (11S) and vicilin (7S) fractions of pulse grains such as pea vicilin and legumin (O'Kane *et al.* 2004; Mession *et al.* 2015; Sha and Xiong 2022), Kidney beans (Shen and Tang 2014), Cowpea vicilin (Rangel *et al.* 2003) Green pea chick pea (Chang *et al.* 2022), Bambara groundnut protein fractions (Diedericks *et al.* 2019; Yang *et al.* 2022).

2.7.1. Protein Solubility

Protein solubility can be defined as the equilibrium between protein-protein (hydrophobic) and protein-solvent (hydrophilic) interactions (Tang and Sun 2011; Lam *et al.* 2018; Kumar *et al.* 2022;). Protein solubility is due to the intermolecular repulsion caused by electrostatic interactions. Electrostatic interactions ionize the interior hydrophobic residues of a protein. It causes the unfolding of the polypeptide chain that results in the exposure of buried functional groups (Malomo and Aluko 2015). The functional properties of protein as a food ingredient for industrial application are dependent on the solubility profile of the protein (Karaca, Low and Nickerson 2011; Cui *et al.* 2020).

Factors such as ionic strength, solvent pH, temperature, and food matrix medium significantly impact the solubility of the protein. In addition, at the isoelectric point of the protein, solubility usually decreased. This is because of the amphoteric nature (containing both acidic and basic functional groups) of the protein. Under these conditions, hydrophobic interactions between neighbouring proteins can lead to aggregation, and once the aggregates are sufficient in size and number, precipitation occurs (Lam *et al.* 2018), thereby leading to minimum protein solubility. For example, the minimum solubility of 12% was reported for Bambara storage protein between pH 4 and 5 (Adebowale *et al.* 2011; Arise *et al.* 2017). and for mung bean and black bean, protein isolates at pH between 4 and 5 (Kudre *et al.* 2013). Kidney bean, red bean, and mung bean vicilin displayed a minimum solubility at pH between 4 and 5 (Tan and Sun 2011). Pea vicilin was reported to be insoluble at pH between 5 and 6 and moderately soluble at pH 4 (65%) (Rangel *et al.* 2003).

Furthermore, the method of extraction employed in the isolation of the protein type may also impact the solubility of the protein as well as the source of the protein (Barac *et al.* 2015;

Shevkani *et al.* 2019). However, the solubility profile of legumin and vicilin extracted from Bambara groundnut has not been fully exploited.

2.7.2. Emulsifying properties

Emulsifying properties of a protein are the ability of the protein to act as an interface between two immiscible components usually oil and water to form stable emulsions (Shevkani *et al.* 2019). Emulsions are thermodynamically unstable because of the increase in the interfacial free energy that increased the interfacial surface tension (Kumar *et al.* 2022). The ability of a protein to adsorb at the interface and form elastic layers surrounding the oil droplets reduced the interfacial tension and thus prevented coalescence in emulsion (Lam *et al.* 2018). This is achievable because of the amphiphilic nature of the protein (existence of both polar and non-polar amino acids residue) (Aryee, Agyei and Udenigwe 2018). The ability of proteins to increase the viscosity of the continuous phase and decrease the rate of movement of oil droplet rate in the emulsion significantly contribute to the stability of the emulsion (Shevkani *et al.* 2019).

The emulsifying capacity of proteins is very essential in food application in the food system and its bio-functional application. This is because of the capacity of the hydrophilic residues of the proteins to interact with water and the hydrophobic residues to interact with oil to form stable emulsions (Shevkani *et al.* 2015). Isoelectric point (pI) and ionic strength of protein have been reported as critical factors that affect the stability of protein emulsion. When the pH of the protein is close to the isoelectric point and the ionic strength is high, emulsions are less stable because the electrostatic repulsion is weak compared to the attractive forces between the droplets (Lam *et al.* 2018). On the other hand, stable emulsions are formed at low ionic strength pH away from the isoelectric point of the protein. This is because the electrostatic repulsive forces are greater which in turn promote stronger interactions between proteins adsorbed to the same droplet to form a robust interfacial film and prevent coalescence. Low temperature retards the stability of emulsions. This is because crystallized water molecules force dispersed droplets closer together, and uneven distribution of the emulsifier on the surface of the droplet. Exposure of hydrophobic residues on the surface of denatured proteins and the use of polar oils can improve the stability of emulsions (Shevkani *et al.* 2015).

Emulsifying properties are generally assessed by two indices known as emulsifying activity (EA) and emulsifying stability (ES). EA measures the amount of oil that can be emulsified per unit of weight of protein, whilst ES measures the ability of the emulsion to resist changes to its

structure over a specified period (Boye, Zare and Pletch 2010). The adsorption and emulsifying properties of proteins are dependent on the solubility and hydrophobicity properties of proteins (Boye, Zare, and Pletch 2010; Lam *et al.* 2018). The relationship between surface hydrophobicity, interfacial tension, and emulsifying properties of proteins was reported by Kato and Nakai (1980). In addition, Yan *et al.* (2021) detailed that the magnitude of the zeta potential of protein (surface charge) together with hydrophobicity significantly affects the adsorption of proteins at the interface. A study revealed that pea vicilin possesses excellent emulsifying properties compared to the corresponding legumin (Kimura *et al.* 2008). This was attributed to the high solubility profile displayed by vicilin (Koyoro and Powers 1987) and its higher surface hydrophobicity (Boye, Zare and Pletch 2010). However, it was observed in a recent study that the kidney bean proteins showed higher EA than proteins from different field pea cultivars, despite lower hydrophobicity and protein solubility.

Furthermore, differences in the emulsifying properties of pulse proteins had been attributed to their composition and molecular flexibility. For instance, higher emulsifying properties displayed by the protein isolate from white cowpea containing a higher amount of vicilin when compared to the isolate from red cowpea with lesser vicilin content was attributed in part to the lower molecular weight and greater flexibility in the structure of vicilin (Yang *et al.* 2022). This made it easy for the isolate to reorient at the interface after being adsorbed there (Shevkani *et al.* 2015).

2.7.3. Foaming properties

Foams are produced when protein films are generated surrounding a gas bubble, and the packing of the gas bubbles into an overall structure (Lam *et al.* 2018). They are formed when proteins form an interface that keeps air bubbles in suspension and prevents their collapse. The surface tension of the solubilized protein decreased as the protein diffused and adsorb to the gas-liquid interface. This results in the unfolding of the protein and orientation of the hydrophobic regions to the gas phase and hydrophilic regions to the liquid phase to assume train and loop formations. Hypothetically, a good foam is signalized by high protein molecular flexibility for quick reorientation, structural disorder, and metastability. Newly created bubbles are thermodynamically stable over a while due to the high surface tension in water (Cohen-Addad and Höhler 2014; Arise 2016; Salahi and Mohebbi 2021).

The foaming properties of pulse proteins are measured as foaming capacity (FC) and foam stability (FS). Foaming capacity is the ability of a protein, under the influence of certain

parameters such as protein concentration, pH, temperature, and salt concentration (Stone *et al.* 2015), to create a certain amount of interfacial area. It depends on the ability of proteins to diffuse to the interface, reorient, and form a viscous film without excessive aggregation (Lam *et al.* 2018; Shevkani *et al.*2019). FC corresponds to the average hydrophobicity of proteins and can be influenced by partial denaturation to increase surface activity. The correlation of the functional property to average hydrophobicity suggests that the proteins exist in a more unfolded state to expose amino acids buried in the core. Since average hydrophobicity is derived using all amino acids in a protein, as opposed to only those exposed to the surface in surface hydrophobicity.

Whilst foaming stability indicates how well protein can stabilize the foam volume over some time (Lam *et al.* 2018; Shevkani *et al.* 2019). Foams that are stable incline to be opposed to gas diffusion, drainage and thinning of lamella fluid, and mechanical shock (Osei-Bonsu, Shokri and Grassia 2015; Afifi *et al.* 2021). Appropriately, stable protein-based foams should have interfacial films that are cohesive through hydrogen bonding and electrostatic and hydrophobic interactions (Lam *et al.* 2018; Xu *et al.* 2020). At the isoelectric pH of a protein, foams are the most stable. This is due to the negligible electrostatic repulsion which strengthens protein-protein interactions and adsorption of protein to the interface, thus promoting viscous film formation and steric stabilization (Stone *et al.* 2015).

Shevkani *et al.* (2015a) reported that a higher solubility profile of cowpea protein isolates enhanced its foaming properties. In addition to this, foaming properties were positively related to the zeta potential (surface charge) and solubility profile for protein isolates from various kidney bean and field pea lines (Shevkani *et al.* 2015b). Greater charge on the surface of proteins was proposed to have contributed to greater foaming properties by weakening hydrophobic interactions, increasing protein solubility and flexibility, which thus allowed for rapid spreading of the protein on the interface and rapidly encapsulating air particles (Amagliani *et al.* 2021). Yang *et al.* (2022) reported that Bambara legumin could not effectively stabilize foam because it formed a weak and easily stretchable interface while vicilin had low foam overrun. Foaming property is an essential functional property of protein in food processing industries.

2.7.4. Gel-forming properties of proteins

A protein gel is defined as a three-dimensional and well-defined network assembled from protein molecules embedded within an aqueous solvent (Lam *et al.* 2018). Gelation property is

a vital functional property of globular proteins due to its importance in elasticity and textural characteristics (Nicolai and Chassenieux 2019). The capacity of the protein to form gel is measured by the least gelation concentration of the protein. Protein gelation can be induced by heat (Fig. 2.3) but extreme heating of proteins at high temperatures greater than 100°C may cause scission/cleavage of peptide bonds which may prevent gelation (Joshi, Timilsena and Adhikari 2017). Gels can also be induced using the cold-set method. The structure of the protein gel and its properties such as strength and stability is influenced by the method and conditions involved in making the gel. Protein gelation is ascribed to the assembly of partially denatured protein molecules, majorly propel by physical interactions in which the aggregation of protein is arbitrarily commonly induced by heat (Resendiz-Vazquez *et al.* 2017; Bangar *et al.* 2022). Heat-induced gelation is a manifold process in which the molecular unfolding of the partially denatured proteins is caused by prompt advances in the temperature of the protein above the protein denaturation temperature to expose buried hydrophobic regions and functional groups (Omura *et al.* 2021). The exposed buried sites form new intermolecular hydrophobic and electrostatic interactions and hydrogen bond that first allows aggregation and then gelation when the amount of aggregated proteins exceeds a critical concentration to form junction zones that leads to a three-dimensional (3D) gel network with apertures filled with water (Resendiz-Vazquez *et al.* 2017; Omura *et al.* 2021). The marked proportion of water entrapped and retained in the gel structure transforms the liquid sample into solids (Al-Ali *et al.* 2021; Bangar *et al.* 2022). Thus, gelation depends on the 3D network formed by proteins, which depends on the protein-protein and protein–solvent interactions (Shevkani *et al.* 2019).

The protein structure including amino acids sequence and composition and secondary and tertiary structure of the protein has been reported to strongly affected gelation properties. It was demonstrated that high molecular weight proteins and amino acids with a high relative amount of hydrophobic side chain groups established a strong network of gel systems (Foegeding and Davis 2011). In addition, gelation conditions such as temperature, protein concentration, pH, and heat treatment have been reported by (Aryee, Agyei and Udenigwe 2018; Al-Ali *et al.* 2021). For instance, gel elastic properties have been related to crosslinking density and stability. The initial network formation in the production of a protein gel is influenced by hydrogen bonds and hydrophobic interactions. Also, covalent interactions strengthen the gel through the presence of cysteine and cystine amino acids (Al-Ali *et al.* 2021). For example, Berghout, Boom and van der Goot (2015) reported that the native 11S glycinin fraction of soy

protein isolate was unfolded during heating and then form a stabilized gel network because of the presence of the S-S bonds and 10.6 μmol/g free sulfhydryl groups in it.

Heat-induced gelation of pulse proteins is studied using a dynamic rheometer. Dynamic rheological measurements of protein suspensions generated parameters including storage (G′) and loss modulus (G″) that are indicators of the gel strength. G′ determines the elastic component of the gel network and indicates the strength of the structure contributing to the three-dimensional network, whilst G″ represents the interactions not contributing to the three-dimensional network (Sun and Arntfield 2010). The thermal denaturation temperature of pulse proteins that determined their thermal stability is commonly lowered than the gelation temperature, as denaturation is a prerequisite for heat-induced gelation (Omura *et al.* 2021). Kidney bean protein isolates had higher gelation temperatures than field pea isolates (Shevkani *et al.* 2015a, b). This was attributed to the relatively higher proportion of β-sheets structure in kidney bean protein isolates that contributed to the higher thermal stability of the proteins through the large surface area and greater intermolecular interaction.

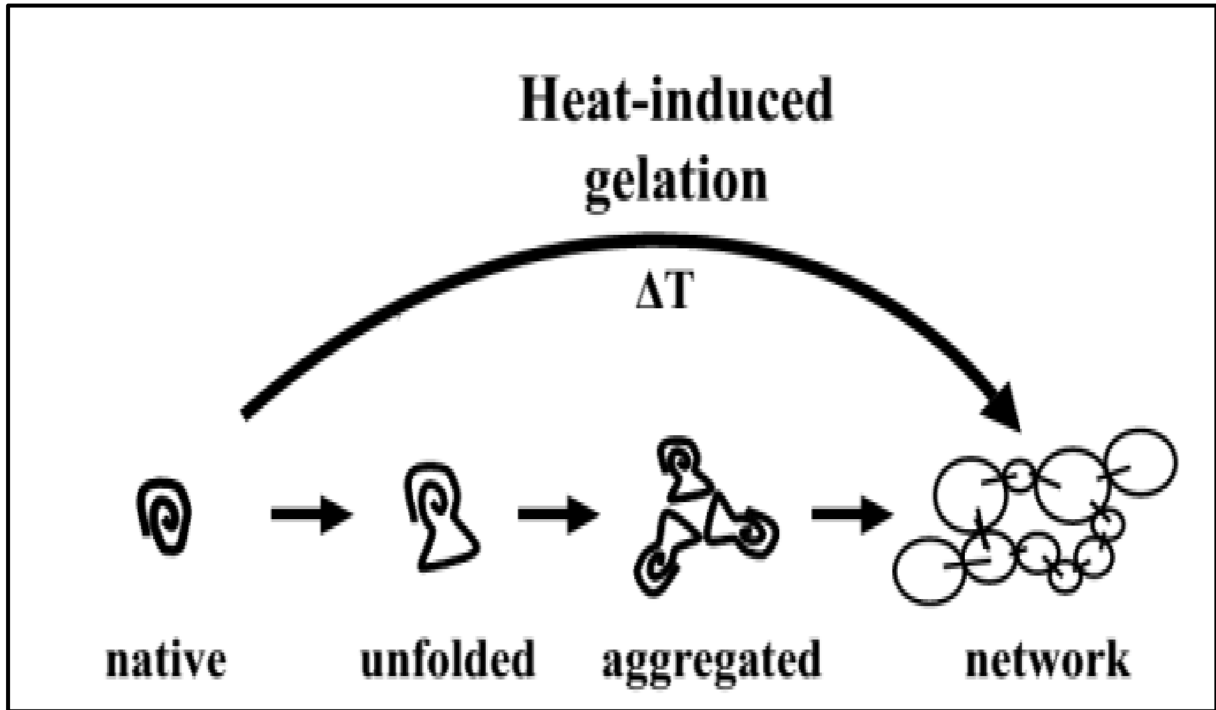


Figure 2. 3: Heat-induced gelation process

Source: Alting (2003)

2.8. Modification of plant proteins

There is a need for alternative sources of protein with the ever-increasing population growth. However, plant proteins are not optimal in their functional properties, and as such, there is a limitation to their application in the food system. Modification of plant-based proteins enhances their application as a food ingredient in food systems by altering their physicochemical and structural properties. The alteration can improve the thermal stability, protein-protein interaction, unfolding, and aggregation, altering interaction strength and bond types (Boye *et al.* 2010). Thus, tackling their limitations. Protein modification can be described as the process of reordering the molecular structure of a protein by specified methods to ameliorate its techno-functionality and bioactive properties. The resulting advancement in the functional properties of protein correlates with the type of interactions promoted in the modification process. Universally, the protein can be modified using physical, chemical, biological (enzymatic), and other novel methods. Physical methods of altering the structure of protein include cold plasma technology.

2.8.1. Cold plasma technology

Cold plasma technology generates a state of matter that contains a compound of reactive oxygen species, reactive nitrogen species and ultraviolet radiations produced when the energy supplied to a gaseous environment dissociates the gas molecular bonds into fully or partially ionized gases called plasma (Thirumdas *et al.* 2018; Ekezie, Cheng and Sun 2019). These particles can break covalent bonds and induce several chemical reactions. The type of gas, energy discharge source (electrical, thermal, optical, electromagnetic), and type of electrode are the factors that influence the properties of the generated plasma. Ionization to generate cold plasma occurs through the external photoelectric effect or by the collision between the electrons at the outer shell and the excited electrons (Filipić *et al.* 2019). For the photoelectric effect, photons are supplied by the outside or emitted by discharge. In the case of collision, excited electrons are provided by the electron beam and accelerated by an external electrical field, electromagnetic field, or radiation (Thirumdas *et al.* 2018).

Cold plasma emits light at discrete wavelengths, corresponding to the transition of electrons in atomic or molecular shells in the visible and ultraviolet (UV) regions of the spectrum (Coutinho *et al.* 2018). For cold plasma generation, temperatures of neutral atoms are comparably lower than that of excited electrons. There is the formation of hot plasma due to high temperature if the electrons gain huge energy to overcome the attraction from its atom.

Various methods have been applied to generate cold plasma, including corona (jet) discharge, dielectric barrier discharge (DBD), microwave, electron cyclotron resonance, and capacity coupled. Plasma can be applied directly to the food surface or indirectly (plasma-activated water or gas to the food surface). Plasma-activated water (PAW) is deionized water containing reactive oxygen species from plasma high-voltage discharge (Porto *et al.* 2018).

2.8.2. Effect of cold plasma treatment on the functional properties of proteins

The subsequent changes in the structure of proteins treated with atmospheric cold plasma resulting in the alteration of their functional properties are achieved through the reaction between reactive oxygen/nitrogen species (ROS/RNS) and the protein molecules. The covalent bonds of the protein molecules were reported to be broken down by the high energy of the plasma. In addition, the reactive species (ROS/RNS) cause oxidation of the sulfur-containing amino acids leading to the cleavage of disulfide (S–S) bonds. This disrupts the conformation of the protein and breaks its polypeptides (Dong *et al.* 2017; Kopuk *et al.* 2022). Exposure of protein to atmospheric cold plasma results in the oxidation of proteins by reactive oxygen species thus leading to the cleavage of peptide bonds (Stadtman and Levine 2003). The cleavage of the alkoxyl radicals and alkyl peroxide derivatives of proteins could be either by the α-amidation or diamide pathways (Fig. 2.4). Glutamyl and aspartyl residues of proteins were oxidized by the reactive oxygen species resulting in the cleavage of the peptide bond (Fig. 2.5). The oxidation of aromatic amino acids is depicted in Fig. 2.6.

Dielectric discharge barrier (DBD) cold plasma treatment has been reported to induce a decline in the micelle's size of zein, thus increasing their solubility (Dong *et al.* 2017). Segat *et al.* (2015) detailed that exposure of whey protein isolates to dielectric discharge barrier cold plasma resulted in significant changes in its emulsifying and foaming properties because of the unfolding of the protein and the increase in the carbonyl functional group. Exposure of protein-rich pea flour to plasma treatment led to the structural modification of protein structure as evidenced by the redshift observed in the fluorescence data (Bußler *et al.* 2015). This was associated with the increased solubility, water, and fat binding capacity recorded for the sample. Ji *et al.* (2018) reported that the functional properties of peanut protein isolate were modified because of the alteration of the side chain of the amino acids in the protein, which was because of the formation of disulfide linkages and the breakage of the covalent bond. However, optimization of the reactive species, generated using various sources of plasma generator such as dielectric barrier discharge (DBD), to induce changes in protein foods is still

challenging as the surface accumulation of these excessive reactive species may cause protein oxidation partially (Qian *et al.* 2021; Wojtyla *et al.* 2016). Excessive oxidation of proteins had been reported to seriously affect their structure and functionality (Tolouie *et al.* 2018).

Protein isolate can be subjected to plasma-activated water (PAW) treatment to avert the problem of excessive oxidation. In this case, protein isolates are hydrated in PAW to allow for uniform and sufficient interactions between reactive species and protein molecules (Bermudez-Aguirre, 2020). Plasma-activated water (PAW) is commonly used to disinfect medical equipment, eliminate microorganisms, and stimulate seed germination and growth (Herianto *et al.* 2021). A recent study reported that plasma-activated water induced the formation of compact chicken myofibrillar protein gel structures with intrinsic antibacterial activity (Qian *et al.* 2021). Furthermore, the impact of plasma-activated water on the morphological, functional, and digestibility characteristics of hydrothermally modified non-conventional talipot starch had been studied by Aaliya *et al.* (2022). However, studies on how plasma-activated water impact plant protein isolate are limited. Therefore, there is a need to exploit the effects of this indirect method of application of the cold plasma process on Bambara globulin.

Figure 2. 4: Cleavage of peptide bond by diamiade and α-amidation pathways

Source: Stadtman and Levine (2003)

Figure 2. 5: (A) Cleavage by oxidation of glutamyi residues and (B) clevage by oxidation of Aspartyl residues

Source: Stadtman and Levine (2003)

Figure 2. 6: Oxidation of aromatic amino acid residues

Source: Stadtman and Levine (2003)

2.8.3. Enzymatic modification of proteins to produce bioactive peptides

The structure of proteins can be changed by enzymes which in turn influences the functional properties (both the techno-functional and bio-functional) of the proteins. Enzymatic hydrolysis of proteins can be described as the catalytic reaction between proteolytic enzymes (such as pepsin, pancreatin, papain, trypsin, and alcalase) and protein substrates that result in the cleavage of peptide bonds in the primary amino acid sequence of the protein and breaking of the long-chain polypeptides substrate into short-chain peptides and amino acids with lower

molecular weights (Eckert *et al.* 2019). Enzymatic hydrolysis of protein can be endogenous (*in vivo*) or exogenous (*in vitro*) to release bioactive peptides from food proteins. The effectiveness of the hydrolysis process is usually dependent on the type of enzyme, the nature of the protein substrate, the enzyme-to-substrate volume ratio, process conditions including pH and temperature, and the presence/absence of proteolytic inhibitors (Ahmadifard *et al.* 2016). The extent of hydrolysis is steered by the degree of hydrolysis (DH), known to be a measure of the percentage of peptide bonds cleaved by the enzymes (Mokni Ghribi *et al*. 2015). The degree of hydrolysis determines the size and amino acid composition of the peptides in addition to the bioactivities of the generated peptides (Udenigwe and Aluko 2012; Marciniak *et al.* 2018). The bioactive properties of protein-derived peptides include antihypertensive, antioxidant, antidiabetic, antimicrobial, anticancer, hypocholesterolemic, immunomodulatory, and anti-inflammatory activities (Nwachukwu and Aluko 2021). These biological properties of food protein-derived peptides were reported to largely depend on the structure, amino acid composition, peptide sequence, peptide chain length, and charge (Girgih *et al.* 2016). For instance, protein-derived peptides with good antihypertensive properties have been reported to contain low molecular weight peptides comprising of branched-chain amino acids such as proline, and aromatic amino acids (Fu *et al.* 2017; Nwachukwu and Aluko 2021). The specific response of the protein-derived antihypertensive peptide to the inhibition of each of the two principal enzymes in the renin-angiotensin pathway (ACE and renin) is not the same (Girgih *et al.* 2016). Succinctly, the inhibition of ACE depends on the presence of peptides containing proline, aromatic, hydrophobic, and branched-chain amino acids whilst there is no positive correlation between the inhibition of renin and the presence of proline. In addition, peptides containing a high acidic amino acid content promote ACE inhibition. However, the inhibition of renin is favoured by the presence of a bulky amino acid residue at the C-terminus and a hydrophobic amino acid residue at the N-terminus.

2.8.4. ACE and renin inhibitory properties of protein

Hypertension is a prominent risk factor and a leading cause of death from cardiovascular diseases such as coronary heart disease, stroke, and heart and kidney failure (WHO 2013; Mazorra-Manzano, Ramírez-Suarez and Yada 2017), accounting for 7.1 million deaths annually (Tanzadehpanah *et al.* 2016). Hypertension can be controlled through the renin-angiotensin system (RAS) that helps to regulate the key enzymes, renin and angiotensin I-converting enzyme (ACE), which are responsible for causing blood pressure (BP) elevation (Crowley and Coffman 2012; Girgih *et al.* 2016; Fu *et al.* 2017).

In the RAS pathway, renin, an aspartyl protease, cleaves angiotensinogen to generate the decapeptide angiotensin I (AT-I), for the subsequent conversion into octapeptide angiotensin II (AT-II) by the angiotensin-1-converting enzyme (ACE) (EC 3.4.15.1) at the C-terminal end (Aluko *et al.* 2015; Girgih *et al.* 2016; Mazorra-Manzano, Ramírez-Suarez and Yada 2017). Angiotensin II (AT-II) is a potent vasoconstrictor that stimulates the release of aldosterone, which increases sodium concentration and contributes to elevated blood pressure. In addition, ACE is reported to hydrolyze bradykinin, a potent vasodilatory peptide, into inactivate peptide fragments (Aluko *et al.* 2015; Salampessy *et al.* 2015; Pan *et al.* 2016). Therefore, inhibition of ACE activity will reduce the formation of AT-II and the destruction of bradykinin, which contributes to the blood pressure-lowering effect. Also, the direct inhibition of renin activity will provide a more effective blockade of the RAS, since renin is known to catalyze the initial rate-limiting step (Azizi 2008; Aluko 2015). It has been reported that prolonged therapy of inhibiting ACE activity leads to increased renal renin secretion that results in high angiotensin I molecules which are converted via non-ACE routes involving chymase, kallikrein, and cathepsin enzymes to cause elevated angiotensin II levels (Malomo *et al.* 2015). Therefore, the simultaneous inhibition of ACE and renin activities will exert a synergistic effect on the efficient treatment of hypertension, compared to the individual enzyme inhibitors that are currently used (Harel *et al.* 2012; Connelly *et al.* 2013; Fu *et al.* 2017).

However, pharmaceutical drugs synthesized chemically are associated with some health-negative side effects such as erectile dysfunction, dry cough, angioedema, taste disturbance, decrease in white blood cells, fatigue, skin rash, and diarrhoea after prolonged use (Chen *et al.* 2013; Aluko 2015; Tao *et al.* 2017). Therefore, there is an urgent need for the development and production of natural food-derived peptides, particularly from plant sources, that can replace or complement synthetic ACE and renin inhibitors. Food-derived bioactive peptides have been reported to have relatively short residue lengths and contain arginine, lysine, and proline (Carbonaro, Maselli, and Nucara 2015), and are more rapidly absorbed with little or no negative side effects because of fast clearance from the blood (Sarmadi and Ismail 2010; Alashi *et al.* 2014). Recently, protein-derived peptides from pulses including hemp seed (Girgih *et al.* 2014), peanut (Jimsheena and Gowda 2011), pea (Li *et al.* 2011; Aluko *et al.* 2015), soy (Rho *et al.* 2009), and flaxseed (Udenigwe *et al.* 2012) had been studied as potential peptides with ACE and renin inhibitory properties.

2.9. Conclusion

Understanding the composition and structure of proteins influences their functional application as a food ingredient in food systems, The reactive oxygen species from direct cold plasma treatment alter the composition, physicochemical and structural properties of a protein, hence improving their functional property. However, there is minimal use of plasma-activated water (an indirect method of cold plasma application) for changing the structural composition of proteins to improve their functional properties. A research study focusing on the use of Bambara groundnut globulin and its subunits as a potential replacement for soy protein isolate and pea protein isolate in food applications such as emulsion, foaming, and gelation is essential to reduce the total reliance on soy and pea proteins for various applications. Information on the ACE and renin inhibitory properties of Bambara globulin and its vicilin fraction is necessary to create opportunities for these proteins in the pharmaceutical industries as potential bioactive functional foods.

2.10. Hypothesis

a. Bambara vicilin will be composed of heterogeneous polypeptides held together by hydrophobic interaction, consisting of varying subunits with no disulfide bond dominated by β-sheet structure. It will show better functional properties (solubility and gelation) than legumin and major storage proteins.

Vicilin had been reported to be composed of heterogeneous polypeptides (Mession *et al.* 2015) with varying subunits as observed in kidney beans (Shevkani *et al.* 2015), mung and black bean (Kudre *et al.* 2013). Vicilin was reported to show better functional properties than legumin owing to its structural flexibility (Lam *et al.* 2016). The presence of carbohydrate moieties was reported for soybean and French bean vicilin (Kimura *et al.* 2008).

b. Modification of Bambara globulin with atmospheric cold plasma-activated water will result in the unfolding of the protein structure through the etching process and influences the functional properties such as emulsion because of the formation of disulfide linkages and breakage of the covalent bond which thus changes the amino acids side chain.

Cold plasma treatment of protein has been reported to cause structural modification of protein (Bußler *et al.* 2015) due to alteration of the side chain of the amino acids in the protein by the

breakage of the covalent bond (Ji *et al.* 2018). These structural changes had been related to the increased solubility, water, and fat binding capacity recorded for the sample.

c. Enzymatic hydrolysis of Bambara vicilin with pepsin and pancreatin simultaneously using membrane size of less than 1 kDa and less than 3 kDa will produce low molecular peptide fragments with potential dual ACE and renin inhibitory properties.

Legume peptides released through enzymatic hydrolysis have been reported to possess antihypertensive activities with smaller sizes having greater ACE inhibitory activities (Alashi *et al*. 2014; Mundi and Aluko 2014). This may be because smaller-size peptides have been suggested to exhibit a high rate of intestinal absorption, without any structural changes (Mundi and Aluko 2014).

2.11. Aim

To characterize and modify Bambara globulin and subfractions for the enhancement of functional properties.

2.12. Objectives

The specific objectives are

a. To determine the composition, structure, and functional properties of vicilin (7S) and legumin (11S) fractionated from Bambara groundnut globulin.
b. To determine the effect of cold plasma-activated water treatment on the composition, structure, physicochemical, and the surface-active properties of Bambara groundnut globulin.
c. To determine the ACE and renin inhibition ability of enzyme hydrolysate of Bambara globulin and its vicilin (7S) fraction.

CHAPTER THREE

3. Composition and some functional properties of Bambara vicilin fraction

Abstract

Bambara groundnut is a drought-tolerant pulse grain and an alternative source of protein. In this study, the Bambara vicilin (7S) protein fraction was extracted and characterized in comparison to the storage protein. The proline content of the vicilin fraction (4.3 g/100 g protein) was almost four times that of the storage protein. Fluorescence and hydrophobicity data suggested a less folded structure for the vicilin fraction when compared to the storage protein. Gel electrophoresis indicated three major polypeptides (50, 70, and 80 kDa) in the vicilin fraction with the presence of disulfide bond(s) in one of the strands. In contrast to the storage protein, which had <40% protein solubility at pH 2-9, vicilin had a significantly ($p < 0.05$) higher solubility with a minimum of 50% at pH 5 and a maximum of 82% at pH 3. Emulsifying stability of the proteins significantly differed ($p < 0.05$) at pH 7. The foaming capacity of the vicilin fraction was significantly ($p < 0.05$) higher than that of the storage protein at pH 3, 7, and 9. It could be concluded that the Bambara vicilin fraction could serve as a potential ingredient in the formulation of food products at acidic pH.

3.1. Introduction

In recent decades, there is a growing interest in the utilization of leguminous grain storage proteins for food and industrial applications (Tavano *et al.* 2008; Nadathur, Wanasundara and Scanlin 2017). Among legume proteins, soybean protein has gained popularity in the food industry as a trusted food ingredient due to its nutritional value and functionality (Makeri *et al.* 2017). However, the inability of soybean to yield better on low fertility poor soil, and extreme drought conditions coupled with the growing demand for plant proteins have resulted in the search for alternative legume protein sources, especially from pulses.

The protein composition of legumes may vary remarkably with the variety or legume type. The major storage proteins in legumes are albumins and globulins (Shen and Tang 2014), with the latter further subdivided into vicilins (7S) and legumins (11S) in peas (Stone *et al.* 2015). Various isoforms of globulins (and vicilin in particular) with different polypeptide constituents may be present even in the same variety (Vasconcelos *et al.* 2010). Vicilin accounted for about 65-80% of the total protein in peas (Mession *et al.* 2015), and 89% in mung beans (Mendoza *et al.* 2001). It consists of 3-5 subunits in pea (Mession *et al.* 2015), 4 subunits in mung bean, and may be glycosylated (Mendoza *et al.* 2001). Vicilins have been found to possess a more hydrophilic surface, which makes them more soluble in aqueous solutions and have potentially better functionality when compared to legumins (Stone *et al.* 2015). For example, purified kidney bean vicilin showed better emulsifying properties than the protein isolates (Shen and Tang 2014). Knowledge of the composition and functionality of major storage proteins and corresponding fractions (e.g. vicilin or legumin) will be critical for the development of protein ingredients with excellent functional attributes such as emulsifying and foaming properties.
Bambara groundnut, a pulse grain of African origin (Mazahib *et al.* 2013) has been of interest due to its ability to grow under extreme drought conditions (Thammarat *et al.* 2015). It is also a good source of proteins (15-27%) (Arise, Amonsou and Ijabadeniyi 2015). In our previous study, we investigated the composition of the major storage proteins in Bambara grain (Arise *et al.* 2017). Based on SDS-PAGE data, vicilin (7S) was tentatively identified as the major protein fraction. Although, the gelation and microstructural properties of Bambara vicilin were recently studied (Diedericks *et al.* 2019), information on protein composition (e.g. amino acid distribution) and functional properties such as emulsifying and foaming properties of fractionated vicilin have not been investigated. Furthermore, the Bambara 7S fraction was not studied in comparison to its storage protein to ascertain that vicilin was successfully fractionated from its storage protein. This research further extends our knowledge of the

composition and functionality of Bambara vicilin (7S), which would be important to facilitate the utilization of Bambara proteins as food ingredients. Therefore, in this study, the Bambara vicilin fraction extracted by the ammonium sulfate precipitation method was subjected to functional characterization.

3.2. Materials and methods

3.2.1. Chemicals

Tris, glycine, sodium hydroxide, hydrochloric acid, sodium phosphate monobasic, sodium phosphate dibasic, potassium bromide, ANS (1,8-anilinonaphthalenesulfonate), β-mercaptoethanol (2-ME), ammonium sulfate, sodium citrate were purchased from Sigma-Aldrich, South Africa. Bradford reagent, BSA standard, and broad range molecular weight protein markers were purchased from Bio-Rad Hercules, USA.

3.2.2. Preparation of defatted flour

Bambara groundnut was obtained from Josini, KwaZulu-Natal province of South Africa, and the flour was defatted as previously described by Arise *et al.* (2015). Briefly, dehulled Bambara seeds were ground into flour and then defatted with n-hexane in the ratio 1:5 (g/ml) (flour: solvent) for 3 h. The defatted flour was left in a fume hood (HEMCO Uniflow LE Airestream, Missouri, USA) overnight to remove residual hexane and then stored at 4°C.

2.2.3. Extraction of Storage protein and vicilin

The method described by Sammour *et al.* (1984) was modified to extract the vicilin fraction. Defatted flour was extracted with 0.1 mol/L phosphate buffer (1 g flour:10 ml buffer) pH 8.0 at 4°C for 2 h, centrifuged at 8000x*g* for 30 min using an Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzig, Germany) and the precipitate discarded. Solid ammonium sulfate (291 g/L) was slowly added to the stirred supernatant at 4°C to obtain 50% saturation. This was centrifuged at 8000x*g* for 30 min after 2 h, and the pellet was discarded. The supernatant collected was adjusted to 70% saturation with ammonium sulfate (125 g/L) and centrifuged again. The supernatant collected was further brought to 90% saturation with ammonium sulfate (134 g/L) and centrifuged. The precipitate from the 70% and 90% saturation described above were collected, respectively, dissolved in 0.1 mol/L phosphate buffer (pH 8.0), dialyzed at 4°C against water, and then freeze-dried (Christ freeze-drier, Niedersachsen, Germany) as the storage protein and vicilin fraction, respectively.

3.2.4. Protein content and yield

Protein contents of Bambara storage protein and vicilin extracts were determined as described by Bradford (1976), respectively. The total protein weight (N×6.25) of the defatted flour was determined by the Kjeldahl method (AOAC 2012). The protein yield was calculated as % protein in the extract to that of the defatted flour.

3.2.5. Amino acid composition analysis

The amino acid profiles of Bambara proteins were determined using the HPLC PICO-TAG system (Waters, Milford, USA) (Bidlingmeyer, Cohen and Tarvin 1984). This method was based on the principle of reverse-phase chromatography with pre-column derivatization following acid digestion. The samples were hydrolyzed with 6 mol/L HCl at 116°C for 24 h before chromatographic analysis. 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 an Agilent ZORBAX Eclipse XDB C18 column, USA (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.

3.2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples were subjected to SDS-PAGE (reducing and non-reducing) according to the method of Laemmli (1970) with slight modifications. Each sample was dispersed (6 mg protein/mL) in Tris/HCl buffer, pH 8.0 containing 0.1 g SDS only (non-reducing buffer) or SDS + β-mercaptoethanol (reducing buffer), followed by heating at 95°C for 10 min, cooled centrifuged (10000x*g*, 15 min) using an Eppendorf microcentrifuge 5425 (Eppendorf Zentrifugen GmbH Leipzig, Germany). After centrifugation, 15 µl of aliquot containing 10 µg protein was loaded onto 4-12% gradient gels, and electrophoresis was performed with a mini-PROTEAN system (BIO-RAD, Hercules, USA). The standard protein marker (10-200 kDa) (broad range molecular weight, Bio-Rad Hercules, USA) was used. The gels were stained with Coomassie brilliant blue.

3.2.7. Surface hydrophobicity determination

Surface hydrophobicity (S_o) of the proteins was determined by the fluorescence method using a hydrophobic probe, 8-anilino-1-naphthalene sulfonic acid (ANS) on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) (Mohan and Udenigwe 2015). Protein stock solution (10 mg/ml) was prepared in 0.1 mol/L sodium

phosphate buffer pH 3.0, 7.0, and 9.0 followed by dilution to 0.0009-0.015 mg/ml. Fluorescence was then measured at excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the plot of fluorescence versus protein concentration was taken to be the hydrophobicity.

3.2.8. Intrinsic fluorescence emission

The method described by Arise *et al.* (2017) was used to record intrinsic fluorescence spectra on the JASCO FP-6300 spectrofluorometer (JASCO, Tokyo, Japan) at 25°C with a 1 cm path length cuvette. Protein stock solution (10 mg/ml) was prepared in 0.1 mol/L sodium phosphate buffer pH 3, 7, and 9. The buffer (pH 3, 7, and 9) was then used to dilute the respective stock solution to 0.002 mg/ml and fluorescence spectra were recorded at excitation wavelengths of 275 nm (tyrosine and tryptophan) with emission recorded from 280 to 450 nm. Emissions of the buffer blanks were subtracted from those of the respective samples to obtain the fluorescence spectra of the sample.

3.2.9. Differential scanning calorimetry (DSC)

Thermal properties of Bambara proteins were studied using a differential scanning calorimeter (model DSC-7, PerkinElmer, Norwalk, CT, USA) according to the modified method of Kudre, Benjakul and Kishimura (2013). The protein sample (1 g) was dispersed in 2.5 ml 0.05 mol/L sodium phosphate buffer (pH 7). The mixture was left for 12 h at 4°C and then 20 mg was accurately weighed into each aluminum pan. The pans were hermetically sealed and heated from 20 to 120°C at a rate of 5°C/min with a sealed empty pan used as a reference.

3.2.10. Protein solubility

The solubility profile of the Bambara proteins was determined as described by Malomo and Aluko (2015). Briefly, 10 mg of the sample was dispersed in 1 ml of distilled water. The pH of the suspensions was adjusted to values ranging from 2 to 9 with either 1 mol/L HCl or 1 mol/L NaOH and measured on a HI 2211 Hanna pH meter (Hanna Instruments, Leighton Buzzard, England). These suspensions were vortexed for 2 min at room temperature and centrifuged at 8000x*g* for 15 min using an Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzig, Germany). The protein content of the supernatant was determined using the Bradford method (1976). Total protein content was determined by dissolving the protein samples in 0.1 mol/L NaOH solution.

The protein solubility was calculated as:

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

3.2.11. Emulsifying activity and stability

The method described for the preparation of oil-in-water emulsion by Arise *et al.* (2014) was modified as follows. Protein slurries of 60 mg/ml concentration were prepared using 5 ml of 0.1 mol/L phosphate buffer pH 3, 7, and 9. The resulting suspensions were each homogenized with 5 ml of sunflower oil using a polytron homogenizer (PT 210: Fisher Scientific, Waterside, UK) at 10,000 rpm for 1 min. The emulsions were centrifuged at 1100 x*g* for 5 min at 4°C using an Eppendorf 5810R centrifuge. The height of the emulsified layer and that of the total content in the tube was measured to calculate emulsifying activity (EA). Emulsion stability (ES) was determined by heating the emulsion at 80°C for 30 min on a water bath (Julabo TW2, PA, USA) after which it was centrifuged at 1100 x*g* for 5 min.

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

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

3.2.12. Foaming capacity and stability

Foams were formed by dispersing a 300 mg protein sample in 5 ml of 0.1 mol/L phosphate buffer at pH 3, 7, and 9 as described by Arise *et al.* (2014). Samples were homogenized at 10,000 rpm for 1 min using a polytron homogenizer (PT 210: Fisher Scientific, Waterside, UK). Volumes of the suspensions before and after homogenization were recorded. Foam stability (FS) was calculated as the volume of foam that remained after standing for 8 h at room temperature. The foaming capacity (FC) was calculated as follows:

$$\text{FC (\%)} = \frac{\text{Volume after homogenization} - \text{Volume before homogenization}}{\text{Volume before homogenization}} \times 100$$

$$\text{FS (\%)} = \frac{\text{Volume after 8 h}}{\text{Initial Volume}} \times 100$$

3.2.13. Statistical analysis

All experiments were done in triplicate unless otherwise stated. Means were computed and Kruskall-Walli's test was used to analyze data ($p < 0.05$).

3.3. Results and discussion

3.3.1. Protein content and yield

The protein content of the Bambara vicilin fraction was 94%. This was substantially higher than that of its storage protein (83%). The high protein content of the vicilin fraction suggested that it is of relatively high purity. The yields of the Bambara storage protein, consisting of albumin and globulin, and its corresponding vicilin fraction were 71% and 81%, respectively. Klassen and Nickerson (2012) reported similar levels of protein content (92%) for crude pea vicilin fraction. The protein content obtained in this study is higher than the values reported for kidney bean, red bean, and mung bean vicilin fractions (Tang and Sun 2011).

3.3.2. Amino acid profile of Bambara storage protein and vicilin

Aspartic and glutamic acids including asparagine and glutamine respectively were the major amino acids of Bambara proteins. The proline content of the vicilin fraction was about four times higher than that recorded for the storage protein (Table 3.1). Proline can impart a rigid structure to vicilin by participating in folding and unfolding processes. The cyclic sidechain of proline introduces a kink in protein structure, which is known to prevent the formation of protein beta-pleated conformation (Damodaran, Parkin and Fennema 2008). This helps in preventing protein aggregation and increases protein solubility as described further in this study. The hydrophobic and aromatic amino acid (e.g. phenylalanine) content was higher in the vicilin fraction than in the storage protein. Bambara proteins had considerable amounts of sulfur-containing amino acids, which are usually limiting in legume seeds. Negatively charged amino acids (NCAA) such as glutamic and aspartic acids have excess electrons, which enhances iron-reducing properties for the prevention of iron-induced lipid oxidation (Nwachukwu and Aluko 2018). The relative content of the positively charged amino acid for the Bambara 7S fraction was lower than that reported for red bean (18.6%), and mung bean (19.5%) vicilin (Tang and Sun 2011).

3.3.3. Gel electrophoresis profile of Bambara vicilin

SDS-PAGE pattern of the Bambara vicilin fraction showed fewer protein bands compared to the storage protein (Fig. 3.1). The storage protein consisted of <28 kDa polypeptides that were mainly absent in the vicilin. Under non-reducing conditions, the major vicilin polypeptide was evident as the most intense band around 50 kDa together with two additional minor polypeptides of molecular weights of 70 and 80 kDa, respectively. The bands at 70 and 80 kDa could represent another subunit of vicilin, which may correspond to the convicilin in peas (Chen *et al.* 2019). The polypeptide around 80 kDa might also correspond to the subunit of legumin in pea protein reflecting cross-contamination from the storage protein (Klassen & Nickerson, 2012). Three major bands (53, 65, and 118 kDa) were reported for Bambara vicilin extracted by the isoelectric precipitation method (Diedericks *et al.* 2019). SDS-PAGE data indicated that vicilin was successfully fractionated from the total seed storage proteins.

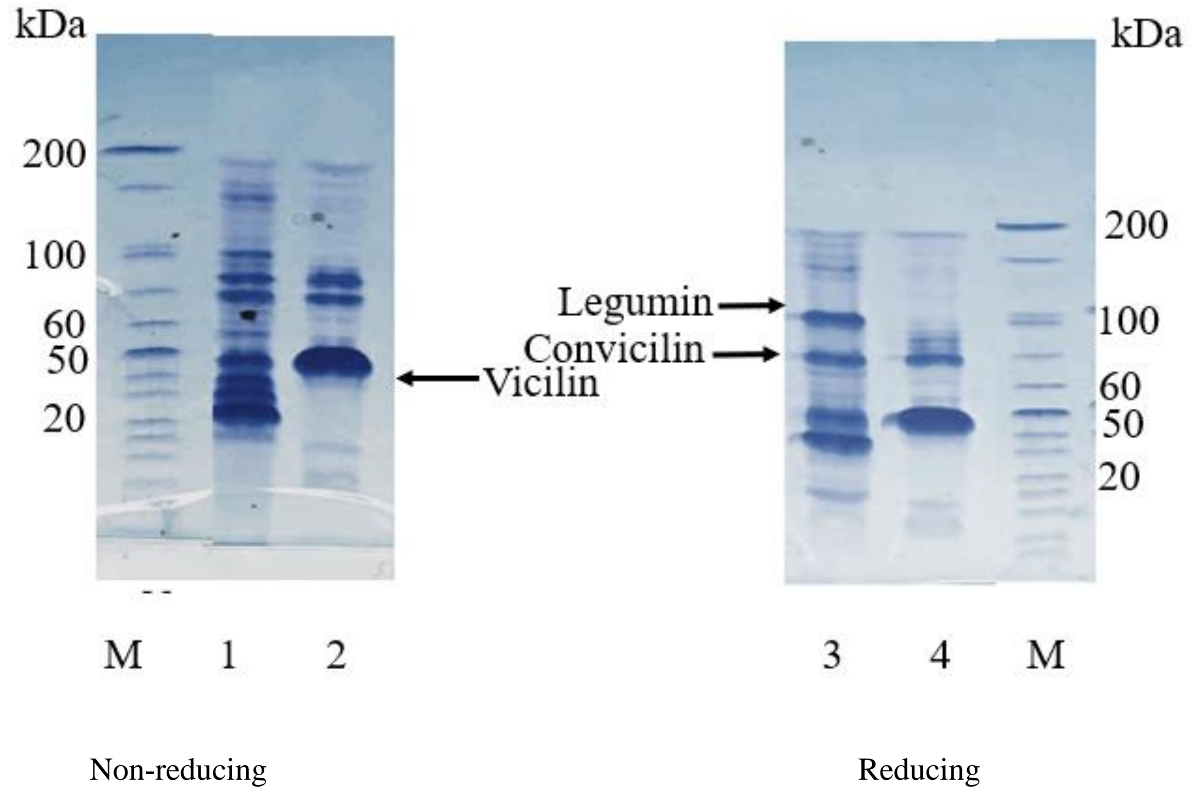
3.3.4. Fluorescence emission spectra of Bambara vicilin

Intrinsic emission fluorescence spectroscopy showed the conformational changes in Bambara proteins at different pH (pH 3, 7, and 9) (Fig. 3.2). The fluorescence spectra showed maximum intensity at 342 and 345 nm, respectively for Bambara proteins at pH 7 and 9 reflecting the dominancy of tryptophan residues. These data further suggest that the tryptophan is more buried in the hydrophobic environment within the protein due to the observed slight blue shift emission at pH 7 (Ajibola *et al.* 2016). The vicilin fraction showed a reduced emission intensity compared to the storage protein, possibly due to the differences in amino acid composition and structural conformation. The vicilin fraction had less tryptophan content (Table 3.1) compared to the storage protein. It also contains higher proline content which could destabilize the protein structure by preventing aggregation (Damodaran, Parkin and Fennema 2008). Weak fluorescence intensity (FI) was recorded for the Bambara storage protein and its vicilin fraction at pH 3. This could result from protein denaturation at acidic pH which completely exposed the tryptophan residues to the hydrophilic environment, hence the total fluorescence quenching.

Table 3. 1: Amino acid composition (g/100 g protein) of Bambara storage protein and its vicilin fraction

Amino acid	Storage protein	Vicilin	FAO/WHO 1991
Asx	12.3 ± 0.0	12.9 ± 0.0	
Thr	4.2 ± 0.0	3.9 ± 0.0	3.4
Ser	6.1 ± 0.0	6.1 ± 0.0	
Glx	18.0 ± 0.0	17.6 ± 0.0	
Pro	1.2 ± 0.0	4.3 ± 0.0	
Gly	4.3 ± 0.0	3.5 ± 0.0	
Ala	4.8 ± 0.0	4.0 ± 0.0	
Cys	1.2 ± 0.0	0.9 ± 0.0	
Val	4.7 ± 0.0	3.8 ± 0.0	3.5
Met	1.6 ± 0.0	1.2 ± 0.0	
Ile	3.6 ± 0.0	3.9 ± 0.0	2.8
Leu	7.2 ± 0.0	7.5 ± 0.0	6.6
Tyr	3.8 ± 0.0	3.9 ± 0.0	1.1
Phe	4.1 ± 0.0	6.7 ± 0.0	6.3
His	4.3 ± 0.0	3.3 ± 0.0	1.9
Lys	8.8 ± 0.0	7.2 ± 0.0	5.8
Arg	7.0 ± 0.0	6.7 ± 0.0	
Trp	1.2 ± 0.0	0.6 ± 0.0	
HAA	33.4 ± 0.0	36.8 ± 0.0	
NCAA	30.3 ± 0.0	30.5 ± 0.0	
PCAA	20.1 ± 0.0	17.3 ± 0.0	

Asx = aspartic acid and asparagine; HAA = hydrophobic amino acid; PCAA = positively charged amino acid; NCAA = negatively charged amino acid.



Non-reducing

Reducing

Figure 3. 1: SDS-PAGE of Bambara proteins.

Lane M: Protein marker, Lanes 1 and 3: Storage protein, Lanes 2 and 4: Vicilin

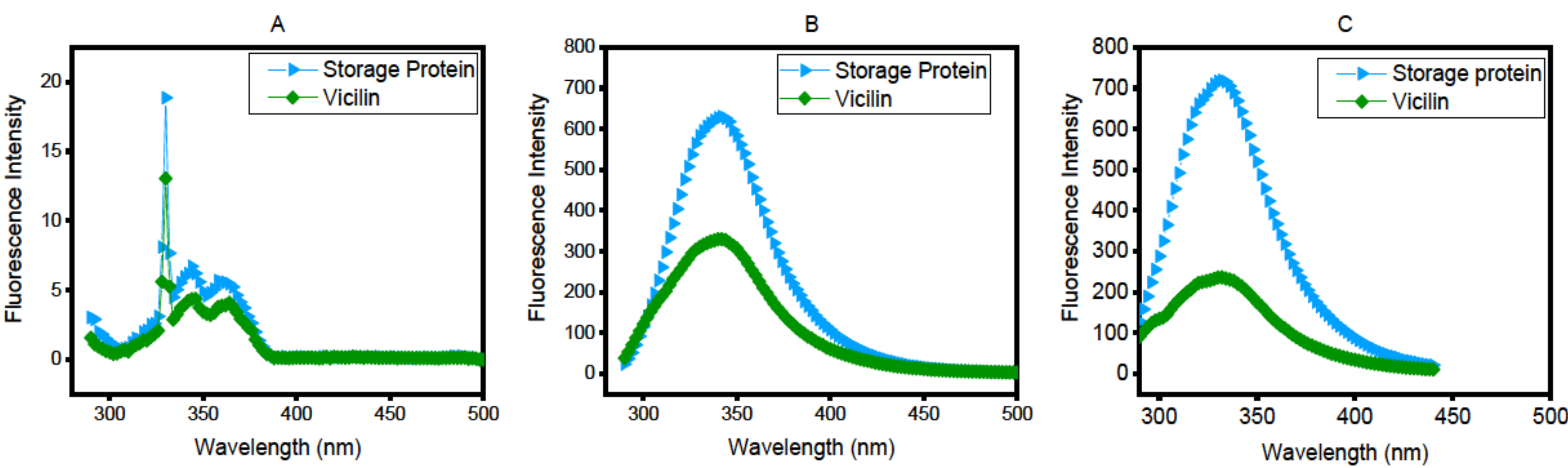


Figure 3. 2: Fluorescence spectra of Bambara storage protein and its vicilin fraction

(A) pH 3 (B) pH 7, and (C) pH 9

3.3.5. Hydrophobicity (S_O) property of Bambara vicilin

Bambara vicilin fraction had the highest So at pH 3 (Fig. 3.3). The hydrophobicity values of the proteins were significantly lowered at pH 7 and pH 9. The hydrophobicity property displayed at acidic pH could be attributed to denaturation, which enhanced the exposure of more hydrophobic patches on the surface of the protein at acidic pH with a concomitant increase in hydrophobicity. This agrees with the complete quenching of fluorescence observed at pH 3 (Fig. 2) (He *et al.* 2014). The So values obtained for Bambara proteins at pH 7 are significantly higher than the values reported by Tang and Sun (2011) for kidney beans (148.6) and mung bean vicilin (259.3). However, even though the protein may be denatured, hydrophobic cavities in the unfolded protein could be responsible for the interaction with ANS and the observed high hydrophobicity.

3.3.6. Differential scanning calorimetry (DSC)

The thermal stability of the proteins using the denaturation temperature (Td) as an indicator was evaluated by the DSC technique. The vicilin fraction showed a single endothermal peak (94℃) when compared to the storage protein, which had two transitions at 79℃ and 94℃, respectively (Fig. 3.4). The high thermal stability of the vicilin fraction might be associated with the high molecular protein subunits as indicated in the gel electrophoresis. The Td reflected the disruption of hydrogen bonds maintaining the conformation of the proteins, particularly the tertiary conformation (Tang and Sun 2011). Higher Td value for the proteins suggested a more compact tertiary conformation of the polypeptides. The Td values (91.4, 80.8, and 85.4 ℃) reported for kidney bean, Mung bean, and Red bean vicilin, respectively (Tang and Sun 2011) were lower than that obtained in this study. The ΔH values obtained for Bambara storage protein and its vicilin fraction in this study are 6.22 J/g and 12.34 J/g, respectively. The enthalpy of denaturation (ΔH) represents the proportion of undenatured protein in a protein sample and is correlated with the extent of the ordered structure of a protein (Ma *et al.* 2018). ΔH also reflects the extent of protein structural unfolding, especially the extent to which the tertiary conformation of a protein is unfolded.

3.3.7. Solubility profile of Bambara vicilin

Bambara storage protein and its vicilin fraction exhibited a V-shaped solubility curve, respectively in the pH 2-9 range (Fig. 3.5). Unlike the storage protein, vicilin fraction displayed a very high solubility (up to 82%) at acidic pH (pH 3). In fact, up to 50% solubility, which is 5

folds more than that of the storage protein was recorded for Bambara vicilin fraction at pH 5. Protein solubility increased when the pH values increased gradually from 5 to 9 or reduced from 5 to 2. This is because, at high acidic or high alkaline pH values, proteins carry net positive or negative charges, respectively. Thus, electrostatic repulsion and ionic hydration could have promoted protein solubilization (Aryee, Agyei and Udenigwe 2018). The solubility pattern of the vicilin fraction might be due to its high proline content. Proline enhances structural flexibility. The percentage solubility value for vicilin fraction at pH 3 correlated with the data (89%) reported for 7S fraction from kidney bean, mung bean, and red bean, respectively at pH 3 (Tang and Sun 2011). Tang *et al.* (2011) also reported 90% solubility for native *phaseolin* at a pH $\leq$ 3.0. The data obtained suggested that Bambara vicilin fraction is a potential ingredient in the food industry, particularly in the acidic beverage industry.

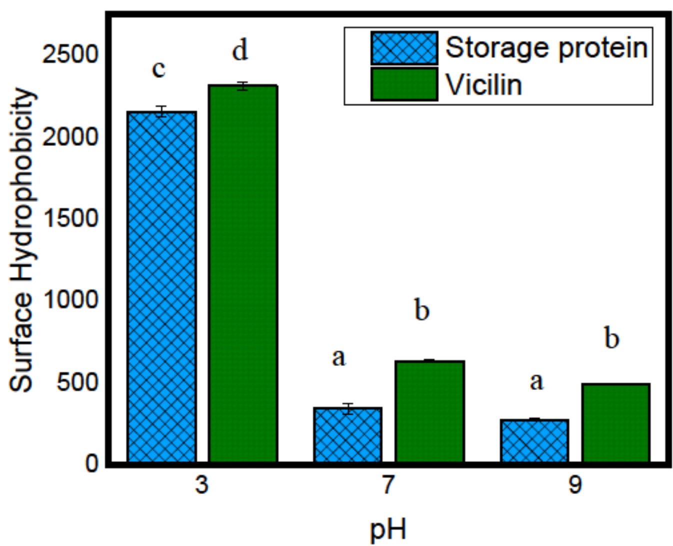


Figure 3. 3: Surface hydrophobicity of Bambara storage protein and vicilin fraction

*Different letters on bars indicate significant differences between the treatment

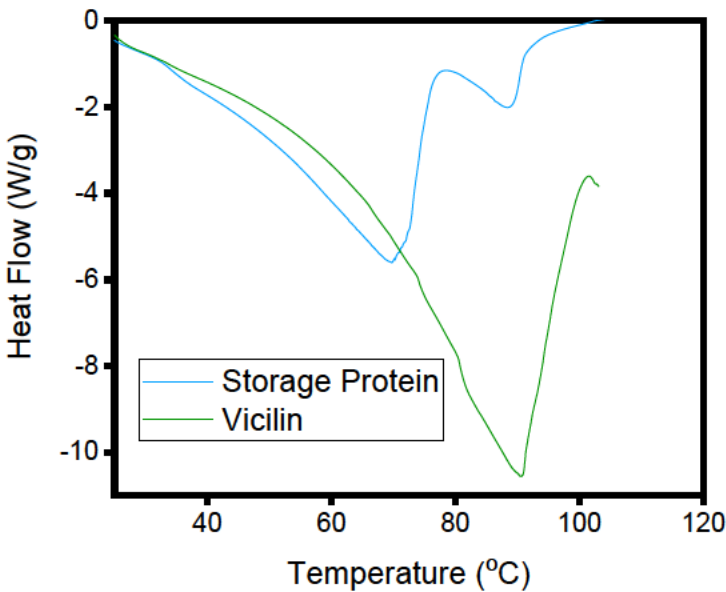


Figure 3. 4: Heat flow pattern of Bambara storage protein and its vicilin fraction.

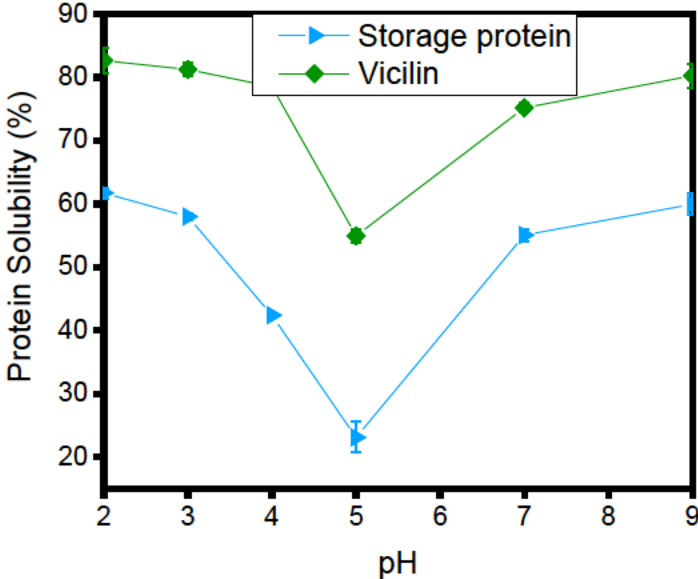


Figure 3. 5: Solubility profile of Bambara storage protein and its vicilin fraction as a function of pH

3.3.8. Emulsifying properties of Bambara vicilin

The percentage emulsifying activity values of the Bambara vicilin fraction and its storage protein increased from pH 3 to pH 9 (Table 3.2). Bambara vicilin fraction had >95% emulsion stability at pH 3, 7, and 9. This could be attributed to its high protein content, solubility (Aryee, Agyei and Udenigwe 2018) and surface hydrophobicity (Ma *et al.* 2018)). High hydrophobicity improves the hydrophobic-hydrophilic balance, which is necessary to form strong interfacial membranes for effective stabilization of the oil-water interface (Lam and Nickerson 2013). High solubility could have enhanced greater diffusion rates and increased protein accumulation at the oil-water interface (Lam and Nickerson 2013). The emulsion stability of Bambara storage protein and its vicilin at pH 3 and 9 did not significantly differ (Table 3). However, vicilin fraction was a more effective emulsion stabilizer at pH 7.0, which suggests stronger interactions with the oil droplets.

3.3.9. Foaming properties of Bambara vicilin

The highest foaming capacity values were observed at pH 3.0 for storage protein and its vicilin fraction (Table 3.3). The highest foaming capacity of crude vicilin fraction at pH 3 is consistent with its percentage solubility. At this pH, the unfolded state of the proteins increased their ability to adsorb at the air-water interface and rearrange to form cohesive viscoelastic films through intermolecular interactions (Houde *et al.* 2018). The high foaming capacity of the vicilin fraction is consistent with its greater solubility, which enables better flexibility, and a more efficient air bubble encapsulation compared to the storage protein. The slight decrease in the foaming capacity at pH 7 and 9 could be attributed to a higher net negative charge. This could have reduced the accumulation of protein molecules at the air-water interface, hence reducing the capacity to encapsulate the air bubbles (Shevkani *et al.* 2015). Unlike the foaming capacity, Bambara vicilin fraction and its storage protein showed good foam stability (up to 81%), which was independent of pH.

Table 3. 2: Emulsifying properties (%) of Bambara storage protein and vicilin fraction

Samples	Emulsifying Activity			Emulsion Stability		
	pH 3	pH 7	pH 9	pH 3	pH 7	pH 9
Storage protein	$37^a \pm 2$	$40^a \pm 2$	$43.2^a \pm 0.8$	$96.9^a \pm 0.4$	$92.7^a \pm 0.4$	$100^a \pm 0.0$
Vicilin	$38^a \pm 1$	$41.7^a \pm 0.2$	$43.7^a \pm 0.8$	$96.9^a \pm 0.1$	$96.4^a \pm 0.0$	$100^a \pm 0.0$

Mean ± SD (n=3). Values along the column followed by the same superscripts are not significantly different.

Table 3. 3: Foaming properties (%) of Bambara storage protein and vicilin fraction

Samples	Foaming Capacity			Foam Stability		
	pH 3	**pH 7**	**pH 9**	**pH 3**	**pH 7**	**pH 9**
Storage protein	$88^{a} \pm 0$	$70^{a} \pm 0$	$71^{a} \pm 1$	$80^{a} \pm 0$	$76^{a} \pm 1$	$79^{a} \pm 1$
Vicilin	$91^{a} \pm 1$	$86^{a} \pm 1$	$88^{b} \pm 0$	$81^{a} \pm 1$	$79^{a} \pm 1$	$80^{a} \pm 0$

Mean ± SD (n=3). Values along the column followed by the same superscripts are not significantly different.

3.4. Conclusions

The impact of amino acids and molecular composition on the functional properties of Bambara vicilin compared to the whole storage protein isolate are reported in this study. Vicilin is the major fraction of the Bambara storage protein isolate with an intense band of around 50 kDa. Bambara vicilin displays better foaming and emulsifying behaviours than the storage protein isolate. Vicilin displays substantially high solubility at acidic pH (pH 3.5) which might create an opportunity for its application in acidified beverages. The observed functional properties of vicilin could be attributed to its high proline content and less folded structure.

3.5. Research outputs

a) Journal article

1. Alabi O.O., Ali, N., Nwachukwu, I.D., Aluko, R.E., Amonson, E.O. 2020. Composition and some functional properties of Bambara vicilin fraction. LWT-Food Science and Technology 125: 109256

b) Conference abstracts

1. Ali, N., **Alabi, O.O.**, Udechukwu, M., Nwachukwu, I.D., Aluko, R.E., Amonsou, E.O. (2018). Fractionation and functionality of Bambara groundnut vicilin (7S) Protein. Presented at the 32nd EFFOST International Conference: Developing innovative food structures and functionality through process and reformulation to satisfy consumers need and expectations, 6th-8th November, Nantes, France.

CHAPTER FOUR

4. Extraction, characterization and rheological properties of vicilin and legumin proteins derived from Bambara globulin

Abstract

Understanding the structure-composition and functionality of pulse proteins and their subunits is essential to facilitate their utilization as a replacement for animal-based proteins. In this study, Bambara globulin was extracted and fractionated into constituent sub-fractions for structural, physicochemical, and heat-induced gel rheological characterization. Although vicilin was the major fraction of Bambara globulin, a legumin-like protein was found in relatively low quantity. Globulin revealed three major protein bands at 50, 58, and 70 kDa, similar to vicilin with predominant β-sheet structures. One basic subunit (22 kDa) was identified in the legumin fraction. Vicilin displayed the highest sol-gel transition temperatures (80°C) followed by legumin (50°C) and globulin (40°C). After the gel point, the G′ and G″ of globulin showed relatively low dependency on heating time, suggesting a rapid establishment of its gel network. Vicilin and legumin displayed a more progressive establishment of their protein, with G′ increasing over the heating time. Vicilin gel consisted of a microporous structure with a small lath sheet-like structure compared to globulin and legumin. The presence of disulfide linkages in globulin and legumin, the number of protein subunits, and protein hydrophobicity appear to be the most influencing factors on gelation of Bambara globulin and its fractions and consequently their application as food protein gels.

4.1. Introduction

Bambara groundnut (*Vigna subterranea*) is an underutilized pulse grain of African origin that can contribute to reducing global food insecurity (Halimi *et al.* 2019). It is a drought-tolerant plant (Halimi *et al.* 2019). As a good source of protein (19-25%) (Chinma *et al.* 2015), Bambara can potentially be used as an alternative to soya protein alongside peas and cowpea. Despite the agronomic and nutritional potentials, Bambara grains are not globally commercialized (Stamp, Messmer and Walter 2012; Yao *et al.* 2015), which may be attributed to limited research in comparison with pea and lack of market value (Mayes *et al.* 2015). Recent studies have shown that Bambara groundnut could be used in the preparation of value-added products like vegetable milk and yogurt (Falade *et al.* 2014), bread (Chinma *et al.* 2015; Abdulrahman *et al.* 2017), and extruded snacks (Oluwole *et al.* 2013). These products could meet the growing demand for healthier products and reduce food insecurity by responding to the hazard of climate change.

The exploration and exposition of structure-function relationships of grain proteins with limited industrial applications are vital to unlocking their potential and facilitating utilization. The interaction of protein grains with their components and ingredients in a complex system is controlled by their structure-function relationship (Joshi *et al.* 2012). The functionality of proteins, such as solubility, rheological behaviour, and gelation properties, is dependent on their molecular and structural composition (Shevkani *et al.* 2019), which is consequently affected by protein extraction method, processing parameters (pH and temperature) (Sun and Arntfield 2012). Globulins including legumin and vicilin are the major storage protein in most leguminous grains (Agarwal 2017). The various isoforms of globulins such as legumin and vicilin may have different polypeptide constituents in the same variety of legumes.

The differences in the polypeptide composition of these isoforms of globulins affect their structural properties and increase the complexity to understand and exploit the properties of these proteins. Thus, limiting their utilization in many food formulations. Previous studies have investigated the structure and molecular composition of globulin proteins, including legumin and vicilin from other grains like mung bean (Tang and Sun 2010), pea, fava bean, and cowpea (Kimura *et al.* 2008). For example, Tang and Sun (2010) reported that the secondary and tertiary structures of mung bean 11S globulin were much more flexible and unfolded compared to the 7S globulin.

Although, chemical and functional properties of Bambara groundnut storage proteins had been reported (Adebowale, Schwarzenbolz and Henle 2011; Arise *et al.* 2017). Some recent studies also generated some information on the gel properties of Bambara vicilin (Diedericks *et al.* 2019) and Bambara storage protein (Ruzengwe, Amonsou and Kudanga 2020). In our previous study, we investigated the composition and some functional properties of Bambara groundnuts vicilin fraction extracted by ammonium sulfate precipitation (Alabi *et al.* 2020). However, structural information including functional attributes, such as gelation and rheological properties, is still lacking in Bambara legumin. Bambara legumin has not been characterized in comparison to vicilin and globulin.

The knowledge of the molecular and structural composition of legumin will be crucial in explaining the functionality of this protein for the development of novel protein ingredients for various food applications. Though the characterization of Bambara groundnut protein has been progressive, additional knowledge on the molecular and structural composition of legumin is required. This study further broadens our understanding of the structure and composition of Bambara legumin fraction compares to vicilin and globulin, to facilitate its utilization. Furthermore, the impact of structure and composition on the gelation process of Bambara legumin fraction relative to vicilin and globulin was also investigated.

4.2. Materials and methods

4.2.1. Materials

Bambara groundnut (Maroon colour) was obtained from Josini, KwaZulu-Natal province of South Africa. All reagents used for the analyses were purchased from Sigma-Aldrich, South Africa, and Bio-Rad Hercules, USA.

4.2.2. Preparation of defatted flour

Bambara seeds were ground into flour and then defatted with n-hexane in the ratio 1:5 (g/mL) (flour: solvent) for 3 h as previously described by Arise *et al.* (2015).

4.2.3. Extraction of Bambara globulin, legumin, and vicilin

The extraction process was adapted from the method reported by Klassen and Nickerson (2012) with slight modification employed. Salt-soluble proteins were extracted with 50 mM K_2HPO_4 buffer, 0.5 M NaCl at pH 7.3, with a flour-to-buffer ratio of 1:10. The mixture was stirred continuously, using a magnetic stirrer, for 1 h at room temperature and centrifuged at 18,000

×g for 25 min at 4°C using an Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzig, Germany). The supernatant was then diluted with cold Milli-Q water in a ratio 1: 4 to facilitate the settling of the salt-soluble protein, adjusted to pH 4.6 using 1 M HCl, and left overnight in a cold room to facilitate the settling of the salt-soluble proteins. The precipitated protein was collected by centrifugation and washed twice with Milli-Q water (pellet-to-water ratio of 1:10) to remove unwanted albumin proteins. The precipitated protein was redissolved in Milli-Q water and dialyzed (dialysis tubing of 6-8 kDa) against water. The dialysis was done for 72 h to remove the salt. The dialysis water was changed three times daily. The pH of the de-salted protein extract was adjusted to 7.0 and then freeze-dried (Christ freeze-drier, Niedersachsen, Germany) as the globulin and stored at -20°C.

For legumin and vicilin extraction, the globulin was re-suspended in the extraction buffer at a volume-weight ratio of 5 mL per gram pellet and stirred continuously for 1 h at 4°C. The protein extract was collected by centrifugation, and this extraction step was repeated twice. The supernatant was pooled together and dialyzed against Mcllvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, pH 4.8) containing 0.2 M NaCl at 4°C for 72 h, with the buffer being changed three times. The legumin precipitate was collected by centrifugation (18,000 ×g for 25 min at 4°C). Vicilin was precipitated from the supernatant at pH 4.5 with 1 M HCl. Legumin and vicilin were washed, re-centrifuged, and dialyzed against water as described above. The pH of legumin and vicilin were adjusted to pH 7.0 before being freeze-dried and stored at -20°C.

4.2.4. Protein content and yields

Protein contents of Bambara globulin, legumin, and vicilin extracts were determined using the modified Lowry method (Markwell *et al.* 1978). The protein yield was calculated as % protein in the extract to that of the defatted flour.

4.2.5. Amino acid analyses

The amino acid profiles of Bambara globulin, legumin, and vicilin were determined using the HPLC PICO-TAG system (Waters, Milford, USA) (Bidlingmeyer, Cohen and Tarvin 1984). The samples were hydrolyzed with 6 mol/L HCl at 116 °C for 24 h before chromatographic analysis. The methionine contents were determined after performic acid oxidation (Gehrke *et al.* 1985). The digests were separated on an Agilent ZORBAX Eclipse XDB C18 column, USA (4.6 x 150 mm) using a gradient of sodium citrate buffers (pH 3.45 and pH 10.85) at 0.45 mL/min flow rate.

4.2.6. Gel electrophoresis

SDS-PAGE (under reducing and non-reducing conditions) was carried out with a 4% stacking gel and 12% resolution gel, as described by Laemmli (1970).

4.2.7. High-performance size-exclusion chromatography

The molecular weight of Bambara globulin, legumin, and vicilin samples was determined using high-performance size exclusion chromatography (Shimadzu Prominence-i LC-2030C, Japan). Briefly, freeze-dried Bambara protein samples were dissolved (10 mg/mL) in sodium phosphate buffer (pH 7), and an aliquot of 10 µL (filtered through 0.2 µm membrane disks) was injected onto a 300 × 7.8 mm Yarra 3 µm SEC 2000 column (Phenomenex, Torrance, CA). Protein samples were eluted from the column at a temperature of 30°C and flow rate of 0.2 mL/min using a linear gradient (0–100%) of water that contained 0.1 ml/100 mL TFA (solvent B) for 60 min. Peak detection was at a wavelength of 214 nm.

4.2.8. Surface hydrophobicity (S_o)

Surface hydrophobicity (S_o) of Bambara proteins was determined on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) using a hydrophobic probe, 8-anilino-1-naphthalene sulfonic acid (ANS), as described by do Evangelho *et al.* (2017). Briefly, protein stock solution (10 mg/mL) was prepared in 0.1 mol/L phosphate buffer (pH 7.0), solubilized for 1 h and then centrifuged at 10,000 ×g for 15 min. The protein stock was diluted to varying concentrations of 0.0009 - 0.015 mg/mL with phosphate buffer to make up 4 mL. To these, 20 µL of ANS (8 mM) was added, and the mixtures were vortexed for 30 s. Fluorescence was measured at excitation and emission wavelengths of 390 and 470 nm, respectively. The fluorescence intensity of the control (ANS + phosphate buffer) was measured and subtracted from that of the sample to obtain the net intensity, which was plotted against protein concentration. Hydrophobicity was calculated as the slope of the plot of fluorescence versus protein concentration.

4.2.9. Intrinsic fluorescence emission

Fluorescence emission spectra of the protein samples were recorded on a JASCO FP-6300 spectrofluorometer (JASCO, Tokyo, Japan) at 25°C with a 1 cm path length cuvette as previously described by Arise *et al.* (2017). Briefly, each protein sample (10 mg) was dissolved in a 0.1 mol/L phosphate buffer (1 mL), pH 7.0. The mixtures were centrifuged at 10,000 ×g

for 15 min. The respective protein stock solution was diluted to 0.002 mg/mL with the buffer, and the fluorescence spectra were recorded at excitation wavelengths of 275 nm (tyrosine and tryptophan) with emission recorded from 280 to 450 nm wavelength scan.

4.2.10. Circular dichroism (CD)

CD spectra of Bambara globulin, legumin, and vicilin were measured in a J-810 spectrofluorometer (JASCO, Tokyo, Japan) at 25℃ using the spectral range of 190-240 nm (far-UV) for secondary structure and 250-320 nm (near-UV) for tertiary structure determinations as previously described by Omoni and Aluko (2006).

4.2.11. Differential scanning calorimetry

Thermal properties of Bambara proteins were studied using a differential scanning calorimeter (model DSC-7, PerkinElmer, Norwalk, CT, USA) according to the modified method of Peng *et al.* (2020). The protein sample (2 mg) (based on protein content) was weighed into an aluminum pan, and 10 µL of 0.05 mol/L sodium phosphate buffer (pH 7) was added. The protein samples were equilibrated at 25℃ for more than 6 h (overnight). The pan was hermetically sealed and heated from 20℃ to 150℃ at a rate of 5℃/min. A sealed empty pan was used as a reference. Onset temperature (To), peak transition or denaturation temperature (Td), and enthalpy change of the endotherm (ΔH) were computed from the thermograms by the Universal Analysis 2000, Version 4.1D (TA Instruments-Waters LLC).

4.2.12. Particle size and zeta potential (ζ)

The zeta potential of Bambara protein fractions was measured over a pH range of 2.0-9.0 at a 1-unit increment using Litesizer Nano ZS (Anton Paar, New Castle, Delaware). The samples (diluted dispersion 2 mg/mL) were hydrated for 1 h and filtered through a 0.45 µm HA Millipore membrane before analysis. The particle size of the samples prepared above was determined at pH 7 using the same instrument.

4.2.13. Protein solubility

The solubility of the protein samples was determined as a function of pH following the method described by Malomo and Aluko (2015). Briefly, 10 mg of the sample was dispersed in 1 ml of distilled water. The pH of the suspensions was adjusted to values ranging from 2 to 9 with either 1 mol/L HCl or 1 mol/L NaOH and measured on a HI 2211 Hanna pH meter (Hanna Instruments, Leighton Buzzard, England). These suspensions were vortexed for 2 min at room

temperature and centrifuged at 8000x*g* for 15 min using Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzig, Germany). The protein content of the supernatant was determined using the Bradford method (1976). Total protein content was determined by dissolving the protein samples in 0.1 mol/L NaOH solution.

The protein solubility was calculated as:

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

4.2.14. Least gelation concentration

The least gelation concentration was determined, as described by Adebowale, Schwarzenbolz and Henle (2011). Bambara protein fractions were dissolved in distilled water at varying concentrations (2-20% based on protein concentration). The mixture was vortexed, adjusted to pH 7, and centrifuged at 8000 ×g for 15 min. The supernatant was heated in a water bath (Julabo TW2, Germany) for 1 h at 95°C. The samples were rapidly cooled with cold running water, and further cooled for 2 h in a refrigerator. The test tubes containing the gels were inverted. The concentration at which the gel does not slip is the least gelation concentration (LGC).

4.2.15. Steady-shear viscosity of Bambara groundnut proteins dispersion

A 10% (w/v) concentration dispersion (based on protein content) was prepared for each sample. The steady-shear viscosity properties of the protein fraction and subunits were determined at 25 °C in a Physica MCR102 rheometer (Anton Paar, New Castle, Delaware) (Berghout, Boom and van der Goot 2015). To evaluate the steady-shear viscosity as a function of shear rate, the shear stress was measured with the application of a given shear rate from 1 s^{-1} to 300 s^{-1} using a unidirectional steady-shear flow.

4.2.16. Preparation of Bambara protein fractions gels

Bambara protein fractions gels were prepared at 22.5% concentration (based on protein content) and at concentrations corresponding to the least gelation concentration of individual protein fractions. The mixture was vortexed, adjusted to pH 7, and centrifuged at 8000 ×g for 15 min. The supernatant was heated in a water bath (Julabo TW2, Germany) for 1 h at 95°C. The samples were rapidly cooled with cold running water.

4.2.17. Rheological properties of Bambara protein fraction gels

The rheological properties of the Bambara groundnut protein fractions gels were determined using a rheometer (Anton Paar MCR 102, New Castle, Delaware) (Berghout, Boom and van der Goot 2015). A frequency sweep test was performed on the gels at constant strain (0.5%) within the linear viscoelastic region (LVR) to obtain the gel fingerprints or mechanical spectra. The test was done using a 25 mm diameter plate-plate geometry, 1 mm zero-gap, and an increasing angular frequency of 0.1-100 rad s^{-1} at 25°C to determine the time scale-dependent viscoelastic response parameters. The edge of the gel samples was coated with a thin layer of paraffin oil to prevent drying out. The gel samples were equilibrated for 5 min.

4.2.18. Scanning electron microscopy (SEM)

The surface morphology of Bambara protein fractions gels prepared at 22.5% concentration was examined using scanning electron microscopy (SEM, Zeiss Ultra Plus FEG SEM, Zeiss, Germany) at an accelerating voltage of 5 kV following a previously reported method (Klost and Drusch 2019).

4.2.19. Gelation of Bambara dispersion

The gelation process of Bambara protein dispersion (22.5% w/v based on protein content) was studied by measuring G' and G" as a function of temperature (Hájovská, Chytil and Kalina 2020). The temperature ramp process was carried out by heating the sample dispersion from 25°C to 95°C, at the rate of 2°C/min, and then isotherm measurement at 95°C for 20 min. The oscillatory frequency was held at the constant value of 1 Hz and the strain was held at 0.5%.

4.2.20. Statistical analysis

All experiments were carried out in triplicate. Data were analyzed using analysis of variance (ANOVA), and means were compared using Fischer's Least Significant Differences Test ($p < 0.05$).

4.3. Results and discussion

4.3.1. Protein content and yield

Bambara globulin extract had a protein content of 89%. Fractionated vicilin from the storage globulin had the highest protein content of 93%, while the least value (67%) was obtained for legumin. These protein contents were higher than those reported for pea-mixed globulin isolate

(85%), legumin (56.5%), and vicilin (54.2%), respectively (Mession *et al.* 2015). Vicilin was the major fraction of Bambara globulin, accounting for approximately 70% of the total protein, which agrees with the findings reported in the previous chapter (chapter three). Differences in solubility behaviours of Bambara globulin fractions as described below could have accounted for the variation in their yield.

4.3.2. Amino acid profile

Aspartic and glutamic acids were the major amino acids of Bambara globulin, vicilin, and legumin fractions (Table 4.1). However, the level of these amino acids appeared relatively low in legumin compared to the globulin and vicilin fractions. For instance, the aspartic acid of legumin was about half those of globulin and vicilin. The glutamic acid contents of globulin and vicilin were not very different, but these were significantly higher, almost double the amount in legumin. The arginine contents of globulin and vicilin were higher than the amount in legumin. A high amount of glutamic acid in synergy with arginine was reported to prevent protein aggregation and thus enhance high protein solubility (Shukla and Trout 2011; Feng *et al.*2019). As expected, Bambara globulin and fractions were good sources of lysine (9.2%) but low in methionine. However, the methionine content of Bambara globulin is ten and five times that reported for mung bean globulin (Tang and Sun 2010) and kidney bean globulin (Mundi and Aluko 2012), respectively. Considering the FAO/WHO (1991) recommended pattern, Bambara globulin contains higher methionine content than the recommended values. Methionine is an essential amino acid that regulates metabolic processes and digestive functioning in the body. The methionine content of Bambara groundnut makes it meet the growing demand for healthier products and reduces food and nutrition insecurity in Sub-Sahara Africa. Bambara vicilin had a considerable amount of proline, which agrees with the findings reported in the previous chapter (chapter three). Proline participates in protein folding and unfolding processes by imparting a rigid structure (Damodaran, Parkin and Fennema 2008). A high proline content helps in preventing protein aggregation and increases protein solubility as described in the previous chapter (chapter three). The amino acid profile of the vicilin fraction appeared much like the globulin, which could be attributed to the fact that it represents the bulk of Bambara globulin (70%).

Table 4. 1: Amino acid composition (g/100 g protein) of globulin, legumin, and vicilin from Bambara groundnut

Amino acid	Globulin	Legumin	Vicilin
Asx	11.37	7.14	13.08
Glx	20.20	11.78	23.81
Ser	5.85	4.40	6.24
Thr	3.29	3.05	3.13
Pro	4.38	3.35	4.90
His	2.77	2.54	3.31
Arg	7.46	5.93	8.02
Gly	3.27	2.95	3.48
Ala	4.33	3.54	4.41
Lys	9.15	5.84	9.83
Tyr	3.99	3.87	4.14
Met	2.99	2.81	2.32
Val	5.02	4.09	5.23
Ile	4.49	3.82	4.75
Leu	8.58	6.43	8.90
Phe	6.54	5.01	6.59
HAA	39.60	32.00	40.58
NCAA	31.57	18.92	36.89
PCAA	19.38	14.31	21.16
AAA	10.53	8.88	10.73

Asx = aspartic acid and asparagine, Glx = glutamic acid and glutamine, HAA = hydrophobic amino acid, PCAA = positively charged amino acid, NCAA = negatively charged amino acid, AAA = aromatic amino acid.

4.3.3. SDS-PAGE of Bambara globulin, legumin, and vicilin

The protein band distribution of Bambara globulin appeared much like that of vicilin, but different from that of legumin extracts (Fig. 4.1). Under the non-reducing condition, Bambara globulin showed three major protein bands, including major broadband at around 50 kDa, similar to the vicilin profile. The legumin showed two major protein bands (70 and 60 kDa) and a minor higher molecular weight protein band at 85 kDa. The 60 kDa band was absent in the vicilin. In the presence of β-mercaptoethanol, no major variation was observed in the vicilin profile, except for the appearance of a band at 12 kDa. However, the disappearance of 70 kDa was noted with two new protein bands emerging at 22 and 12 kDa, respectively, in the legumin profile, suggesting that some of its proteins were stabilized by disulfide linkages.

Further, the 22 kDa low molecular weight band could correspond to basic legumin by comparison with pea legumin (Chen *et al.* 2019a) and soy protein isolate (Chen et al., 2019b). The presence of some vicilin bands in the legumin fraction and vice versa could be attributed to cross-contamination, which is very common, especially when using the isoelectric precipitation procedure to fractionate proteins (Klassen and Nickerson 2012). SDS-PAGE data indicated three protein subunits, which lack disulfide linkage for the vicilin fraction. Although vicilin is the major fraction, Bambara globulin contained some small proportion of legumin, which is stabilized by disulfide bonds.

4.3.4. Size-exclusion chromatography

The high-performance size-exclusion chromatography profile of Bambara vicilin displayed one major peak corresponding to the molecular weight of 120 kDa (Fig. 4.1). Legumin had one major peak in addition to the two minor peaks corresponding to the molecular weight of 140 and 95 kDa. Two predominant peaks with molecular weights of 414 kDa and 119 kDa were identified for globulin. The molecular weight of 119 kDa recorded for Bambara globulin corresponded to the high molecular weight of 118 kDa identified for Bambara vicilin in SDS-PAGE carried out under non-reducing conditions (Fig. 4.1). These values recorded for globulin and vicilin correspond to the α-subunit of vicilin, which was similar to that reported for cowpea 7S protein (Abdel-Shafi *et al.* 2019). This data indicated that vicilin is the major protein fraction in Bambara globulin. A molecular weight of 196 kDa was reported for Bambara vicilin isolated using the Osborne extraction method (Diedericks *et al*. 2019). The recorded molecular weights of 414 kDa for Bambara globulin correspond to the hexameric structure of legumin. This datum

is similar to the reported values in the range of 330-410 kDa for the 11S pea globulin (legumin) (Mession *et al.* 2015).

4.3.5. Surface Hydrophobicity of Bambara globulin and its fractions

The hydrophobicity of Bambara proteins increased in the order of globulin < legumin < vicilin (Fig. 4.3A). The high hydrophobicity of Bambara vicilin suggested that it might have a fairly open or unfolded structure compared to legumin and could be indicative of the high solubility observed for vicilin (Fig. 4.6B). The differences in the hydrophobicity of Bambara globulin, legumin, and vicilin might be related to variations in amino acid composition. The hydrophobicity value obtained for globulin is higher than 162.10 reported for peanut protein isolate but lower than 746.4 reported for soybean isolate (Ma *et al.* 2018). Bambara vicilin could be a potential ingredient in the formulation of food emulsions since a high hydrophobicity value is an indicator of good emulsifying properties.

4.3.6. Fluorescence emission spectra of Bambara globulin

The fluorescence spectrum of Bambara globulin showed a maximum peak intensity (λmax = 346 nm), similar to the vicilin and legumin fractions, which could be attributed to tryptophan emission (Fig. 4.3B). However, in comparison to these two fractions, the fluorescence peak intensity was significantly reduced for the globulin. In the native state, tryptophan is generally located in the core of the protein. When unfolded, it becomes exposed to solvent, resulting in reduced emission maximum and a more significant quenching (Ma *et al.* 2018). However, based on hydrophobicity data (Fig. 4.3A), the reduced fluorescence spectrum of Bambara globulin cannot be linked to an unfolded structure. Presumably, the interaction of tryptophan within the globulin peptide chain, as aromatic amino acids such as phenylalanine and tyrosine, can interact with tryptophan when in proximity leading to tryptophan quenching (Arai and Kuwajima 1996; Mune Mune *et al.* 2020). For instance, the interaction of tryptophan with other amino acids such as phenylalanine and tyrosine within the peptide chain might have reduced the amount of absorbed light energy and enhanced fluorescence quenching (Royer, 2006). Legumin showed the highest maximum (λmax) value compared to vicilin, perhaps due to its more compact structure. SDS-PAGE data indicated the presence of disulfide linkage in legumin (Fig. 4.1), which could be contributing to its stability in an aqueous solvent. In addition to protein conformation, the difference in fluorescence spectra among Bambara storage protein fractions could be associated with variations in amino acid composition (Table 4.1).

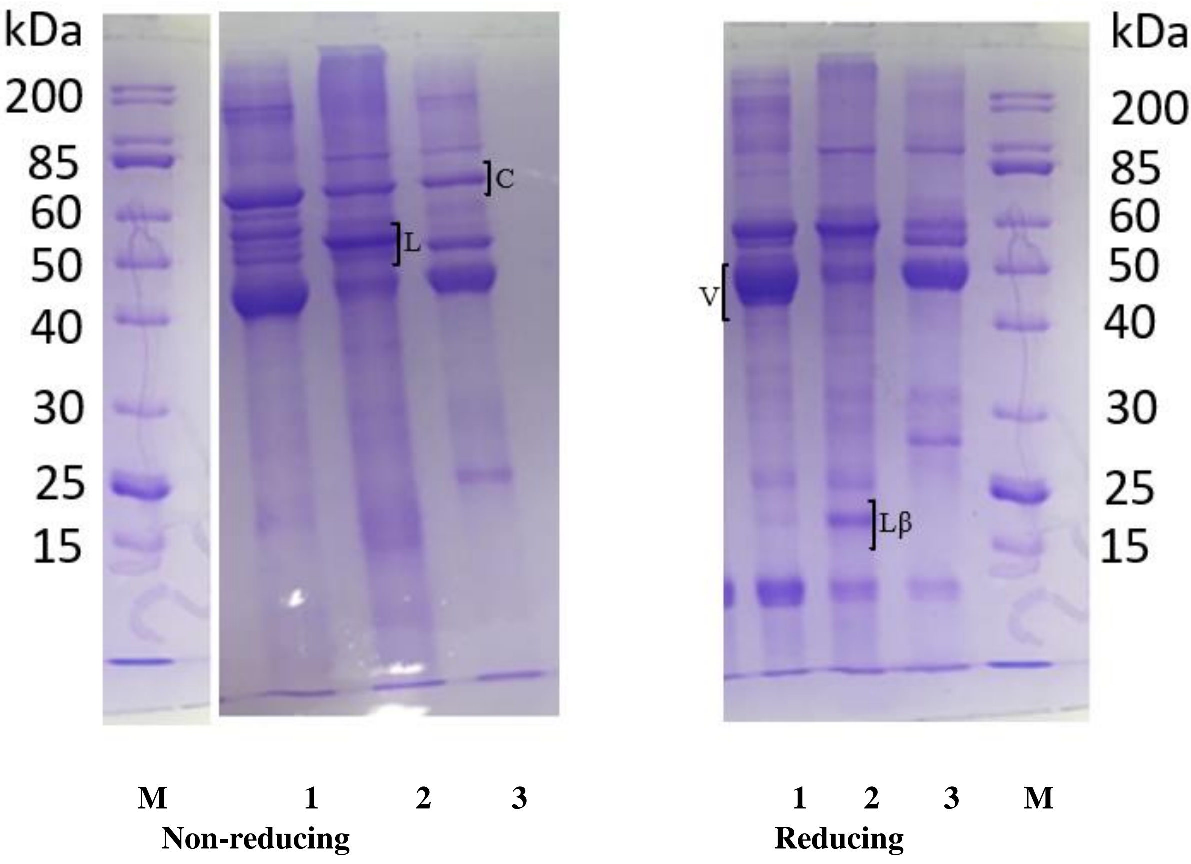


Figure 4. 1: SDS-PAGE pattern of Bambara globulin, legumin, and vicilin.

Lane M: Protein marker, Lanes 1: vicilin; Lane 2: legumin; Lane 3: globulin. Lβ: Basic subunits of legumin; V: Vicilin subunit; C: Convicilin subunit; L: Legumin subunit.

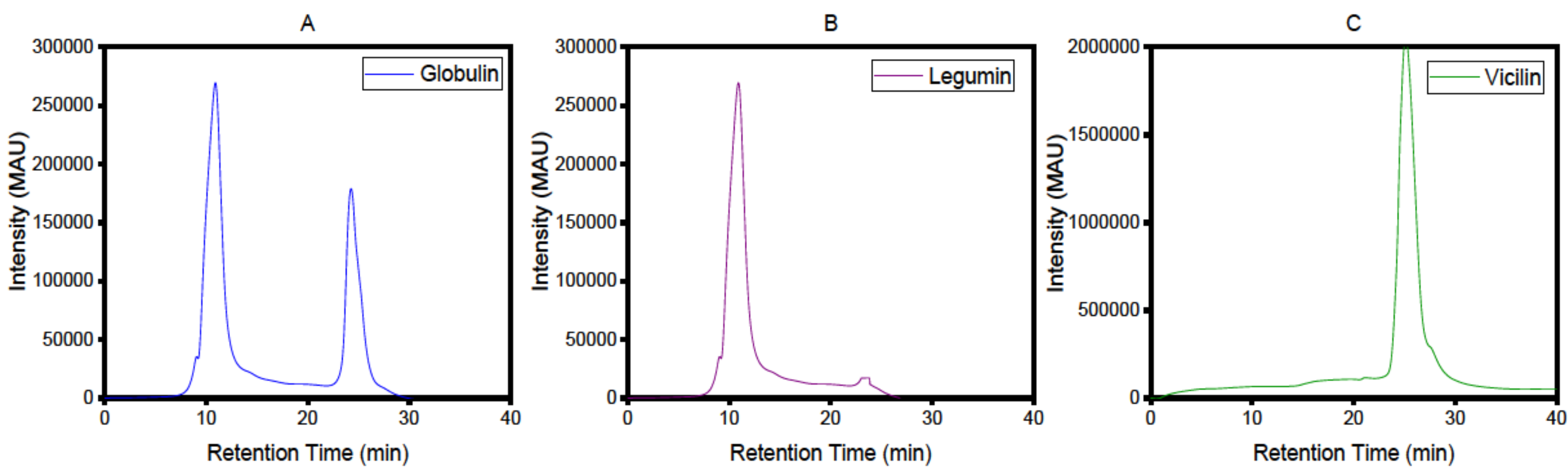


Figure 4. 2: Size exclusion chromatography elution profile of Bambara protein fractions

(A) Globulin **(B)** Legumin **(C)** Vicilin

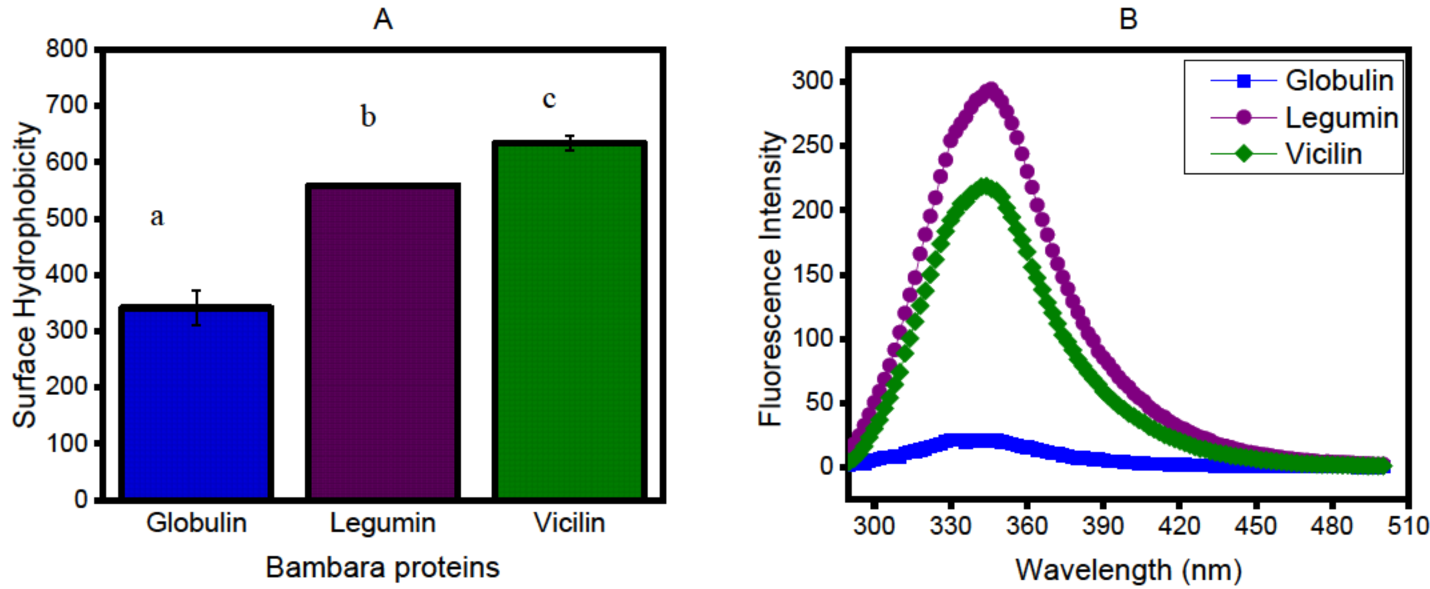


Figure 4. 3: Physicochemical properties of Bambara groundnut protein fractions measured at pH 7.

(A) Surface Hydrophobicity **(B)** Fluorescence Intensity

4.3.7. CD Spectra

Globulin and vicilin showed similar far UV spectra with a single positive at 195 nm and a negative minimum and broader peak ranging between 210-220 nm (Fig. 4.4A). These peaks depict the dominance of the β-sheet structure or the presence of ordered secondary structure elements including α-helix and β-sheet (Zhong and Xiong 2020). However, the legumin spectrum displayed a single positive peak at 194 nm and a minimum negative peak at 208 nm suggesting the dominance of helical structure (Sun *et al.* 2017; Yang *et al.* 2020). The dominance of the β-sheet structure (a protein conformation that provides greater exposure to the polar surrounding) in globulin and vicilin agrees with the fluorescence data (Fig. 4.3B) that showed lower fluorescence intensity values when compared to the legumin dominated by helical structure. The data obtained for globulin are similar to previous reports indicating the dominance of the β-sheet structure in most plant proteins compared to the helical and unordered secondary structure (Malomo and Aluko 2015; Tan *et al.* 2019).

The near-UV CD spectrum of Bambara globulin showed a prominent positive peak between 262 and 272 nm. Legumin and vicilin fractions showed a positive peak at 266 nm and 268 nm, respectively, with vicilin having a higher mean residue ellipticity (Fig. 4.4B). These prominent peaks reported for Bambara globulin and its fractions were consistent with phenylalanine residues within a non-polar surrounding. However, globulin showed very low ellipticity, suggesting that its aromatic residues are closer to the hydrophilic environment (Ijarotimi *et al.* 2018) compared to legumin and vicilin. The near-UV CD data obtained in this study showed that the exact shape and size of the near-UV CD spectrum of a protein is determined by the aromatic amino acid profile, as shown in Table 4.1, the interaction of these amino acids, and the type of their surroundings as reported by Kelly, Jess and Price (2005).

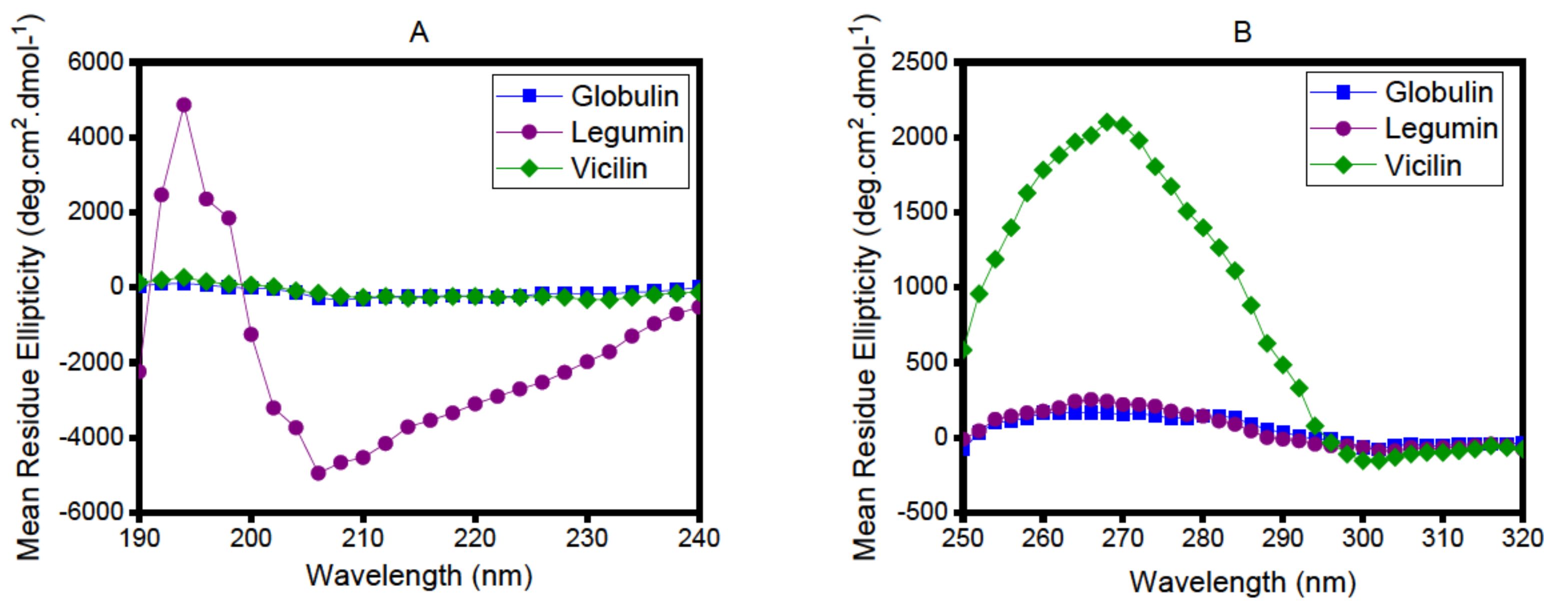


Figure 4. 4: Circular Dichroism spectra of Bambara groundnut protein fractions measured at pH 7.

(A) Far-UV CD spectra and (B) Near-UV CD spectra

4.3.8. Differential scanning calorimetry

Bambara globulin showed a single endothermal peak at 106°C, which was similar to the vicilin fraction (99°C), whilst the legumin had two transitions peak at 96°C and 112°C, respectively (Fig. 4.5). The change in enthalpy (ΔH) value obtained was in increasing order of globulin (6.52 J/g) < vicilin (12.34 J/g) < legumin (22.0 J/g). The temperature of denaturation, which is indicative of the thermal stability of protein, obtained for the vicilin subunit is in close range to that of globulin. This might be because vicilin represents the bulk of Bambara globulin as described above. The high denaturation temperature of globulin and its subunits (legumin and vicilin) might be associated with their lower molecular weight polypeptides as indicated in the gel electrophoresis (Fig. 4.1) (Peng *et al.* 2020). In addition, this might be due to a stable hydrogen bond, increased helical content as reported for legumin (Fig. 4.4A), and greater hydrophobic content. The highest enthalpy of denaturation (ΔH) recorded for legumin indicated a more folded structure in the subunit since ΔH represents the proportion of undenatured protein and is correlated with the extent of the folded structure of a protein (Ma *et al.* 2018). This suggests that legumin extract is more stable because of the presence of disulfide linkages as indicated by the SDS-PAGE (Fig. 4.1). The lowest ΔH value recorded for Bambara proteins might be due to an increase in the stability of hydrophobic interaction as corroborated by the hydrophobicity data (Fig. 4.3A) and fluorescence data (Fig. 4.3B). The thermal property of vicilin is in agreement with our previous report (Alabi *et al.* 2020). The thermal properties recorded for Bambara globulins are significantly higher than that reported for mung bean globulins (Tang and Sun 2010). The thermal property of protein is an essential factor for its heat-induced aggregation and gelation behaviours. '

4.3.9. Surface charges (ζ) and particle size distribution

Bambara globulin, legumin, and vicilin fraction showed similar ζ profiles at studied pHs (Fig. 4.6A). The surface charge values gradually changed from negative to positive as the pH decreased from 9.0 to 2.0. The ζ values are consistent with the fact that the protonation of carboxyl groups and deprotonation of amino groups of proteins gradually changed their electrostatic repulsion pattern from negatively charged proteins to positively charged proteins as the pH decreased (Wei *et al.* 2020). The isoelectric point (pH at ζ=0; pI) of legumin is around 4.6, while globulin and vicilin were around 5.0. At the isoelectric point of the protein, aggregation occurs because of the prevalence of attractive force over the electrostatic repulsive force, resulting in minimal solubility. The pI of Bambara globulin appeared to be higher than

that reported for pea globulin (Stone *et al.* 2015), while that of legumin was similar to that reported for legumin-like protein from lentils (Jarpa-Parra *et al.* 2015).

Bambara globulin displayed a typical monomodal particle size distribution pattern, similar to legumin (Table 4.2). In contrast to this, vicilin showed an aggregation peak at 0.006 μm in addition to its prominent peak, thus having a multimodal size distribution. The polydispersity index values for all the protein fractions were lower than 0.3, reflecting that the protein fractions contained uniform particle size (Boachie *et al.* 2019).

4.3.10. Solubility profile of Bambara globulins and fractions

The Bambara globulin showed minimum solubility at pH 5, similar to the legumin and vicilin (Fig. 4.6B). However, the difference in solubility profile occurs at low acid and alkaline pH ranges. Bambara vicilin displayed the highest solubility, approximately 82%, which is more than those of globulin and legumin at pH 3.5. The variation in the solubility pattern of Bambara globulin, legumin, and vicilin fractions might be attributed to the differences in their amino acid composition and protein structure. Vicilin showed higher content of positively and negatively charged amino acids than the legume and globulin (Table 4.1). These amino acids were reported to enhance protein solubility by stabilizing protein against aggregation (Feng *et al.* 2019). Also, vicilin lacks disulfide linkages when compared to legumin (Fig. 4.1). Disulfide linkages influence intermolecular hydrogen and hydrophobic bond formation, which are presumably responsible for lower solubility in legumin. The solubility of Bambara globulin was higher than that reported for hemp seed globulin (Malomo and Aluko 2015) at the same pH ranges.

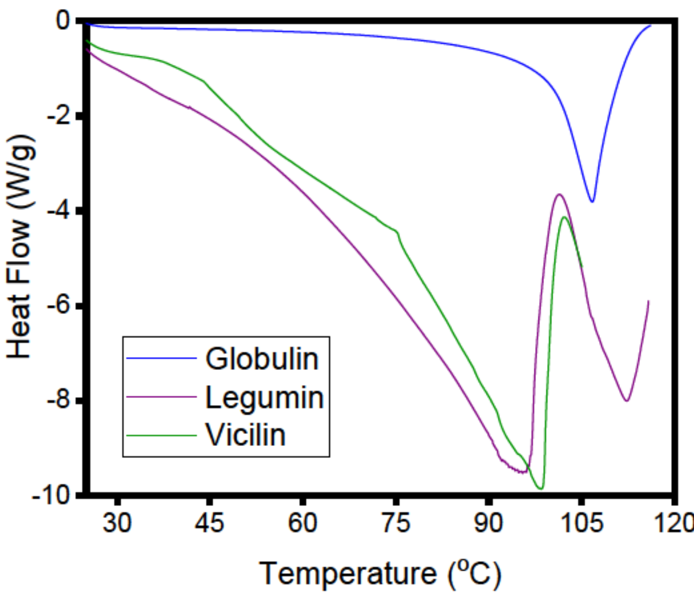


Figure 4. 5: Thermal properties of Bambara globulin, legumin, and vicilin.

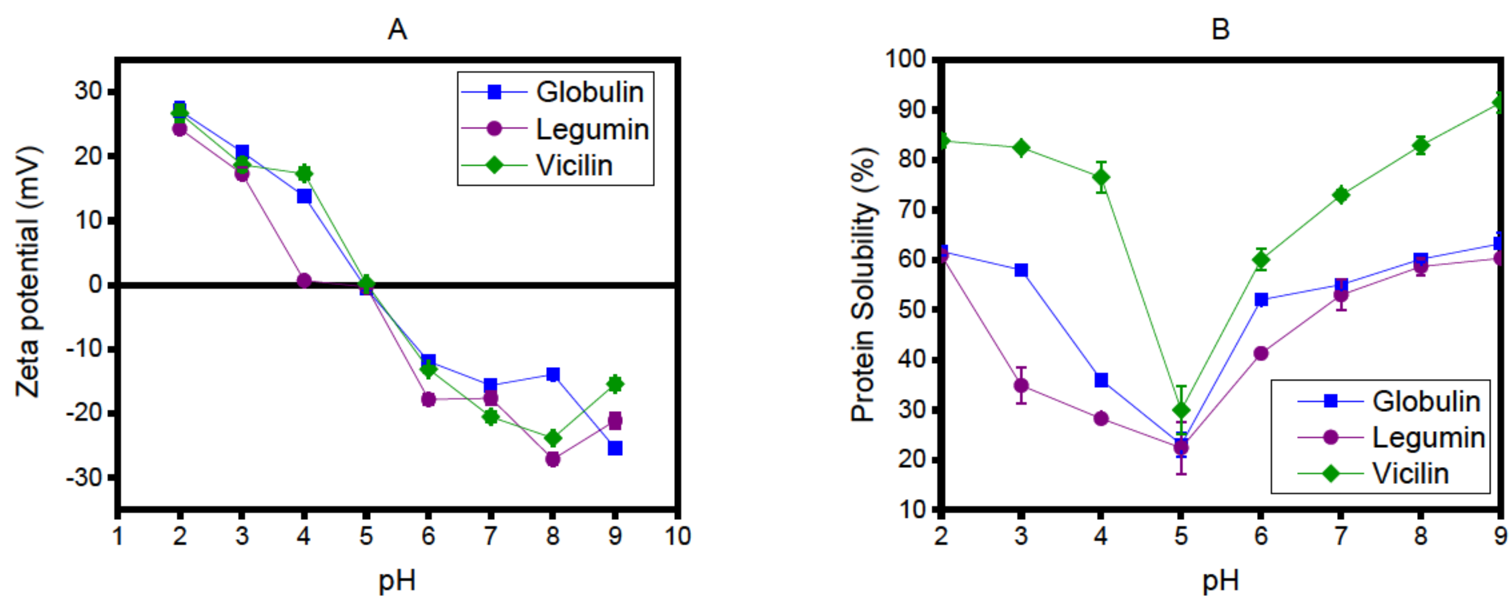


Figure 4. 6: Zeta potential and solubility profile of Bambara groundnut protein fractions.

(A) Zeta potential **(B)** Solubility profile

Table 4. 2: Particle size distribution of Bambara globulin and its legumin and vicilin fractions

Samples	Peak Intensity 1 (µm)	Peak Intensity 2 (µm)	Polydispersity Index (%)	Hydrodynamic Diameter (µm)	Size Distribution (µm)		
					D_{10}	D_{50}	D_{90}
Globulin	$0.168^{c} \pm 0.08$	-	13.0^{a}	0.139^{a}	0.065^{a}	0.137^{a}	0.298^{c}
Legumin	$0.132^{a} \pm 0.03$	-	17.3^{a}	0.135^{a}	0.086^{b}	0.123^{a}	0.177^{a}
Vicilin	$0.149^{b} \pm 0.05$	0.006 ± 0.00	20.3^{b}	0.132^{a}	0.073^{a}	0.130^{a}	0.225^{b}

Values along the column followed by the same superscripts are not significantly different.

4.3.11. Critical protein concentration for gel formation

The least gelation concentration (LGC) was determined for the respective protein fractions to establish their critical protein concentrations before rheological measurement. LGCs were significantly different among protein fractions, with vicilin showing the highest value (20%) followed by legumin (18%) and globulin (8%) at pH 7. Although vicilin is similar to globulin in composition and structure (Fig. 4.1), vicilin alone requires more protein to form a gel. Critical gelation concentration may be influenced by several factors including the variation in amino acid and subunit composition, and the degree of interaction in the heat-induced process (Totosaus *et al.* 2002; Ogawa *et al.* 2006; Sun and Holley 2011). The formation of heat-induced gel is mainly governed by hydrophobic interaction and disulfide linkage. Vicilin lacks disulfide linkage therefore, its gelation may be primarily driven by hydrophobic interaction. Furthermore, the reduced gelling capacity (high LGC) of Bambara vicilin might be attributed to the increased inter-electrostatic repulsion between protein molecules over the electrostatic attraction forces that bond molecular network as observed in its solubility profile (Fig. 4.6B) (Makeri *et al.* 2017). The dominance of repulsive force in vicilin dispersion could be attributed to the high content of negatively charged amino acid which corroborated with its high solubility (Fig. 4.6B) compared to other fractions. The slightly low LGC of legumin compared to vicilin may be due to its higher molecular weight (Sun and Holley 2011) and the presence of disulfide linkage. Synergistic effects of vicilin and legumin subunits may be responsible for globulin gelation at significantly reduced protein concentration, where less than half of what is needed for vicilin gelation is required for gel formation in globulin. In comparison with other legumes, the LCG for Bambara globulin is lower than that reported for winged bean (14%), soybean (12%) globulin (Makeri *et al.* 2017), and lupin protein isolate (18%) (Berghout, Boom and van der Goot 2015).

4.3.12. Steady-shear viscosity of Bambara groundnut proteins dispersion

Bambara globulin displayed a shear thinning behaviour typical for dispersion (10% protein concentration), similar to the legumin and vicilin fractions (Fig. 4.7). Most food biopolymers present this type of flow behaviours on shearing, with high viscosity profile, legumin had the least viscosity profile. The viscosity profile of globulin is much similar to that of vicilin. This is because as described above, vicilin is the major fraction of Bambara globulin which coincides with the similarities in their amino acid profile. The viscosity of the proteins drops relatively at a low shear rate and flattens out at a higher shear rate. This might be due to the

strong orientation of the particles of the inner phase of the protein dispersion which occurs at a low shear rate (Berghout, Boom and van der Goot 2015). In this case, most of the particles are aligned in the direction of the shear, and thus cannot be orientated further resulting in less flow resistance.

4.3.13. Mechanical spectra of Bambara protein gels

The frequency sweep data revealed some differences in the mechanical spectra of protein gels prepared at their critical protein concentration (LGC). An elastic character of the gels was dominant as the $G' > G''$. Vicilin prepared at LGC had the highest G′ followed by legumin and globulin, which could majorly be attributed to the protein concentration effect on protein aggregation and crosslinking (Fig. 4.8A and 4.8B). Frequency sweep analyses were therefore performed at a concentration of 22.5% for comparison and to minimize the protein concentration effects. Protein gels showed frequency-dependent behaviours of both the storage and loss moduli. G′ was greater than G″ in the entire frequency but the difference between these moduli was smaller than 1 log cycle, indicative of the formation of weakly structured gels (Kamal, Foukani and Karoui 2017) (Fig. 4.9A and 4.9B). The highest G′ of vicilin gel could be attributed to the increased number of intermolecular cross-links in its integral matrix. This also indicates more rigidity or firmness of the vicilin gel compared to globulin and legumin. Hydrophobic interaction and disulfide linkages are the main forces that participate in the stabilization of the structure of the gel. The data obtained in this study suggested that the more pronounced and stable structure development observed in vicilin gel after cooling was primarily due to hydrophobic interaction, hydrogen bond formation, and possible oxidation of the sulfhydryl group. The legumin appeared to contain disulfide linkage, which contributes to structural stability. The Tan δ values of gels from globulin, legumin and vicilin were less than 1 (Fig. 4.8C and 4.9C), indicating the dominance of an elastic structure and formation of weakly structured in the gels (Peyrano *et al.* 2019). A power law model was used to fit the G′ and G″ with the angular frequency with equations (1) and (2).

$$G' = Go' . \omega^{n'} \quad (1)$$

$$G'' = Go'' . \omega^{n''} \quad (2)$$

Where Go′ and Go″ are storage and loss moduli respectively, n' and n'' exponents denote the frequency dependence of G′ and G″ (Table 4.3). At 22.5% concentration, $G' > G''$ and are frequency dependent in Bambara globulin and vicilin gels, whilst the $G'' > G'$ in legumin gels

with the n' and n'' values showing frequency independence because they are low and similar. The G′ and G″ values for globulin and vicilin indicated greater gel firmness in terms of the elastic resistance to deformation (Nijenhuis 1997; Moreno *et al.* 2020). For globulin and vicilin, the rate of decrease of G′ was lower than in G″ with decreasing frequency as indicated in their n' and n'' values.

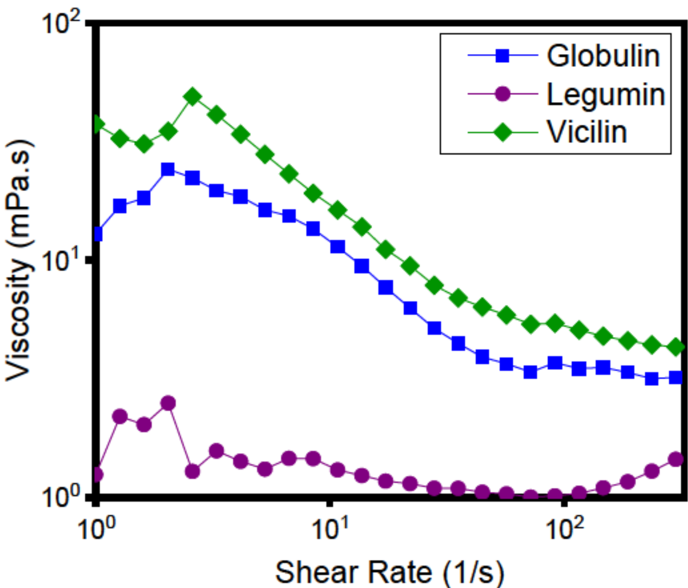


Figure 4. 7: Steady-shear viscosity of Bambara globulin, legumin, and vicilin

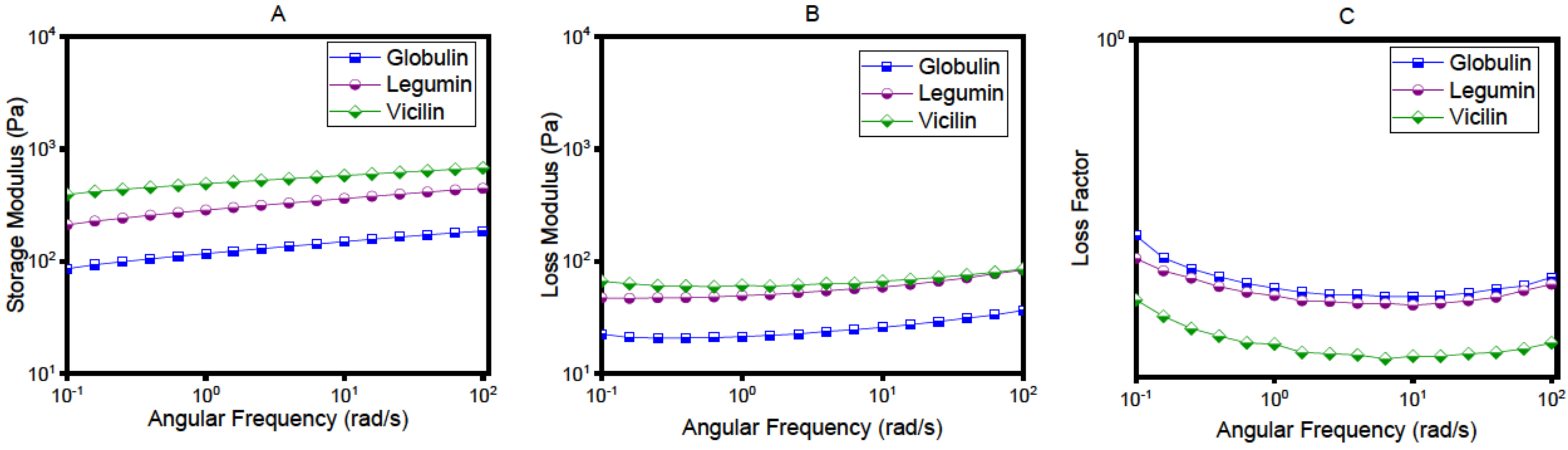


Figure 4. 8: Frequency sweep data of gels prepared from Bambara groundnut protein fractions at their critical protein concentration (LGC) **(A)** Dynamic Storage modulus (G′), **(B)** Dynamic Loss modulus (G″), **(C)** Tan δ.

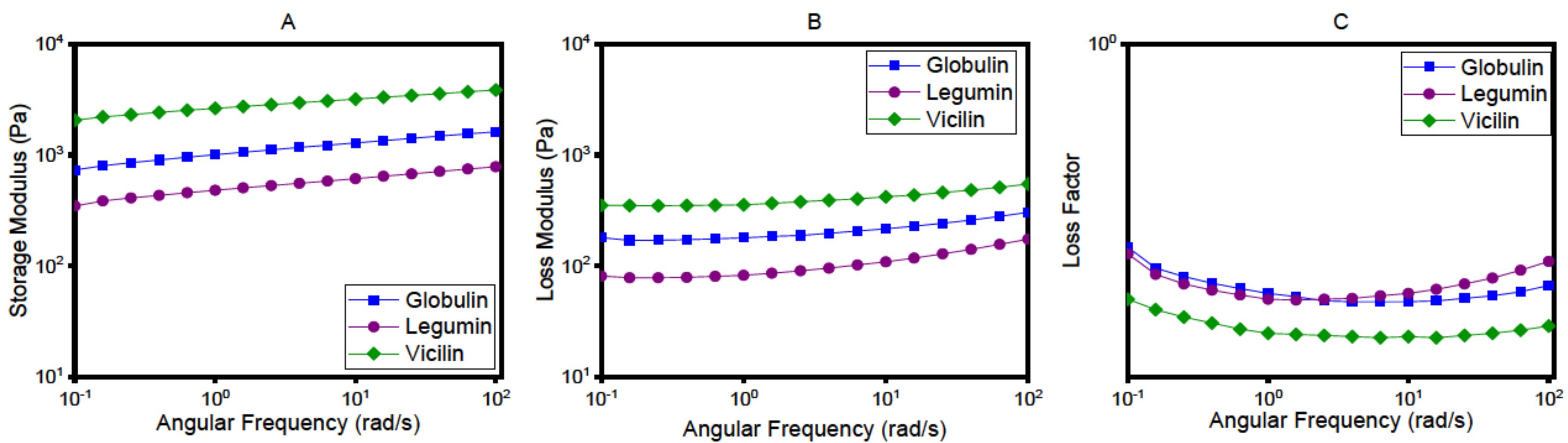


Figure 4. 9: Frequency sweep data of gels prepared from Bambara groundnut protein fractions at a protein concentration of 22.5% **(A)** Dynamic Storage modulus (G′), **(B)** Dynamic Loss modulus (G″), **(C)** Tan δ.

Table 4. 3: Power law parameters for gels from Bambara globulin and its subunits at 22.5% protein concentration

Samples	Go′ (Pa)	n′	r^2	Go″ (Pa)	n″	r^2	G* (Pa)	n*	r^2
Globulin	$992.6^b \pm 4.73$	$0.1084^b \pm 0.00$	0.9970^a	$184.8^a \pm 4.11$	$0.0899^a \pm 0.08$	0.9009^a	$1010^b \pm 3.86$	$0.1078^b \pm 0.00$	0.9980^a
Legumin	$473.9^a \pm 2.11$	$0.1097^b \pm 0.00$	0.9974^a	$992.6^c \pm 4.73$	$0.1084^b \pm 0.00$	0.9970^b	$481.8^a \pm 1.75$	$0.1104^b \pm 0.00$	0.9983^a
Vicilin	$2603^c \pm 8.46$	$0.0860^a \pm 0.00$	0.9976^a	$369.8^b \pm 5.88$	$0.0698^a \pm 0.01$	0.9096^a	$2629^c \pm 7.61$	$0.0857^a \pm 0.00$	0.9980^a

Mean ± SD. n=2   Values along the column followed by the same superscripts are not significantly different

4.3.14. The microstructure of Bambara protein gels

Scanning electron micrographs showed that Bambara globulin and legumin have large and collapsed sheet-like structures (Fig. 4.10). This could be indicative of the formation of weakly structured gels, which collapsed during freezing before conducting the microscopic analyses. Similar observations were made for psyllium gel dried under the same condition as described in this study (Farahnaky *et al.* 2010). The vicilin fraction showed the formation of defined laths sheet-like structure with numerous interconnected micropores. Bambara vicilin thus produced a more stable structured gel compared to globulin and legumin fractions. The microstructure display of Bambara proteins is consistent with the frequency sweep data where the vicilin gel appeared more rigid (high G′) than globulin and legumin. Although the rheological properties of heat-set protein gels are important in probing their structure, these analyses do not provide any information on the gelation process before gel setting. We, therefore, investigate the gelation process to monitor the structure development and identified gel points by conducting temperature ramp studies at 22.5% protein concentration.

4.3.15. Gelation profile of Bambara dispersion

Bambara protein dispersion was further assessed to determine the gel point and overall response to heating. The criteria based on the intersection between the tangent line of storage moduli and baseline were used (Sun and Arntfield 2012; Moreno *et al.* 2020). Bambara protein fractions displayed viscoelastic profiles that are typical of a biopolymer solution in which G′ is greater than G″ at room temperature (Monteiro *et al* 2013; Monteiro and Lopes-da-Silva 2017). These data suggested the presence of cross-links and some level of structure in these protein suspensions before heating. Vicilin displays the highest sol-gel transition temperature (80°C) followed by legumin (50°C) and globulin (40°C) (Fig. 4.11 A, B, and C). Differences in the gel points may be attributed to variations in amino acid and subunit composition including the thermal unfolding properties of the protein fractions, and the presence of disulfide linkages in globulin and legumin. The low gelling point for globulin might be related to the synergistic effects of thermal interaction between legumin and vicilin subunits. Bambara globulin had a lower gelling point compared to salt-extracted pea protein isolate with a sol-gel temperature of 85°C (Sun and Arntfield 2010). The lower gel point of globulin and legumin might be due to the contribution from both hydrophobicity and disulfide linkages which facilitate the formation of hydrophobic interaction (Sun and Arntfield 2010; Zhang *et al.* 2020).

Furthermore, the gelation patterns during heating were also significantly different among protein fractions before or after the gel point. Vicilin showed a breakdown in structure as demonstrated by a slight decrease in G′ from 40°C up to 65°C before the transition point. This was followed by a progressive increase in the G′ up to 95°C, marking the end of the heating profile. The decrease in the G′ of vicilin might be linked to the disruption of the protein network and dissociation of protein subunits at low heating temperatures (Comfort and Howell 2002; Nicolai 2019). Legumin subsequently showed progressive structural development throughout the heating period as shown by the continuous increase in the G′ and G″ with the heating time, and the difference between these moduli remains almost constant after the gel point. The increase in the G′ is due to the gradual development of the gel network during heating mainly through hydrophobic interaction and hydrogen bonds (Sun and Arntfield 2010). The continuous increase in the moduli might be linked to the incorporation of more protein within the protein network or further rearrangement of the protein network resulting from dominant hydrophobic interactions and disulfide bond formation (Monteiro and Lopes-da-Silva 2017). Globulin displayed a marginal increase in the G′ after the sol-gel transition with a constant phase angle (G′ having a weak slope) thus suggesting a rapid setting and establishment of the gel network. The complex modulus showed that vicilin produced a stiffer gel compared to globulin and legumin (Fig. 4.11D). This property might be related to the dominancy of the β-sheet structure (Guo *et al.* 2018) and the higher crosslink density of gel in vicilin. Bambara proteins can be used as a thickener and gelling agent in the food industry.

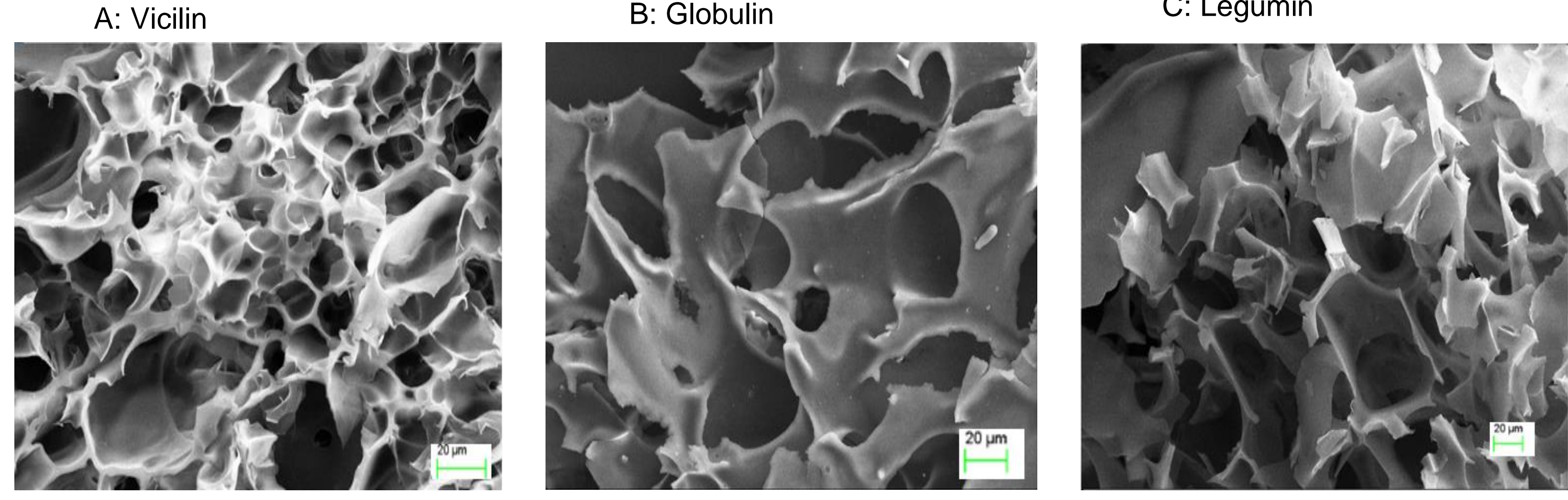


Figure 4. 10: Scanning electron microscopy images of gels from Bambara protein fractions.

(A) Vicilin **(B)** globulin and **(C)** legumin

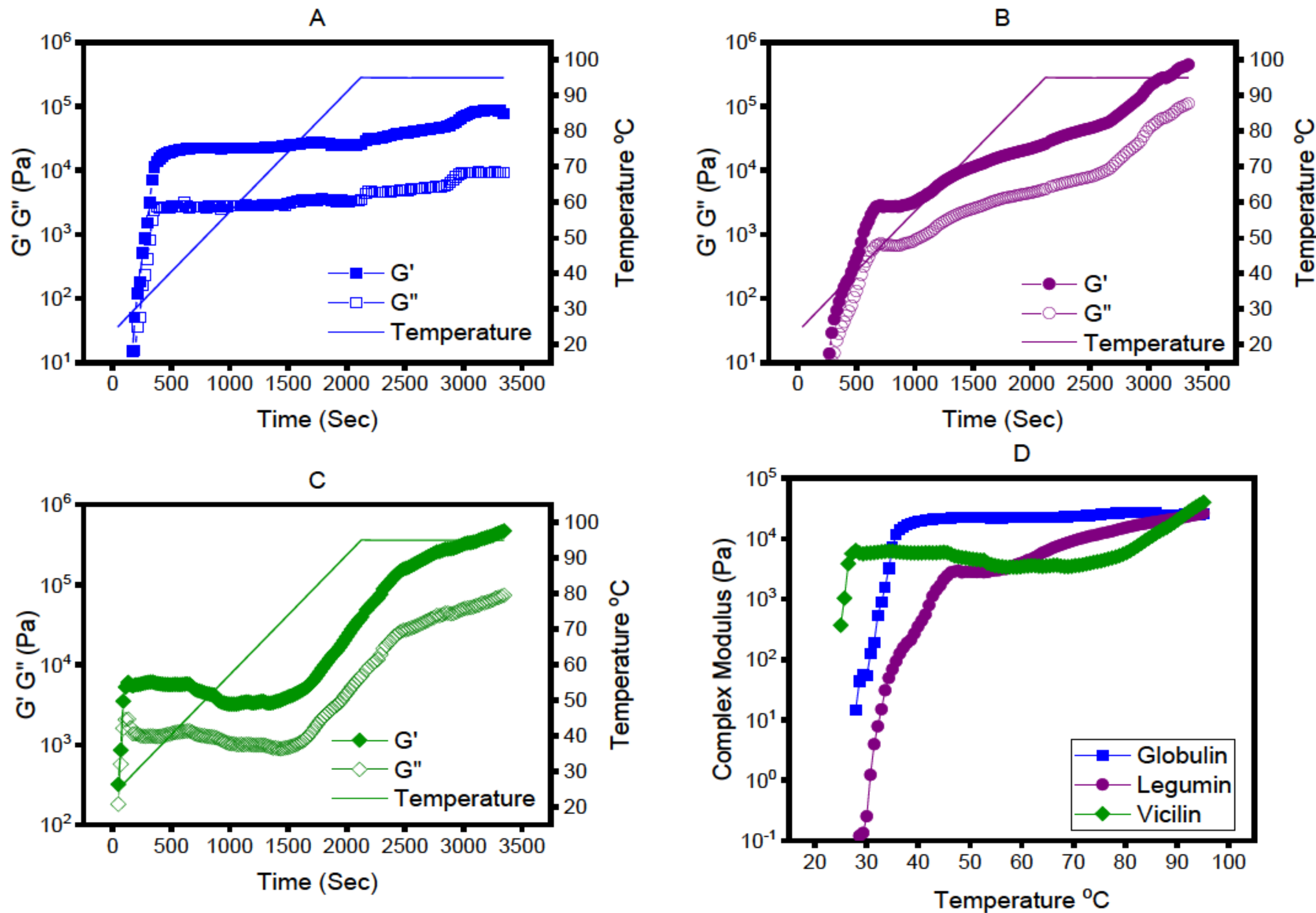


Figure 4. 11: Temperature dependence of G′ and G″ moduli of Bambara proteins at 22.5% concentration during heating ramp. **(A)** Globulin **(B)** Legumin **(C)** Vicilin and **(D)** Complex modulus

4.4. Conclusions

Bambara globulin contains about 70% vicilin fraction with a limited proportion of legumin protein stabilized by a disulfide bond. The dominant secondary structure of the Bambara globulin and vicilin was the β-sheet structure, except for the legumin fraction, which has a helical structure. The presence of disulfide linkage in globulin and legumin including the differences in their secondary structure and protein subunit composition might have significantly contributed to their low sol-gel transition temperatures and gel structure development. The microstructure of vicilin consists of a microporous structure with a small lath sheet-like structure compared to globulin and legumin.

4.5. Research outputs

a) Journal article

1. Opeyemi Alabi, Eric O. Amonsou. Impact of structure-composition on gel microstructures and rheological properties of vicilin and legumin-like proteins derived from Bambara globulin. Submitted to Food Research International Journal: under review

b) Conference abstract

1. Alabi, O.O. and Amonsou, E.O. (2021). Impact of structure-composition on gel microstructures and rheological properties of vicilin and legumin-like proteins derived from Bambara globulin. Presented at the 2021 AOCS Annual Meeting and Expo: Advancing the science and technology of oils, fats, proteins, surfactants and related material, enriching the lives of people everywhere, 3rd-14th May, Chicago, USA.

CHAPTER FIVE

5. Modification of Bambara globulin with plasma-activated water.

Abstract

The functional application of plant proteins as food ingredients which is limited when in their native form can be improved by altering their composition and structure. In this study, the impact of plasma-activated water (PAW) on the structure composition and functionality of Bambara groundnut globulin was investigated. Bambara globulin was treated with PAW (pH 3.4 and conductivity of 200 mV) at 4°C overnight. Plasma treatment resulted in the loss of helical structure and over a 3-fold increase in β-turns. Amino acid data showed a 20% reduction in glutamic acid after treatment. PAW-treated Bambara globulin showed a slight redshift in fluorescence intensity suggesting an unfolding of the protein structure, which also correlated with the observed increased hydrophobicity. Crosslinking was not evident in the gel electrophoresis and no major change in protein surface charge and solubility profiles. Average oil droplet sizes were increased with increasing protein concentrations from 10-20 mg/ml, suggesting that Bambara globulin had reduced emulsifying capacity after treatment with PAW. However, foaming capacities were significantly better and stable at up to 15 mg protein/mL. PAW modification could be a promising strategy for the enhancement of the foaming properties of plant proteins.

5.1. Introduction

The need for innovation has necessitated the search for newer sources of proteins. Based on this, proteins from plant sources have been identified to improve flexibility and satisfy the nutritional needs of the growing population (Galanakis *et al.* 2021; Basak and Annapure 2022) when food security and sustainability are of utmost importance. In addition to the nutritional needs, the biodiversity and green environment offered by the plant proteins are of importance to the consumers. Plant proteins are not included in the food matrices due to their poor functional properties such as low solubility and emulsification. Of such plant proteins, are many from underutilized grain legumes of African origin, such as Bambara groundnut (*Vigna subterranean*). Bambara groundnuts have a high protein content of about 19-25% (Chinma *et al*. 2015). Bambara groundnut protein is rich in essential amino acids such as lysine (Mune mune 2015; Alabi *et al.* 2020), and particularly methionine (1.62 g/100g), which is limited in most legumes (Kudre, Benjakul and Kishimura 2013; Alabi *et al.* 2020). The functional properties and commercial applications of Bambara groundnut protein as food ingredients in the food system may be enhanced through the modification process.

Several modification methods such as physical, chemical, and the use of enzymes can be employed to effect changes in the composition and structural attributes of Bambara groundnut proteins. Enzymatic hydrolysis and chemical methods involving the acylation process have been used on Bambara groundnut proteins as a modification technique (Lawal, Adebowale and Adebowale 2007; Mune mune 2015). Though these methods were reported to enhance the functional properties of Bambara groundnut protein, the processes involved were complex, expensive, and time-consuming. Furthermore, the chemical residual effect from chemical modification poses a potential danger to human health. Recently, non-thermal processes such as cold plasma technology, a green method, have been of interest to the food industry for food applications because it has no undesirable effects such as loss of color (Misra *et al.* 2015; Thirumdas *et al.* 2018). It is also environmentally friendly, cost-effective, energy and water-saving technology (Šimončicová *et al.* 2019).

Cold plasma (CP) is considered a partially ionized gas consisting of positive and negative ions, radiation energetic ions, atoms, electrons, reactive species containing reactive oxygen species (ROS), reactive nitrogen species (RNS), and ultraviolet photons generated by ionizing gases with electricity (Misra *et al.* 2015; Coutinho *et al.* 2018; Thirumdas *et al.* 2018; Ekezie, Cheng, and Sun, 2019; Luo et al., 2020). These particles can break covalent bonds and induce several

chemical reactions. Previous studies have reported that plasma species from dielectric barrier discharge (DBD) can interact with proteins in various ways, causing structural modification, which influences their functionality in food systems (Bußler *et al.* 2015; Segat *et al.* 2015; Dong *et al.* 2017). Dielectric discharge barrier (DBD) cold plasma treatment has been reported to induce a decline in zein micelles size increasing their solubility (Dong *et al.* 2017). The exposure of whey protein isolates to dielectric discharge barrier cold plasma resulted in significant changes in its emulsifying and foaming properties as a result of the unfolding of the protein and the increase in the carbonyl group (Segat *et al.* 2015). Alteration in the functional properties of peanut protein isolate by the breakage of covalent bond and modification of its amino acid side chain through disulfide linkages formation as a result of ROS attack was reported by Ji *et al.* (2018). Generally, the impact of plasma treatment on proteins are considered to be caused by structural changes, formation of disulfide bonds and hydrophobic interactions, stabilization by hydrogen bonds, and electrostatic interactions (Kato *et al.*1986; Sekul, Vinnett and Ory 1997; Ekezie *et al.* 2019). However, optimization of the reactive species, produced in DBD plasma, to induce changes in protein foods is still challenging as the surface accumulation of these excessive reactive species may cause protein oxidation partially (Qian *et al.* 2021; Wojtyla *et al.* 2016). Excessive oxidation of proteins can seriously affect their structure and functionality (Tolouie *et al.* 2018). To prevent excessive oxidation of proteins, protein isolate can be subjected to plasma-activated water (PAW) treatment which can be regarded as a less severe green modification approach for functional property improvement as opposed to direct treatment by cold atmospheric plasma. In this case, protein isolate. In this case, protein isolates are hydrated in PAW to allow for uniform and sufficient interactions between reactive species and protein molecules (Bermudez-Aguirre 2020). PAW is deionized water containing reactive oxygen species from plasma high-voltage discharge (Porto *et al.* 2018).

Currently, PAW is widely used to disinfect medical equipment, eliminate microorganisms, and stimulate seed germination and growth (Herianto *et al.* 2021). A previous study showed that plasma-activated water induced the formation of compact chicken myofibrillar protein gel structures with intrinsically antibacterial activity (Qian *et al.* 2021). Also, the influence of plasma-activated water on the morphological, functional, and digestibility characteristics of hydrothermally modified non-conventional talipot starch had been studied (Aaliya *et al.*2022). However, research on the effect of PAW on plant protein isolate is minimal. Presently, limited research has been conducted to investigate how PAW impacted on conformational,

physicochemical, and functional properties of Bambara globulin. Therefore, the purpose of this study was to examine the effects of PAW on the structure, physicochemical and functional properties of Bambara globulin.

5.2. Materials and methods

5.2.1. Preparation of defatted flour

Bambara groundnut was obtained from Josini, KwaZulu-Natal province of South Africa, and the flour was defatted as previously described by Arise *et al.* (2017).

5.2.2. Extraction of Bambara Globulin

Globulin was extracted with 50 mM K_2HPO_4 buffer, 0.5 M NaCl at pH 7.3, with a flour-to-buffer ratio of 1:10 as adapted from the method reported by Klassen and Nickerson (2012). The mixture was stirred continuously, using a magnetic stirrer, for 1 h at room temperature and centrifuged at 18,000 ×g for 25 min at 4°C using an Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzig, Germany). The supernatant was then diluted with cold Milli-Q water, adjusted to pH 4.6 using 1 M HCl, and left overnight in a cold room to facilitate the settling of the salt-soluble proteins. The precipitated protein was collected by centrifugation and washed twice with Milli-Q water (pellet-to-water ratio of 1:10) to remove unwanted albumin proteins. The precipitated protein was redissolved in Milli-Q water and dialyzed (dialysis tubing of 6-8 kDa) against water. The dialysis was done for 72 h to remove the salt. The dialysis water was changed three times daily. The pH of the de-salted protein extract was adjusted to 7 and then freeze-dried (Christ freeze-drier, Niedersachsen, Germany) as the globulin and stored at -20°C.

5.2.3. Preparation of plasma-activated water and treatment of Bambara globulin with plasma

Plasma-activated water (PAW) was prepared using an atmospheric pressure plasma jet (APPJ) system. PAW was obtained by the generation of plasma discharge over the surface of 1 L sterile deionized water for 1 h at room temperature and was kept at 4°C. The distance between the end of the plasma jet and the water surface was 5 mm. The input power was set at 750 W, and Nitrogen was used as the carrier gas. The pH and the electrical conductivity of the plasma-activated water were measured to be 3.4 and 200 mV, respectively. Freeze-dried Bambara globulin was dispersed in PAW at a solid: solvent ratio of 1:5 and stirred for 1 h, kept overnight at 4°C. Plasma-treated Bambara globulin was neutralized to pH 7 and then freeze-dried.

5.2.4. Protein content

The protein content of Bambara globulin was determined using the modified Lowry method (Markwell *et al.* 1978).

5.2.5. Amino acid composition analysis

The amino acid profiles of plasma-treated Bambara globulin were determined using the HPLC PICO-TAG system (Bidlingmeyer, Cohen and Tarvin, 1984). This method was based on the principle of reverse-phase chromatography with pre-column derivatization following acid digestion. The samples were hydrolyzed with 6 M HCl at 116°C for 24 h before chromatographic analysis. The methionine contents were determined after performic acid oxidation (Gehrke *et al.* 1985). 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 a 0.45 mL/minute flow rate.

5.2.6. Gel electrophoresis

Plasma-treated Bambara globulin was subjected to SDS-PAGE (reducing and non-reducing) according to the method of Laemmli (1970) with slight modifications. Each sample was dispersed (6 mg protein/ml) in Tris/HCl buffer, pH 8.0 containing 10% (w/v) SDS only (non-reducing buffer) or SDS + 10% (v/v) β-mercaptoethanol (reducing buffer), followed by heating at 95°C for 10 min, cooled centrifuged (10000x*g*, 15 min). After centrifugation, 15 µl of aliquot containing 10 µg protein was loaded onto 4-12% gradient gels, and electrophoresis was performed with a mini-PROTEAN system (BIO-RAD). A mixture of protein standards (10-200 kDa) was used as the molecular weight marker and the gels were stained with Coomassie brilliant blue.

5.2.7. Intrinsic fluorescence

The method described by Arise *et al.* (2017) was used to record intrinsic fluorescence spectra on the JASCO FP-6300 spectrofluorometer (JASCO, Tokyo, Japan) at 25°C with a 1 cm path length cuvette. Fluorescence spectra of the protein solution (0.002% (w/v)) prepared in 0.1 M sodium phosphate buffer pH 7 were recorded at excitation wavelengths of 275 nm (tyrosine and tryptophan) with emission recorded from 280 to 450 nm.

5.2.8. Surface hydrophobicity

Surface hydrophobicity (S_o) of the plasma-treated Bambara globulin was determined by the fluorescence method using a hydrophobic probe, 8-anilino-1-naphthalene sulfonic acid (ANS) (Mohan and Udenigwe 2015). Protein stock solution (10 mg/ml) was prepared in 0.1 M sodium phosphate buffer pH 7 followed by dilution to 0.0009-0.015%. Fluorescence was then measured at excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the plot of fluorescence versus protein concentration was taken to be the hydrophobicity.

5.2.9. Fourier transform infrared spectroscopy (FTIR)

The secondary structure of the Bambara protein was analyzed by an infrared spectrophotometer (Agilent Cary 630 FTIR, CA, USA). The sample was scanned at 4 cm resolution from 4000 cm^{-1} to 400 cm^{-1} and read from 32 cycles.

5.2.10. Particle size and zeta potential (ζ)

The zeta potential of plasma-treated Bambara globulin was measured over a pH range of 2.0-9.0 at a 1-unit increment using Litesizer Nano ZS (Anton Paar, New Castle, Delaware). The samples (diluted dispersion 2 mg/mL) were hydrated for 1 h and filtered through a 0.45 µm HA Millipore membrane before analysis. The particle size of the samples was determined using the same instrument. The sample was prepared at pH 7 as described for zeta potential.

5.2.11. Protein solubility

The solubility profile of the plasma-treated Bambara proteins was determined as described by Malomo and Aluko (2015). Briefly, 10 mg of the sample was dispersed in 1 ml of distilled water. The pH of the suspensions was adjusted to values ranging from 2 to 9 with either 1 M HCl or 1 M NaOH. These suspensions were vortexed for 2 min at room temperature and centrifuged at 8000x*g* for 15 min. The protein content of the supernatant was determined using the modified Lowry method (Markwell *et al.* 1978). Total protein content was determined by dissolving the protein samples in 0.1 M NaOH solution.

5.2.12. Emulsifying formation and oil droplet size measurement

The oil-in-water emulsion was prepared as adapted from the method reported by Adebiyi and Aluko (2011), with slight modifications. Protein slurries of 10, 15, or 20 mg/ml concentrations were prepared using 5 ml of 0.1 M phosphate buffer pH 7 followed by the addition of 1 ml of pure canola oil. The oil/aqueous mixture was homogenized at 20,000 rpm for 2 min, using the

20 mm non-foaming shaft on a Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The oil droplet size ($d_{3,2}$) of the emulsions was determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as the dispersant. Under constant shearing, the emulsion sample taken from the emulsified layers of the samples was added to about 100 ml of water contained in the small volume wet sample dispersion unit (Hydro 2000S) attached to the instrument until the required level of obscuration is attained. The instrument was set to automatically measure the oil droplet size of each emulsion in triplicate and each sample was prepared in triplicate. The emulsion formed was kept at room temperature for 30 min without agitation and the particle size distribution and mean particle diameter was measured again to assess emulsion stability (ES), which was calculated as the percentage ratio of oil droplet size at time zero to oil droplet size measured at 30 min.

$$ES = \frac{\text{Particle size at 0 min } (d_{3,2})}{\text{Particle size at 30 min } (d_{3,2})} \times 100$$

5.2.13. Foaming capacity (FC) and stability (FS)

FC was determined as described by Adebiyi and Aluko (2011) using slurries that were prepared as 10, 15, and 20 mg/ml (protein weight basis) sample dispersions in 50 ml graduated centrifuge tubes containing 0.1 M phosphate buffer, pH 7. Sample slurry was homogenized at 20,000 rpm for 1 min using a 20 mm foaming shaft on the polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The foaming capacity was calculated using the mean of three measurements:

$$FC\ (\%) = \frac{\text{Volume after homogenization} - \text{Volume before homogenization}}{\text{Volume before homogenization}} \times 100$$

The ability of the sample to retain air for a certain period (foam stability) was calculated by measuring the foam volume after storage at room temperature for 30 min and expressed as a percentage of the original foam volume.

$$FS\ (\%) = \frac{\text{Volume after 30 min}}{\text{Initial Volume}} \times 100$$

5.2.13. Statistical analysis

All experiments were carried out in triplicate. Data were analyzed using analysis of variance (ANOVA), and means were compared using Fischer's Least Significant Differences Test ($p < 0.05$).

5.3. Result and discussion

5.3.1. Amino acid composition

Plasma treatment significantly impacted the amino acid composition of Bambara globulin (Table 5.1). The amount of glutamic acid in Bambara globulin was reduced by about 20% due to a deamination reaction compared to the plasma-treated globulin. There was an apparent increase in the tyrosine and phenylalanine content of plasma-treated globulin compared to the untreated, suggesting these amino acids were minimally affected. This could be attributed to the reduced hydroxylation since plasma was not directly applied to the protein as opposed to observed studies (Takai *et al.* 2014). Methionine was significantly reduced in plasma-treated globulin because of a hydroxylation reaction whilst serine, glycine, and threonine were unaffected by plasma treatment. Treatment of Bambara groundnut protein with PAW did not significantly alter the lysine content of the protein, which is good to maintain the quality of the protein. Also, the lysine content was still within the recommended standard stated by the FAO/WHO. This could also have a good impact on the functionality. The changes in the amino acid composition of Bambara globulin on exposure to plasma treatment might be attributed to the oxidation reaction induced by the reactive oxygen species on the α-carbon amino acid, the protein polypeptide backbone, and the aliphatic side chains of the hydrophobic amino acid residues. These results indicate that electron-rich groups in amino acids were modified by the various kinds of active species generated by the plasma treatment.

5.3.2. Gel electrophoresis profile of plasma-treated Bambara globulin

Under the non-reducing conditions, plasma-treated Bambara globulin showed three protein bands (55, 63, and 70 kDa) similar to the untreated globulin (Fig. 5.1). The protein band pattern also remained unchanged under reducing conditions. Although we anticipated protein crosslinking, SDS-PAGE data suggested the plasma treatment did not result in any major changes in the molecular weight distribution of the protein. Simultaneous formation of dityrosine crosslinks and breakdown of protein-protein bonds were reported for plasma-treated grass pea protein isolate (Mehr and Koocheki 2021). The bands at 55 and 63 kDa in Bambara protein could be vicilin and legumin, respectively whilst the band at 70 kDa might denote the α-subunit of vicilin (O′Kane *et al.* 2004; Peng *et al.* 2016), which may correspond to the convicilin in grass pea (Feyzi *et al.* 2018).

Table 5. 1: Amino acid compositions (g/100g) of plasma-treated Bambara globulin

Amino acid	Plasma Globulin	Globulin	FAO/WHO,1991
Asx	8.35	11.37	
Glx	14.17	20.20	
Ser	5.17	5.85	
Thr	3.28	3.29	
Pro	3.19	4.38	
His	2.04	2.77	1.9
Arg	6.57	7.46	
Gly	3.16	3.27	
Ala	3.39	4.33	
Lys	8.02	9.15	5.8
Tyr	5.09	3.99	1.1
Met	1.43	2.99	
Val	4.12	5.02	3.5
Ile	3.42	4.49	2.8
Leu	6.74	8.58	6.6
Phe	8.09	6.54	6.3
HAA	35.24	39.60	
NCAA	22.52	31.57	
PCAA	16.63	19.38	
AAA	13.18	10.53	

Asx = aspartic acid and asparagine, Glx = glutamic acid and glutamine, HAA = hydrophobic amino acid, PCAA = positively charged amino acid, NCAA = negatively charged amino acid, AAA = aromatic amino acid.

5.3.3. Fluorescence emission spectra of plasma-treated Bambara globulin.

The fluorescence emission spectrum of the plasma-treated Bambara globulin at pH 7 was characterized by λmax of 360 nm (Fig. 5.2A) reflecting the dominancy of tryptophan residues. A redshift in the wavelength was observed for the Bambara globulin after treatment with plasma-activated water. These changes in the fluorescence properties of globulin after plasma treatment indicated that tryptophan residues had changed to a more polar environment (Malik, Sharma and Saini 2017; Ji *et al.* 2019; Mehr and Koocheki 2020). Redshift observed in the fluorescence spectrum of tyrosine and tryptophan residues was accompanied by the unfolding and degradation of protein-protein bonds in protein molecules (Liu, Pu and Sun 2017; Ekezie *et al.* 2019; Han, Cheng and Sun 2019). Furthermore, the etching process, resulting in the dissociation of the Bambara globulin subunit, from plasma treatment increased the surface cavity of proteins thereby altering its tertiary structure (Mehr and Koocheki 2021). In addition, changes in the tertiary structure of Bambara globulin after plasma treatment could have resulted from the dissociation of disulfide bonds as shown in SDS-PAGE and the unfolding of the protein structure since disulfide bonds played an important role in the preservation of protein tertiary structures (Jespersen *et al.* 2014; Dong, *et al.* 2017; Mehr and Koocheki 2021).

5.3.4. Hydrophobicity (S_O) property of plasma-treated Bambara globulin

The hydrophobicity of Bambara globulin at pH 7 significantly increased after treatment with plasma-activated water (Fig. 5.2B). The high hydrophobicity property displayed by the plasma-treated globulin could be attributed to the dissociation of the reversible protein aggregates and protein subunits by the etching process of plasma treatment. In this case, more hydrophobic amino-acids residues initially buried in the interior of the protein were exposed during the treatment of Bambara globulin with plasma-activated water because of protein unfolding (Mohseni-Shahri *et al.* 2014; Li *et al.* 2017). Thus, making more hydrophobic amino acid residues accessible to the ANS binding. Furthermore, the by-products of the oxidation of protein by reactive oxygen species or reactive nitrogen species (ROS/RNS) such as dityrosine increase the surface hydrophobicity of plasma-treated proteins as reported by Liu *et al.* (2015) and Radi (2013). However, the high hydrophobicity of plasma-treated Bambara globulin might be attributed to the dissociation of its legumin subunit as observed in the gel electrophoresis (Fig. 1). Chang *et al.* (2015) reported that the dissociation of protein subunits contributed to the higher So. These data agreed with the fluorescence data which showed that plasma treatment induced the unfolding of the protein. Plasma-treated Bambara globulin could be a

potential ingredient in the formulation of food emulsion since hydrophobicity is an important surface-related property of the protein.

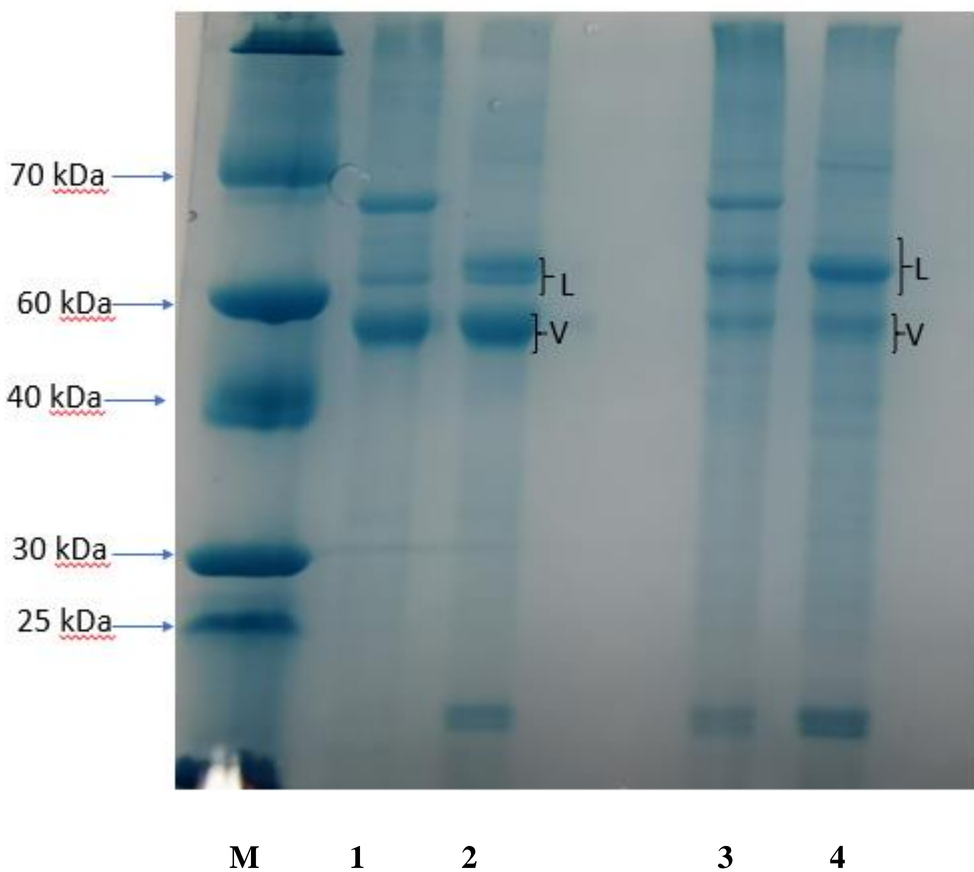


Figure 5. 1: SDS-PAGE of plasma-treated Bambara globulin.

Lane M: Protein marker, lanes 1 and 2: untreated globulin, Lanes 3 and 4: plasma globulin. Lanes 1 and 3 were carried out under non-reducing conditions while Lanes 2 and 4 were carried out under reducing conditions.

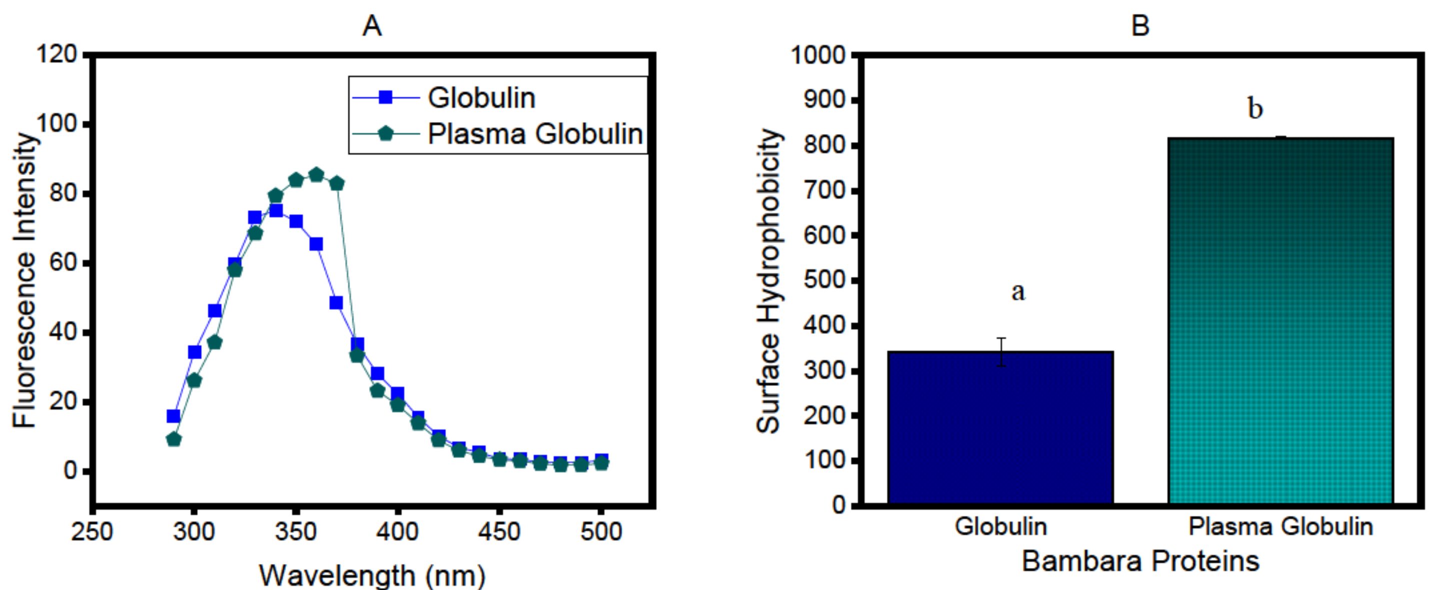


Figure 5. 2: Physicochemical properties of plasma-treated Bambara globulin

(A) Fluorescence Intensity; (B) Surface Hydrophobicity

5.3.5. Fourier transform infrared spectroscopy

The secondary structure of plasma-treated Bambara globulin was estimated from the amide I region as it represented the most prominent and sensitive vibrational band of the protein backbone, which is due to the predominant C=O stretching band with little contribution from C-N stretching. According to the previous studies (Jia *et al.* 2018), the band in the region of 1650–1660 cm^{-1} is assigned to α-helix; 1618–1640 and 1670–1690 cm^{-1} corresponded to β-sheet; 1660–1670 and 1690–1700 cm^{-1} to β-turn; and 1645 cm^{-1} to random coils. In contrast with the untreated Bambara protein, the α-helices content of the plasma-treated protein decreased by 86%, and β-sheet and β-turn components increased by 13% and 21%, respectively (Table 5.2). This data reflected the degradation of α-helix structures and rearrangement of β-structures as evidenced by the hydrophobicity and fluorescence data (Fig. 5.2A and Fig. 5.2B). The disruption of hydrogen bonding that maintains the stability of the α-helices component by plasma treatment enhanced the formation of β-sheets (Misra *et al.* 2016). This could alter the functional and physicochemical properties of proteins.

5.3.6. Surface charges (ζ-potential) and particle size distribution

Plasma-treated Bambara globulin showed similar ζ profiles to the untreated globulin at studied pHs (Fig. 5.3A). Plasma treatment did not change the isoelectric point (pH at ζ=0; pI) of Bambara globulin which was around 5.0. A significant reduction was observed at pH 7.0 in the ζ-potential value of plasma-treated globulin from -15.6 mV in untreated globulin to -26.9 mV in plasma-treated globulin. This change might be attributed to the higher rate of deprotonation reactions at higher pH values due to the oxidation of charged amino acids by the reactive oxygen species. Positively charged amino acids were converted into neutral amino acids, which were often oxidized into negatively charged forms (Stadtman and Levine 2003; De Graf Hazoglou and Dill 2016).

Plasma globulin displayed a typical multimodal particle size distribution pattern, compared to untreated globulin (Table 5.3). Treatment of Bambara globulin with plasma-activated water significantly increased its polydispersity index (PDI) value close to 0.3. This might be due to the formation of aggregates as influenced by the attachment of water micelles to the protein molecules by the reaction of the reactive species. In the present study, oxidizing reactive species from plasma-activated water might have promoted hydrophobic interactions and the formation of intermolecular disulfide bridges. The data agreed with that reported by Ekezie *et al.* (2019).

5.3.7. Solubility profile of Plasma Bambara globulin

Plasma treatment slightly increased the solubility of globulin at pH 2 and 3 compared to the untreated globulin (Fig. 3B). However, there are no major changes in the solubility profile of plasma-treated globulin and the untreated as both showed minimum solubility at pH 5 (pH around isoelectric point). The increase in the solubility profile of plasma-treated globulin might be attributed to the interaction of polar amino acids with water molecules through electrostatic interaction and hydrogen bonding. Treatment of Bambara globulin with plasma-activated water exposed the non-polar and polar amino acids buried inside the protein molecule to its surface, thereby increasing the protein hydrophilicity properties (Pankaj *et al.* 2014; Zhu *et al.* 2021). The lower content of hydrophobic amino acids as recorded in Table 1 in plasma globulin might have contributed to its better solubility profile. Bußler *et al.* (2015) reported an increase in the solubility of pea protein fractions on exposure to DBD plasma.

Table 5. 2: Secondary structure of plasma-treated Bambara globulin

Samples	β-sheet (%)	Random coil (%)	α-helix (%)	β-turns (%)
Plasma Globulin	69.15^{b} ± 0.12	-	-	29.52^{b} ± 1.19
Globulin	56.57^{a} ± 1.70	20.75 ± 0.09	14.40 ± 1.85	8.28^{a} ± 0.07

Mean ± SD (n=3). Values along the column followed by the same superscripts are not significantly different.

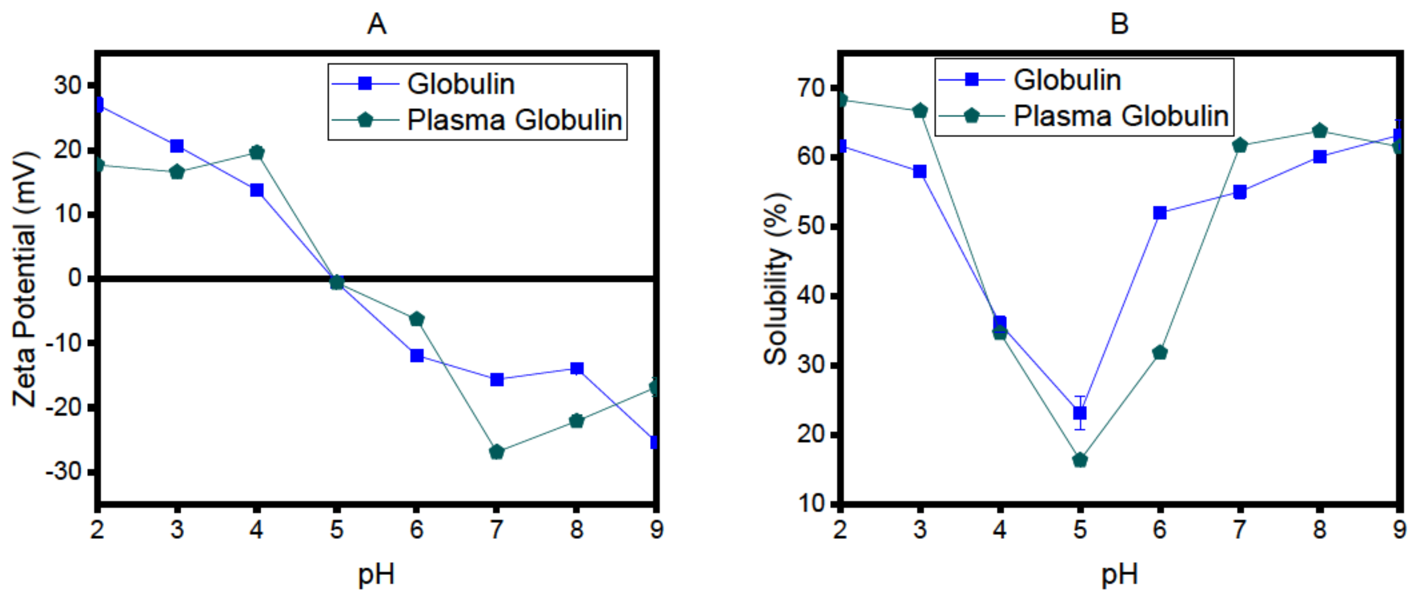


Figure 5. 3: (A) Zeta potential and **(B)** Solubility profile of plasma-treated Bambara globulin

Table 5. 3: Particle size distribution of plasma-treated Bambara globulin

Samples	Peak Intensity (nm)	Polydispersity Index (%)	Hydrodynamic Diameter (nm)	Size Distribution (nm)		
				D_{10}	D_{50}	D_{90}
Globulin	144.33[a] ± 0.08	18.70[a]	139.34[a]	84.60[a]	132.43[a]	203.30[a]
Plasma Globulin	184.31[b] ± 0.03	26.20[b]	160.69[b]	92.00[b]	163.92[b]	261.30[b]

Mean ± SD (n=3). Values along the column followed by the same superscripts are not significantly different.

5.3.8. Emulsifying properties

Emulsion capacity (EC) was measured as the oil droplet size since smaller oil droplet size reflected the high emulsifying capacity of a protein (Fig. 5.4A). The lower oil droplet size was observed for Bambara globulin at the measured concentrations compared to the plasma-treated globulin. This suggested the formation of a stronger and higher number of interfacial membranes as more proteins were available at the oil-water interface (Ijarotimi *et al.* 2018). However, the oil droplet size formed by plasma-treated globulin (5.43 ± 0.12 µm) was higher than that of the untreated globulin (3.41 ± 0.09 µm) indicating a poor emulsifying capacity of the Bambara globulin treated with plasma-activated water. The data showed that treatment of Bambara globulin with plasma-activated water did not improve its emulsifying capacity but rather significantly reduced it. Pérez-Andrés *et al.* (2019) reported that cold atmospheric plasma significantly reduced the emulsion capacity of hemoglobin and pork gelatin. Furthermore, the high oil droplet size recorded for plasma-treated Bambara globulin could be attributed to the changes in the structure of the protein. In this case, the amphipathic nature of the protein decreased because of the exposure of the hydrophilic groups on the protein surface shielding the hydrophobic groups buried in the inner portion of the protein. Thus, preventing interaction with the non-polar phase of the emulsion, and negatively impacting the emulsifying capacity of protein.

Emulsion stability (ES) increased as the protein concentration increased for plasma-treated globulin (Fig. 5.4B). Treatment of Bambara globulin with plasma-activated water significantly increased the emulsion stability as the protein concentration increased to 15 mg/mL and 20 mg /mL. This could be attributed to the protein concentration effect, which improved the hydrophobic-hydrophilic balance that is necessary to form strong interfacial membranes for effective stabilization of the oil-water interface (Lam and Nickerson 2013). The ability of plasma-treated globulin to stabilize its emulsion was significantly lowered at 10 mg/mL compared to the untreated globulin. Bambara globulin had the least ES at the concentration of 15 mg/mL compared to plasma-treated globulin. This could be attributed to the formation of a very weak interfacial membrane that was less resistant to coalescence. Emulsions formed by plasma-treated globulin exhibited bimodal droplet size distribution similar to the untreated globulin at all protein concentrations (Fig. 5.5). This reflected the inability of both treated and untreated globulin in producing uniform size oil droplets during homogenization. The emulsions formed contained oil droplets in the range of 100-1000 µm range.

5.3.9. Foaming properties

The ability of plasma-treated globulin solution to create foam significantly differed from the untreated globulin at 15 mg/mL (Fig. 5.6A). There seemed to be a slight increase in the foaming capacity of Bambara globulin at 10 mg/mL following plasma treatment. An increase in the foaming capacity of Bambara globulin after plasma treatment might be attributed to its unfolded structure as confirmed by the high hydrophobicity (Fig. 5.2B). The exposure of hydrophobic residues enhanced protein-air interaction. Duan *et al.* (2018) reported that high surface hydrophobicity might be positively correlated with the foaming capacity of food proteins. Mostly, oxidation of amino acids and structural changes in plasma-treated globulin might have induced higher protein chain flexibility as a result of faster adsorption at the air-water interface, which then impacts more elasticity on the adsorbed layer. In this study, a significant increase was not reflected in the foaming capacity of Bambara globulin at 10 mg/mL and there was a significant decrease in the foaming capacity at 20 mg/mL following plasma treatment, though oxidizing species were able to induce unfolding of protein structure which might facilitate/enhance foaming power. These results probably might be due to the excessive oxidation of the protein/amino acids accompanied by weak interfacial adsorption that caused foam bubbles to easily coalesce or collapse.

A slight increase was observed in the foaming stability (FS) of plasma globulin at 10 and 15 mg/mL compared to plasma globulin at a concentration of 20 mg/mL (Fig. 5.6B). The increase in the FS might be because of the unfolding of globulin, due to oxidation reaction by ROS as shown in the hydrophobicity data (Fig. 5.2B), which could probably strengthen the hydrophobic interaction and thus increase the foam stability of the protein. In this case, interfacial films would be more resistant, and the protein network constituted a mechanical barrier toward the rupture of the bubbles and coalescence. Furthermore, the increment could be related to the presence of the small-size aggregates which enhanced the formation of films with high rigidity due to the high packing density and strong intermolecular interaction. The decrease in the FS of globulin at a concentration of 20 mg/mL after plasma treatment observed could be attributed to the formation of larger aggregates causing steric impediments and thus preventing the formation of a closely packed interfacial layer (Amagliani and Schmitt 2017).

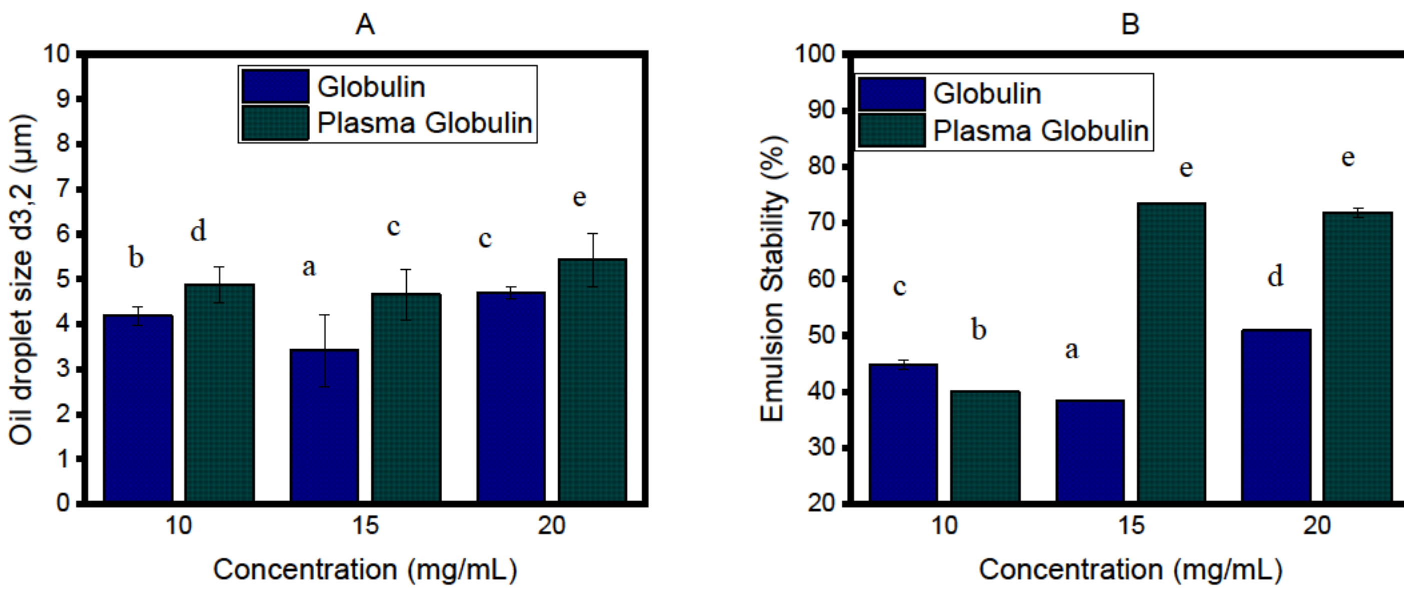


Figure 5. 4: Emulsifying properties of plasma-treated Bambara globulin at varying concentrations.

(A) Oil droplet size and (B) Emulsion Stability. Globulin Plasma Globulin

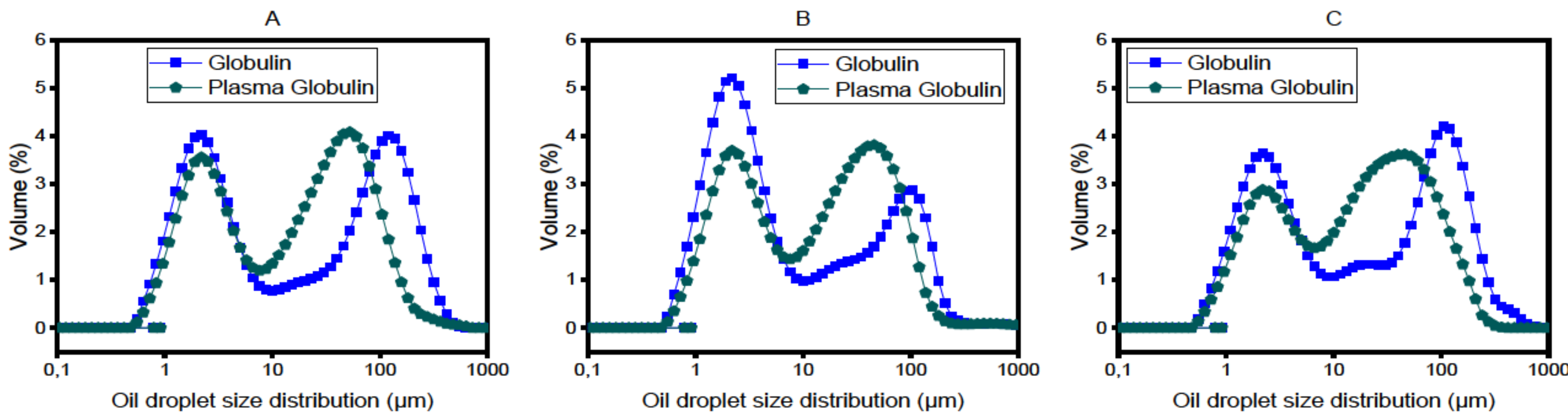


Figure 5. 5: Oil droplet size distribution properties of emulsion formed at pH 7 with plasma-treated Bambara globulin. Protein concentrations: (A) 10 mg/mL, (B) 15 mg/mL, and (C) 20 mg/mL

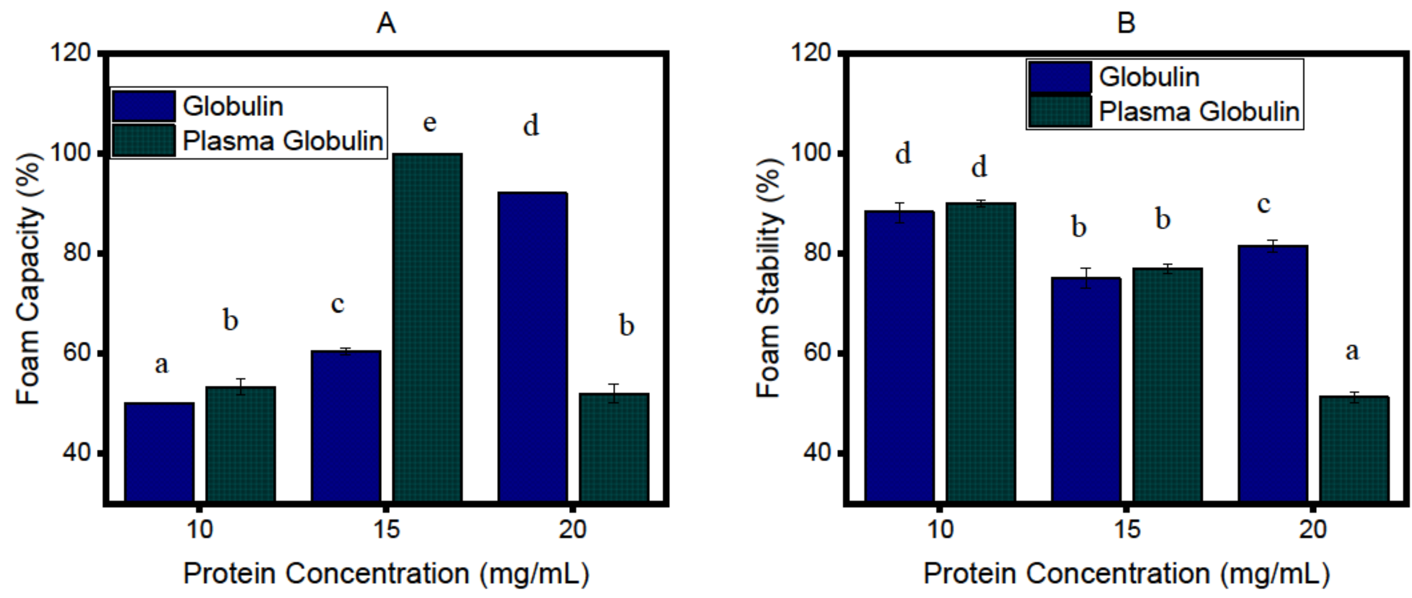


Figure 5. 6: Foaming properties of plasma-treated Bambara globulin at varying concentrations.

(A) Foaming Capacity (B) Foaming Stability

5.4. Conclusion

The impact of cold plasma-activated water treatment on the composition and molecular structure of Bambara groundnut protein and how PAW modification may affect its emulsifying and foaming properties are reported in this study. The hydration of Bambara groundnut globulin with plasma-activated water reduces the proportion of acidic amino acids and increases the proportion of β-sheet content and surface hydrophobicity of the treated protein compared with the native protein isolate. Although PAW treatment disrupted the conformation of Bambara groundnut globulin, the molecular weight distribution patterns of the proteins by gel electrophoreses appear similar. Cold plasma treatment significantly improves the foaming properties of the Bambara groundnut globulin only. This study findings provide some insight into the application of PAW as a promising green approach to improve some functional properties of proteins and may deserve further investigations to tailor the functionality of major biopolymers derived from pulse grains for food applications.

5.5. Research outputs

a) Journal article

1. Alabi, O.O., Annor, G., and Amonsou, E.O. 2023. Effect of cold plasma-activated water on physicochemical, and functional properties of Bambara groundnut globulin. *Food Structure*, 36:100321

b) Conference abstract

1. Alabi, O.O. and Amonsou, E.O. 2021. Impact of plasma-activated water treatment on the physicochemical and functional properties of Bambara globulin. Presented at the 2022 AOCS Annual Meeting and Expo: Advancing the science and technology of oils, fats, proteins, surfactants and related material, enriching the lives of people everywhere, 3rd-14th May, Atlanta, USA.

CHAPTER SIX

6. Inhibition of renin and angiotensin-I-converting enzyme by Bambara vicilin hydrolysate fractions

Abstract

The global impact of hypertension has made it a serious public health issue. This study investigated the ACE and renin inhibitory properties of globulin and vicilin hydrolysates including their peptide fractions. Hydrolysates from both globulin and vicilin had similar protein content. The protein content of the membrane fractions (<1 and <3 kDa) increased with increasing peptide size for both Bambara globulin and vicilin hydrolysates. The proportion of hydrophobic amino acid residues in both protein hydrolysates is high indicating the property that enhances lipid interaction, which thus improves entry of their peptides into target organs via hydrophobic associations. The rate of hydrolysis with pepsin and pancreatin showed an upward trend throughout the hydrolysis reaction. Surface hydrophobicity increased significantly ($p<0.05$) with an increase in peptide size from <1 to <3 kDa with that of vicilin hydrolysate and membrane fractions having the highest values. The low molecular weight peptide fractions of globulin hydrolysate showed the strongest ACE inhibitory activity at 1 mg/mL concentration compared to vicilin. Unlike ACE inhibition, a high molecular peptide fraction of vicilin hydrolysate favoured renin inhibitory activity at the same concentration. Bambara globulin and vicilin hydrolysate with low molecular weight peptides could be used as functional ingredients because of their ACE and renin inhibitory properties.

6.1. Introduction

The growing global burden of hypertension threatening an estimated one billion individuals (approximately 14.9% of the global population) has made it a serious public health issue in both developed and developing countries (Bavishi, Bangalore and Messerli 2016; Fu *et al.* 2017). Hypertension is a leading risk factor for coronary artery disease and its associated complications including heart failure, stroke, renal disease, and diabetes (Abegaz *et al.* 2017; Mazorra-Manzano, Ramírez-Suarez and Yada 2017). Hypertension is the state in which the blood pressure in the body is abnormally high for a prolonged period with the systolic blood pressure (SBP) recording ≥140 mmHg and the diastolic blood pressure (DBP) reading ≥90 mmHg (Muntner *et al.* 2019). It is one of the most significant causes of mortality worldwide. It has been predicted that 1.5 billion people will suffer from hypertension by 2025 with more than 7.1 million annual deaths (Tanzadehpanah *et al.* 2016). Hypertension can be controlled through the renin-angiotensin system (RAS) that helps to regulate the key enzymes, renin and angiotensin I-converting enzyme (ACE), which are responsible for causing blood pressure (BP) elevation (Crowley and Coffman 2012; Girgih *et al.* 2016; Fu *et al.* 2017). In the RAS pathway, renin, an enzyme synthesized in the kidney, cleaves angiotensinogen to generate the decapeptide angiotensin I (AT-I), for the subsequent conversion into octapeptide angiotensin II (AT-II) by the angiotensin-1-converting enzyme (ACE) at the C-terminal end (Aluko *et al.* 2015; Girgih *et al.* 2016; Mazorra-Manzano, Ramírez-Suarez and Yada 2017). Angiotensin II (AT-II) is a potent vasoconstrictor that stimulates the release of aldosterone, which increases sodium concentration and contributes to elevated blood pressure. In addition, ACE is reported to hydrolyze bradykinin, a potent vasodilatory peptide, into inactivate peptide fragments (Aluko *et al.* 2015; Salampessy *et al.* 2015; Pan *et al.* 2016). Therefore, inhibition of ACE activity will reduce the formation of AT-II and the destruction of bradykinin, which contributes to the blood pressure-lowering effect. Also, the direct inhibition of renin activity will provide a more effective blockade of the RAS, since renin is known to catalyze the initial rate-limiting step (Azizi 2008; Aluko 2015). It has been reported that prolonged therapy of inhibiting ACE activity leads to increased renal renin secretion that results in high angiotensin-I molecules which are converted via non-ACE routes involving chymase, kallikrein, and cathepsin enzymes to cause elevated angiotensin II levels (Malomo *et al.* 2015). Therefore, the simultaneous inhibition of ACE and renin activities will exert a synergistic effect on the efficient treatment of hypertension, compared to the individual enzyme inhibitors that are currently used (Harel *et al.* 2012; Connelly *et al.* 2013; Fu *et al.* 2017).

The global impact of hypertension encourages investigation into antihypertensive medications and new therapeutic alternatives from food sources (Muntner *et al.* 2019; Shi *et al.* 2017). At present, different types of antihypertensive medications including angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and calcium channel blockers owing to the physiological mechanisms of blood pressure control involving cardiac output, peripheral vascular resistance, and circulating blood volume have been developed (Siddiqi and Shatat 2020). However, these medications are associated with some negative side effects such as erectile dysfunction, dry cough, angioedema, taste disturbance, decrease in white blood cells, fatigue, skin rash, and diarrhea after prolonged use (Chen *et al.* 2013; Aluko 2015; Tao *et al.* 2017). In addition to the negative effects of medications for antihypertensive, ACE inhibitors, angiotensin receptor blockers, and calcium channel blockers have been reported to cause upper respiratory tract abstraction and angioedema in adults and children and may contribute to the development of cancer (Hom, Hirsch, and Elluru 2012; Shapovalov, Skryma, and Prevarskaya 2013). Therefore, there is an urgent need for the development and production of natural food-derived peptides to replace or complement synthetic ACE and renin inhibitors due to the high cost and known negative side effects (Udenigwe and Aluko 2012). Food-derived bioactive peptides have been reported to be effective in hypertension control (Niaz, Hafeez, and Imran 2017). This is because of their relatively short residue length and are more rapidly absorbed with little or no negative side effects because of fast clearance from the blood (Sarmadi and Ismail 2010; Alashi *et al.* 2014). The hydrophobic and bulky amino acids had been reported as structural requirements for ACE-inhibitory peptides (Udenigwe *et al.* 2012). This was in conformation to the recorded high percentage of ACE-inhibitory activity for Bambara hydrolysate from alcalase (Arise *et al.* 2016). However, the ACE-inhibitory activity of hydrolysate from pepsin and trypsin respectively was reduced. Bambara globulin and vicilin had been reported to contain a high amount of hydrophobic and aromatic amino acids (Alabi *et al.* 2020). Therefore, combination of pepsin and pancreatin endopeptidases which would give hydrolysates containing high amount of hydrophobic and bulky amino acids including positively charged amino acids would be essential to inhibit the ACE properties.

Although, a previous study suggested Bambara protein hydrolysates have dual potential antioxidant and antihypertensive properties (Arise *et al.* 2016), there is a paucity of information on the amino acid sequence of salt-extracted Bambara protein, globulin, and its subunit, vicilin, which contributes to identification or development of potent peptide inhibitors of Bambara vicilin fraction-derived peptide with ACE and renin inhibitory properties. The evaluation of

the globulin and vicilin components of Bambara will unlock its potential in various food applications.

6.2. Materials and methods

6.2.1. Preparation of defatted flour

Bambara seeds were ground into flour and then defatted with n-hexane in the ratio of 1:5 (g/mL) (flour: solvent) for 3 h as previously described by Arise, Ijabadeniyi, and Amonsou (2015). The defatted flour was left in a fume hood (HEMCO Uniflow LE AireStream, Missouri, USA) overnight to remove residual hexane and then stored at 4°C.

6.2.2. Extraction of Bambara globulin and vicilin

The extraction method adapted from the method reported by Klassen and Nickerson (2012) with slight modification was employed. Salt-soluble proteins were extracted with 50 mM K_2HPO_4 buffer, 0.5 M NaCl at pH 7.3, with a flour-to-buffer ratio of 1:10. The mixture was stirred continuously, using a magnetic stirrer, for 1 h at room temperature and centrifuged at 18,000 ×g for 25 min at 4°C using an Eppendorf 5810R centrifuge (Eppendorf Zentrifugen GmbH Leipzig, Germany). The supernatant was then diluted with cold Milli-Q water, adjusted to pH 4.6 using 1 M HCl, and left overnight in a cold room to facilitate the settling of the salt-soluble proteins. The precipitated protein was collected by centrifugation and washed twice with Milli-Q water (pellet-to-water ratio of 1:10) to remove unwanted albumin proteins. This was dialyzed (dialysis tubing of 6-8 kDa) against water, to remove salt, for 72 h. The dialysis water was changed three times daily. The pH of the de-salted protein extract was adjusted to 7.0 and then freeze-dried (Christ freeze-drier, Niedersachsen, Germany) as the globulin and stored at -20°C.

For vicilin extraction, the globulin was re-suspended in the extraction buffer at a volume-weight ratio of 5 mL per gram pellet and stirred continuously for 1 h at 4°C. The protein extract was collected by centrifugation, and this extraction step was repeated twice. The supernatant was pooled together and dialyzed against Mcllvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, pH 4.8) containing 0.2 M NaCl at 4 °C for 72 h, with the buffer being changed three times. The supernatant was collected by centrifugation (18,000 ×g for 25 min at 4°C). Vicilin was then precipitated from the supernatant at pH 4.5 with 1 M HCl. Vicilin was washed, re-centrifuged, and dialyzed against water as described above. The pH of vicilin was adjusted to pH 7.0 before being freeze-dried and stored at -20°C.

6.2.3. Protein content and yields

The protein contents of Bambara globulin and vicilin extracts with their hydrolysates and membrane fractions were determined using the modified Lowry method (Markwell *et al.* 1978), respectively.

6.2.4. Preparation of hydrolysate from Bambara globulin and vicilin with their peptide fractions

Enzymatic hydrolysis of Bambara globulin and vicilin isolate was carried out using pepsin and pancreatin enzymes successively with reaction conditions as described by Alashi *et al.* (2014) with some modifications. Briefly, Bambara globulin and vicilin, respectively, (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 pepsin (37°C and pH 3) and pancreatin (40°C and pH 8). Each enzyme was added to the protein slurry at an enzyme-to-substrate ratio (E/S) of 1:100, per the protein content of the sample. Digestion was carried out for 6 h (2 h and 4 h for pepsin and pancreatin digestion, respectively (pH was kept constant by the addition of 1 M NaOH or 1 M HCl). The enzymes were inactivated at the end of the hydrolysis by heating the mixture in a water bath at 95°C for 10 min. The mixture was then cooled to room temperature before centrifugation at 8000 xg for 60 min to precipitate the undigested proteins at the end of the hydrolysis period. The supernatant containing target peptides was collected, and a portion of it was freeze-dried to obtain Bambara globulin hydrolysate (BGH) and Bambara vicilin hydrolysate (BVH), respectively, while the remaining portion was passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1 kDa and 3 kDa sequentially in an Amicon 8400 stirred ultrafiltration cell (Millipore Corp., Billerica, MA, USA). The permeate from each MWCO membrane (<1 and 1–3 kDa, respectively) was collected, freeze-dried, and stored at -20°C until required for further analysis. The protein contents of the freeze-dried Bambara protein hydrolysates and membrane fractions were also determined using the modified Lowry method (Markwell *et al.* 1978).

6.2.5. Amino acid analyses

The amino acid profiles of Bambara globulin and vicilin hydrolysate were determined using the HPLC PICO-TAG system (Waters, Milford, USA) (Bidlingmeyer, *et al.* 1984). The samples were hydrolyzed with 6 mol/L HCl at 116°C for 24 h before chromatographic analysis. The cysteine and methionine contents were determined after performic acid oxidation (Gehrke,

et al. 1985) while the tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992). The digests were separated on an Agilent ZORBAX Eclipse XDB C18 column, USA (4.6 x 150 mm) using a gradient of sodium citrate buffers (pH 3.45 and pH 10.85) at a 0.45 mL/min flow rate.

6.2.6. Degree of Hydrolysis (DH)

The available free amino groups in the Bambara protein samples upon hydrolysis were determined using the *o*-phthalaldehyde (OPA) method as described by Wanasundara *et al.* (2002) with slight modifications. The freshly prepared OPA reagent consisted of 6 mM of OPA dissolved in methanol, 0.1 M of sodium tetraborate decahydrate containing 0.2% (w/v) of sodium-dodecyl-sulfate (SDS), and β-mercaptoethanol. Bambara protein samples/standard (10 µL) were mixed with 200 µl of OPA reagent in a 96 well-clear plate and incubated for 100 sec at 37°C before measuring the absorbance at 340 nm on a microplate reader. The number of free amino groups in the hydrolysate was calculated as serine-NH_2 moieties using Gly-Gly as a standard. The total number of primary amino groups in the globulin and vicilin was determined by acid hydrolysis using 6 M of HCl at 110°C for 24 h. This represented complete hydrolysis of all peptide bonds of the protein. The DH (%) was calculated as follows:

$$= 100 * \frac{\text{(OPA)hydrolysate}}{\text{(OPA)acid digest}}$$

6.2.7. Surface hydrophobicity (So)

Surface hydrophobicity (So) of Bambara globulin and vicilin hydrolysates and their membrane fractions (<1 kDa and < 3 kDa), respectively, was determined by the fluorescence method using a hydrophobic probe, 8-anilino-1-naphthalene sulfonic acid (ANS) on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) as described by do Evangelho *et al.* (2017). Briefly, protein stock solution (10 mg/mL) was prepared in 0.1 mol/L phosphate buffer (pH 7.0) and then centrifuged at 10,000 ×g for 15 min. This was diluted to varying concentrations of 0.0009 - 0.015 mg/mL with phosphate buffer to make up 4 mL. To these, 20 µL of ANS (8 mM) was added and the mixtures were vortexed for 30 s. Fluorescence was measured at excitation and emission wavelengths of 390 and 470 nm, respectively. The fluorescence intensity of the control (ANS + phosphate buffer) was measured. The slope of the plot of fluorescence versus protein concentration was taken to be the hydrophobicity.

6.2.8. ACE inhibition assay

The *in-vitro* inhibition property of ACE in Bambara globulin and vicilin hydrolysate, and their membranes permeates, respectively was measured using the method described by Udenigwe *et al.* (2009) with slight modification. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5, and kept at 37°C) was mixed with 10 µL of ACE (final activity of 25 mU) and 20 µL of Bambara protein samples dissolved in the Tris–HCl buffer (1 mg/mL final concentration). The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. Tris–HCl buffer was used as the assay blank. The ACE activity was expressed as the change in the rate of reaction (ΔA/min) and inhibitory activity was calculated using the equation below:

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

Where $(\Delta A/min)(blank)$ and $(\Delta A/min)(sample)$ are ACE activities in the absence and presence of samples, respectively.

6.2.9. Renin inhibition assay

Renin Inhibitor Screening Assay Kit was used to determine the *in-vitro* inhibition *of* human recombinant renin activity of Bambara protein samples as previously described by Alashi *et al.* 2014 with slight modification. Briefly, Bambara samples and Renin buffer were diluted using 50 mM Tris–HCl, pH 8.0 containing 100 mM NaCl. The renin protein was diluted at a ratio of 1:20 using the assay buffer before pre-warming to 37°C. Before the reaction, (1) Background wells were filled with 20 µL of renin substrate, 151 µL of assay buffer, and 19 µL Milli-Q water (2) Blank wells were filled with 20 µL of renin substrate, 141 µL of assay buffer, and 19 µL Milli-Q water (3) The inhibitor wells were filled with 20 µL of renin substrate, 141 µL of assay buffer and 19 µL samples (1 mg/mL final concentration based on protein content). The reaction was then initiated by adding 10 µL of renin enzyme to the blank and sample wells. The mixtures in the microplate were shaken and incubated at 37°C for 15 min and the fluorescence intensity was measured using a spectrofluorimeter plate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) at an excitation and emission wavelengths of 340 and 490 nm, respectively. Percentage inhibition of renin activity was calculated as follows:

$$\text{Inhibition of renin inhibition (\%)} = x = \frac{(FI\ of\ blank\ well\text{-}FI\ of\ sample\ well)}{(FI\ of\ blank\ well)} * 100$$

6.2.10. Statistical analysis

All experiments were carried out in triplicate. Data were analyzed using analysis of variance (ANOVA) and means were compared using Fischer's Least Significant Differences Test ($p < 0.05$).

6.3. Result and discussion

6.3.1. Protein content

Bambara globulin had a lower protein content than its subunit, vicilin (Table 6.2). In contrast, hydrolysates from both globulin and vicilin had similar protein content. There was a significant ($p < 0.05$) decrease in the protein content of the hydrolysates. This might be attributed to the extent of hydrolysis by the proteases employed (pepsin-pancreatin) as reported by Olagunju *et al.* (2018) for pigeon pea hydrolysates obtained by alcalase and pancreatin digestion. The protein content of the membrane fractions (< 1 and < 3 kDa) increased with increasing peptide size for both Bambara globulin and vicilin hydrolysates. The low protein content recorded for the <1 kDa permeate during the sequential membrane ultrafiltration might be attributed to the presence of impurities such as salt formed during the protein hydrolysis process (Girgih *et al.* 2014). The data obtained in this study followed a similar trend to that reported for canola hydrolysates by Alashi *et al.* (2014).

Table 6. 1: The protein content of hydrolysates from Bambara globulin and vicilin and their membrane fractions.

Samples	Protein Content (%)
Bambara Globulin	$89.02^{c} \pm 0.09$
BGHppc	$80.83^{b} \pm 0.21$
<1 kDa BGHppc	$66.68^{a} \pm 0.92$
< 3 kDa BGHppc	$80.07^{b} \pm 0.62$
Bambara Vicilin	$96.00^{d} \pm 0.91$
BVHppc	$80.40^{b} \pm 2.31$
<1 kDa BVHppc	$63.69^{a} \pm 0.06$
< 3 kDa BVHppc	$89.92^{c} \pm 0.22$

n=3. Values along the column followed by the same superscripts are not significantly different. BGHppc: Bambara globulin hydrolyzed with pepsin and pancreatin; BVHppc: Bambara vicilin hydrolyzed with pepsin and pancreatin; <1 kDa BGHppc: less than 1 peptide size from Bambara globulin hydrolyzed with pepsin and pancreatin; <3 kDa BGHppc: less 3 peptide size from Bambara globulin hydrolyzed with pepsin and pancreatin <1 kDa BVHppc: less than 1 peptide size from Bambara vicilin hydrolyzed with pepsin and pancreatin; <3 kDa BVHppc: less than 3 peptide size from Bambara vicilin hydrolyzed with pepsin and pancreatin

6.3.2. Amino acid

The amino acid composition of Bambara globulin hydrolysate is similar to that of vicilin (Table 6.1). Glutamic and aspartic acids were the most abundant amino acids in both Bambara protein hydrolysates. The total hydrophobic amino acid (HAA) residues in both protein hydrolysates are similar while the negatively charged amino acids in vicilin hydrolysate are slightly higher than that of the globulin hydrolysate. The high amounts of negatively charged amino acids (NCAA) in the Bambara proteins hydrolysates are an index of antioxidant quality. NCAAs had been reported to possess free electrons that could be donated to neutralize and limit the destructive ability of free radicals (Aondona *et al.* 2021; Da Rocha *et al.* 2018). The presence of high amounts of HAAs in the Bambara globulin and vicilin hydrolysates suggested properties that promote lipid interaction, which enhance the entry of their peptides into target organs via hydrophobic associations (Sarmadi and Ismail 2010). The high amount of arginine in the Bambara proteins hydrolysates is a positive development because the presence of this amino acid in substantial quantity could make it serve as a precursor for the production of nitric oxide (NO). Nitric oxide promotes vasodilation through the enzyme endothelial nitric oxide synthase. Khalaf *et al.* (2019) reported that elevated blood pressure in both humans and animals was due to the inadequate production of NO by the body. The high amount of positively charged amino acids (PCAA) recorded for Bambara protein hydrolysates had been associated with strong radical scavenging activity due to the presence of the imidazole ring (Aondona *et al.* 2021; Samaranayaka and Li-Chan 2011). For utilization of protein hydrolysates in functional foods, the ingredients must present adequate nutritional value, in addition to bioactive properties. In this regard, the amino acids content of Bambara proteins hydrolysates was comparable to the FAO/WHO (1991) recommended pattern for amino acids.

Table 6. 2: Amino acid composition (g/100 g protein) of hydrolysates from Bambara globulin and vicilin

Amino Acids	Globulin Hydrolysate	Vicilin Hydrolysate	FAO/WHO (1991)
HIS	3.01	3.31	1.90
SER	5.66	5.75	
ARG	7.03	7.19	
GLY	3.38	3.11	
ASP	12	11.95	
GLU	19.47	21.73	
THR	3.25	2.71	3.40
ALA	3.87	3.42	
PRO	4.62	4.71	
CYS	0.46	0.32	
LYS	7.28	7.29	5.80
TYR	3.79	3.67	
MET	1.29	1.11	2.50
VAL	5.4	5.16	3.50
ILE	4.38	4.03	2.80
LEU	8.22	7.79	6.60
PHE	5.87	5.84	6.30
TRP	1.04	0.9	1.10
AAA	10.69	10.41	
PCAA	17.31	17.79	
NCAA	31.46	33.67	
HAA	35.06	33.54	

Asx = aspartic acid and asparagine, Glx = glutamic acid and glutamine, HAA = hydrophobic amino acid, PCAA = positively charged amino acid, NCAA = negatively charged amino acid, AAA = aromatic amino acid,

6.3.3. Degree of Hydrolysis (DH)

The degree of hydrolysis values of Bambara globulin and vicilin hydrolysates showed an upward trend throughout the hydrolysis reaction. The rate of hydrolysis with pepsin and pancreatin was very fast during the first 30 min, with the rate higher for Bambara vicilin than globulin (Fig. 6.1 A and B). The results suggested that the enzymes were in close contact with the substrate at the beginning of the reaction. After this, the hydrolysis proceeded more slowly for both protein samples. The slowdown of the rate of hydrolysis observed may be due to the disappearance of proteins and large molecular size peptides in addition to the prolongation of the time of hydrolysis. This might have resulted in a decrease in the availability of substrates or in enzyme activity. Pepsin enzyme is an endoprotease, having more catalytic sites to destroy the protein peptide bonds, such as aromatic amino acids and hydrophobic amino acids (Tyr, Trp, Phe, and Leu) (Tavano 2013). The Bambara globulin and vicilin hydrolysates by a combination of pepsin and pancreatin showed the highest degree of hydrolysis value at 6 h. The degree of hydrolysis affects the size, structure, and amino acid composition of peptides generated during hydrolysis (Liu *et al.* 2016; Wang *et al.*2021). The data followed a similar trend to that reported by Hu, Singh, and Chan (2018) and Zou *et al.* (2016).

6.3.4. Surface Hydrophobicity

Surface hydrophobicity data recorded for vicilin hydrolysate, and its membrane fraction were higher than that of globulin hydrolysate with its membrane fraction (Fig. 6.2). So was directly proportional to the peptide size because the values increased significantly ($p<0.05$) with an increase in peptide size from <1 to <3 kDa. The hydrophobicity of the Bambara globulin and vicilin hydrolysate was the highest. This might be a result of subsequent exposure to hydrophobic amino acids that were buried inside the core of the folded structure of native protein molecules (Olagunju *et al.* 2018). The surface hydrophobicity of peptides could be considered an important determinant of bioactivity when interaction with hydrophobic biological targets is required. Surface Hydrophobicity of food protein-derived peptides played important roles in their bioactivities (Ajibola *et al.* 2011).

6.3.5. ACE Inhibitory properties of Bambara proteins hydrolysates and membrane fractions

The inhibitory activity of the Bambara globulin hydrolysate peptide fractions was molecular weight-dependent similar to that of vicilin (Fig. 6.3). The Low molecular weight peptides (LMW) (<1 kDa) fractions from both globulin and vicilin hydrolysate showed a higher

percentage of ACE inhibitory activity than the high molecular weight (HMW) peptides (<3 kDa). Generally, all peptide fractions from Bambara proteins hydrolysates including the hydrolysate itself had a higher percentage of Angiotensin-converting enzymes (ACE) inhibitory activity at 1 mg/mL concentration. Lower ACE inhibitory activity was observed at 0.5 mg/mL concentration of the Bambara proteins. The minimum inhibition activity was observed at 0.5 mg/mL concentration for the protein hydrolysates. This result suggested that LMW peptides were more active at inhibiting ACE activity when compared to HMW peptide proteins. The data obtained in this study corroborated the fact that the best ACE-inhibitory activity was attributed to LMW fractions as reported for sesame hydrolysate peptide fractions by Aondona *et al.* (2021). In addition, the high hydrophobic and aromatic amino acids of pepsin and pancreatin hydrolysate could have resulted in the high ACE-inhibitory activity as these amino acids had been reported as the structural requirement for ACE-inhibitory peptides (Udenigwe *et al.* 2012). Similar ACE-inhibitory activity behaviour of peptides was also reported for kidney bean peptide fractions (Mundi and Aluko 2014).

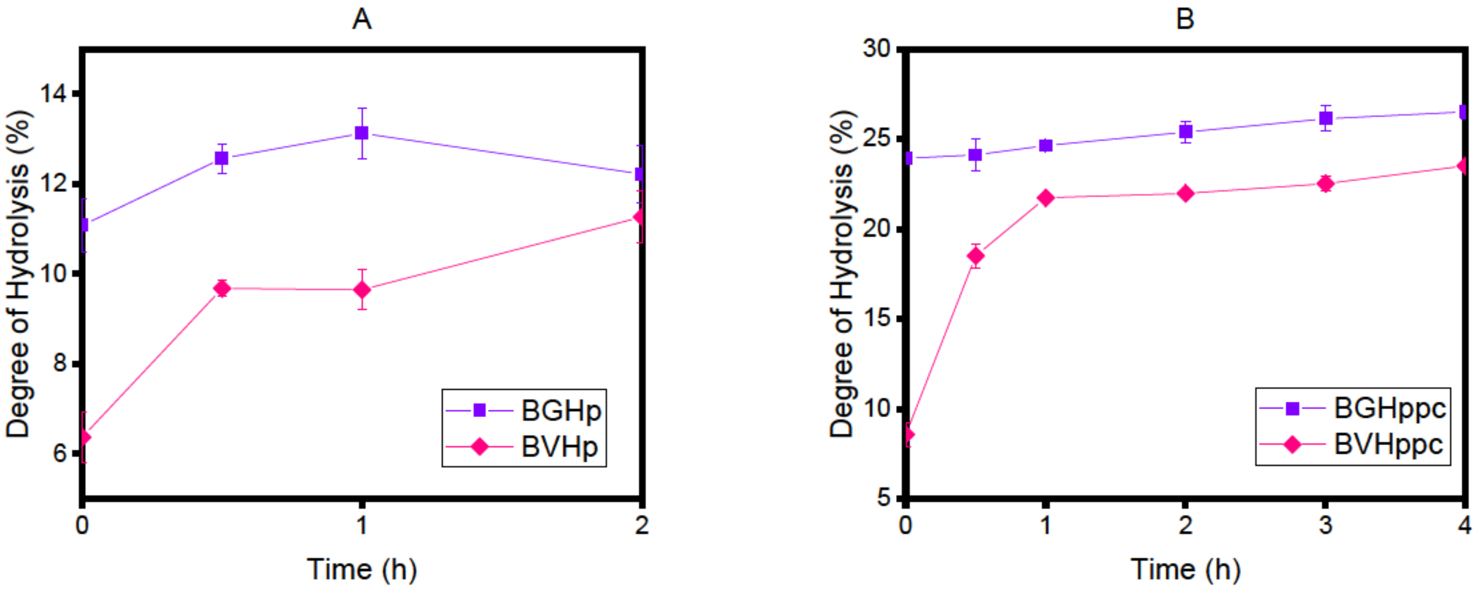
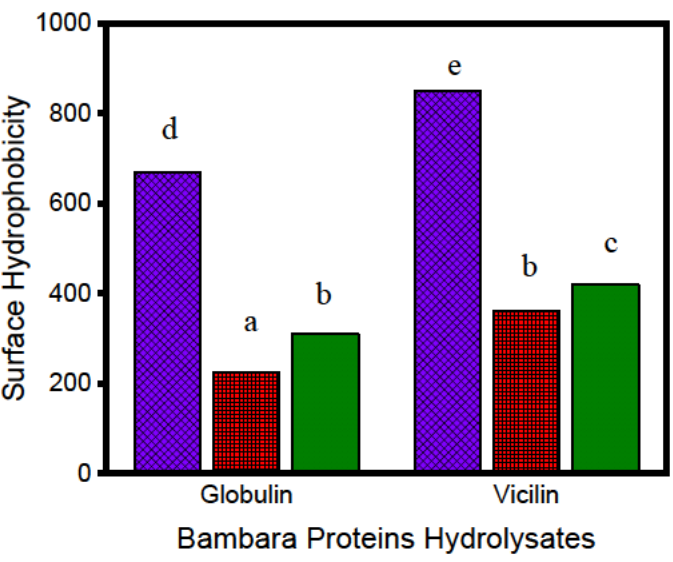


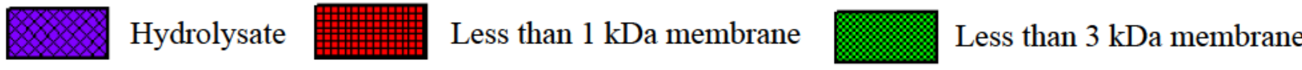
Figure 6. 1: Enzymatic hydrolysis curve on Bambara globulin and vicilin isolate by proteases.

(A) Degree of hydrolysis of Bambara protein hydrolysates using pepsin enzyme.

(B) Degree of hydrolysis of Bambara protein hydrolysates using a combination of pepsin and pancreatin enzymes.

BGHppc: Bambara globulin hydrolyzed with pepsin and pancreatin; BVHppc: Bambara vicilin hydrolyzed with pepsin and pancreatin





*Bars that contain different letters are significantly different at $p<0.05$

Figure 6. 2: Surface hydrophobicity of Bambara globulin and vicilin isolate with their membrane fractions.

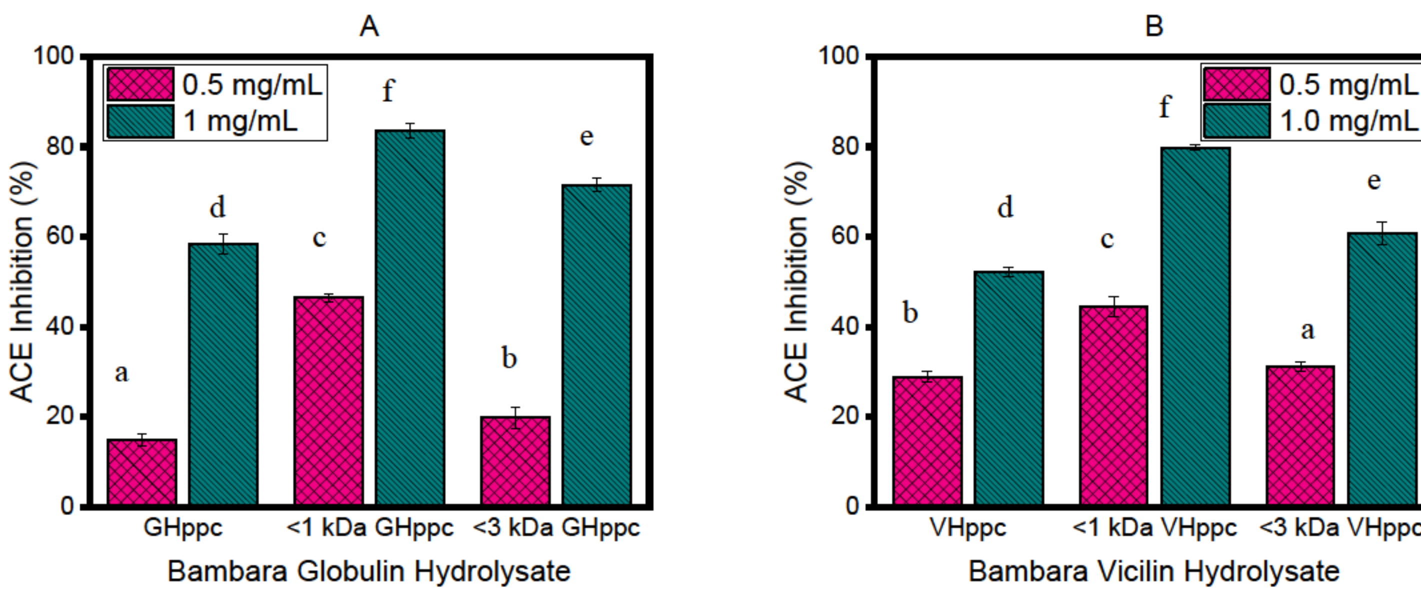


Figure 6. 3: Inhibition of angiotensin-converting enzyme (ACE) by enzymatic Bambara protein hydrolysates and membrane ultrafiltration fractions at 0.5 mg/mL and 1.0 mg/mL, respectively.

Error bars (mean ± standard deviation, n = 3) with different alphabets have mean values that are significantly different ($p < 0.05$). GHppc: Bambara globulin hydrolyzed with pepsin and pancreatin; VHppc: Bambara vicilin hydrolyzed with pepsin and pancreatin; <1 kDa GHppc: less than 1 peptide size from Bambara globulin hydrolyzed with pepsin and pancreatin; <3 kDa GHppc: less 3 peptide size from Bambara globulin hydrolyzed with pepsin and pancreatin <1 kDa VHppc: less than 1 peptide size from Bambara vicilin hydrolyzed with pepsin and pancreatin; <3 kDa VHppc: less than 3 peptide size from Bambara vicilin hydrolyzed with pepsin and pancreatin

6.3.6. Renin Inhibitory properties of Bambara proteins hydrolysates and membrane fractions

The peptide fractions were better renin inhibitors than the Bambara globulin and vicilin hydrolysates (Fig. 6.4). Renin inhibitory activity was not observed for Bambara vicilin hydrolysate obtained from the combination of pepsin and pancreatin enzymes at both 0.5 and 1 mg/mL concentration. Globulin hydrolysate showed a very low renin inhibitory activity at 1mg/mL concentration. The ultrafiltration membrane peptide fraction (<3 kDa) for globulin hydrolysate showed higher renin inhibition activity similar to the vicilin hydrolysate. The strongest renin inhibitory activity was recorded for high molecular weight peptide fractions for globulin and vicilin at 1 mg/mL concentration. Unlike ACE inhibition, where the low molecular peptide fraction favoured its inhibition properties. High molecular weight peptide fractions with hydrophobic side chains had been reported to enhance the inhibition of renin activity (Aderinola *et al.* 2018; Yuan, Wu, and Aluko 2007). This is because these fractions were better accommodated at the renin active site. The type of enzyme used significantly impacted the renin inhibitory potential of protein hydrolysates. This result agreed with the previous studies that showed that the inhibition of renin activity was favoured by high molecular weight peptide fractions (Aondona *et al.* 2021; Mundi and Aluko 2014). Renin is a rate-limiting enzyme in the renin-angiotensin system responsible for converting angiotensinogen to angiotensin I, which is a substrate for ACE.

6.4. Conclusion

The negatively charged, and the hydrophobic amino acids were high in both globulin and vicilin hydrolysates which is an index of antioxidant quality and properties that promotes lipid interaction. Hydrophobicity significantly increased with peptide size which is an important determinant of bioactivity. The degree of hydrolysis values of Bambara globulin and vicilin hydrolysates showed an upward trend throughout the hydrolysis reaction. The Low molecular weight peptides (<1 kDa) fractions from both globulin and vicilin hydrolysate at 1 mg/mL showed a higher percentage of ACE inhibitory activity. Smaller size peptides exhibit better bioactivity because of the higher possibility for an increased rate of intestinal absorption with no structural degradation and entry into cells when compared with the larger-sized peptides. High molecular weight peptide (<3 kDa) fractions showed greater inhibition of renin activity.

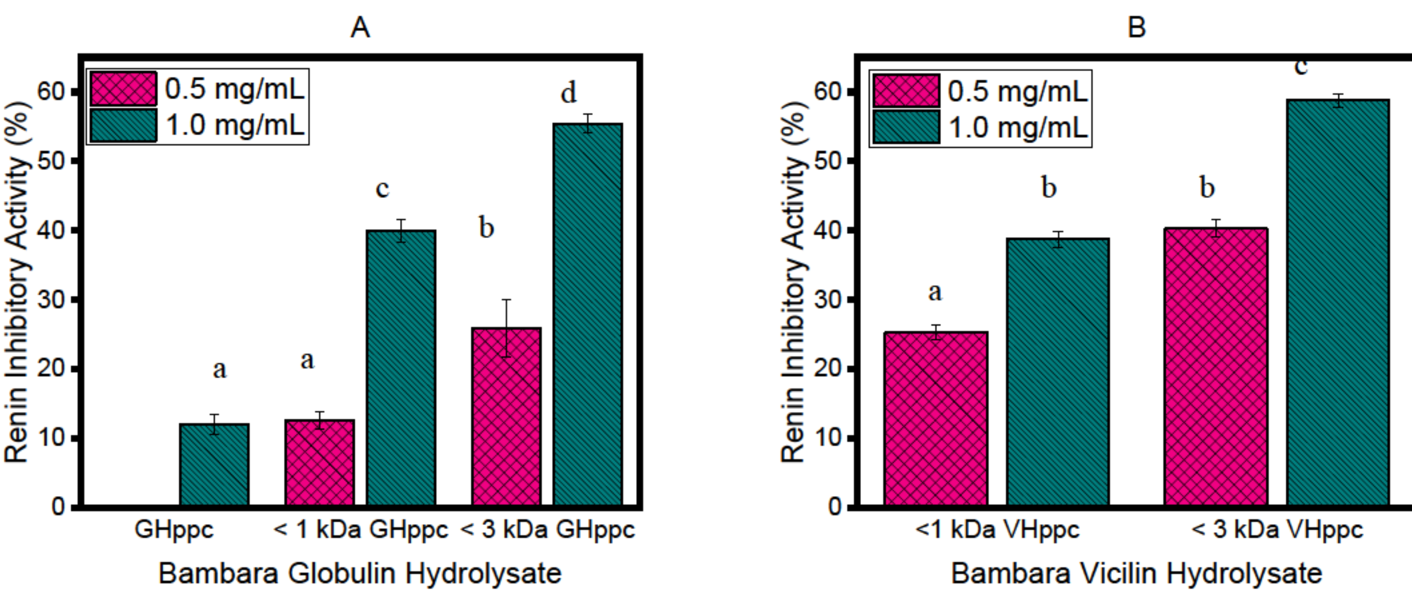


Figure 6. 4: Inhibition of renin by enzymatic Bambara protein hydrolysate and membrane ultrafiltration fractions at 0.5 mg/mL and 1.0 mg/mL, respectively.

CHAPTER SEVEN

7. General Discussion

In this thesis, Bambara storage protein (globulin) was isolated, fractionated into subunits known as legumin and vicilin, and characterized. Bambara globulin was then modified with plasma-activated water, a green, environmentally friendly technology. The first part of the thesis reported the composition and some functional properties of vicilin fraction from Bambara groundnut (Chapter 3). The subsequent chapter discussed the impact of structure composition on gel microstructures and rheological properties of vicilin-like and legumin-like proteins fractionated from Bambara globulin (Chapter 4). The succeeding chapter described the effect of plasma-activated water on the structural, physicochemical, and functional properties of Bambara globulin (Chapter 5). The last section of the thesis detailed the inhibition of angiotensin-I-converting and renin enzymes by Bambara globulin and vicilin hydrolysate fractions (Chapter 6).

There is variation in the protein composition of legumes which is dependent on the sources and type of the legume. The major storage proteins in legumes are albumins and globulins (Shen and Tang 2014). Globulin is subdivided into vicilins (7S) and legumins (11S) based on their sedimentation coefficient. The composition and functional properties of Bambara vicilin, an isoform of globulin, in comparison with its storage protein were investigated to advance knowledge on the development of protein ingredients with excellent functional attributes, which would be important to facilitate the utilization of Bambara proteins as food ingredients. The major vicilin polypeptide was evident as the most intense band around 50 kDa. The proline content of the Bambara vicilin fraction was about four times that of the storage protein. A high amount of proline was reported to help in preventing protein aggregation and increase protein solubility. This is because proline prevents the formation of protein beta-pleated conformation. Bambara storage proteins and vicilin contain a considerable amount of sulfur-containing amino acids, which are usually limiting in legume seeds. The fluorescence spectra showed maximum intensity at around 345 nm reflecting the dominancy of tryptophan residues. Fluorescence and hydrophobicity data suggested a less folded structure for the vicilin fraction when compared to the storage protein. This was attributed to the higher proline content recorded for the vicilin fraction which could prevent aggregation by destabilizing the protein structure (Damodaran et al., 2008). Bambara storage protein had two transitions at 79℃ and 94℃, respectively compared to the vicilin fraction which showed a single endothermal peak at 94℃. The high

thermal stability of the Bambara proteins might be associated with their high molecular protein subunits as indicated in the gel electrophoresis. Vicilin had a significantly ($p<0.05$) higher solubility (82%) at pH 3. The emulsion activity of Bambara storage protein and vicilin were below 45% whilst higher emulsion stability (>95%) was recorded for these proteins at pH 3, 7, and 9, respectively. The foaming capacity of the vicilin fraction was significantly ($p < 0.05$) higher than that of the storage protein at pH 3, 7, and 9. The high foaming capacity of the vicilin fraction was consistent with its greater solubility, which enables better flexibility, and a more efficient air bubble encapsulation compared to the storage protein. These functional attributes were attributed to the high protein content, solubility, and surface hydrophobicity.

The interaction of plant proteins with their subunits and ingredients in a complex system is controlled by their structure-function relationship (Joshi *et al.* 2012). The functional properties of proteins, such as solubility, rheological behaviour, and gelation properties, majorly depend on their molecular and structural composition (Shevkani *et al.* 2019). The differences in the polypeptide and subunit composition including legumin and vicilin of plant storage proteins such as globulins affect their structural properties and determine their applications in the food system. The impact of the structure-composition of vicilin and legumin-like proteins fractionated from Bambara globulin on their gel microstructures and heat-induced gel rheological properties were studied to unlock their potential and facilitate their utilization as a replacement for animal-based proteins. Bambara legumin had the least protein content compared to globulin and vicilin. Bambara globulin is being stabilized by disulfide linkages from legumin as shown by the SDS-PAGE despite containing about 70% of vicilin. The methionine content of Bambara globulin and its fractions is higher than the FAO/WHO recommended value. Legumin had the least amino acid content including the negatively charged amino acids which was almost half of the values recorded for globulin and vicilin.

The maximum peak intensity of the fluorescence spectrum of Bambara globulin and its subunit fractions (legumin and vicilin) was attributed to tryptophan emission. Legumin fraction showed the highest maximum (λmax) value compared to the vicilin fraction and the parent protein, globulin, which might be due to its more compact structure. The presence of disulfide linkage was a contributing factor to the stability of legumin in an aqueous solvent. The fluorescence peak intensity for the globulin was significantly reduced. However, this reduced fluorescence intensity could not be linked to denaturation based on the hydrophobicity data recorded. Probably, the reduced intensity could be linked to the effect of tryptophan quenching as

aromatic amino acids such as phenylalanine and tyrosine, can interact with tryptophan when in proximity to destruct tryptophan. The interaction of aromatic amino acids with tryptophan might have reduced the amount of absorbed light energy and thus enhanced fluorescence quenching. The differences in the fluorescence and hydrophobicity data of Bambara globulin, legumin, and vicilin were related to variations in amino acid composition. The Legumin spectrum displayed a single positive peak at 194 nm and a minimum negative peak at 208 nm which suggested the dominance of helical structure. The dominance of the helical structure agreed with the fluorescence data that showed higher fluorescence intensity.

Globulin had the highest gelling capacity (low least gelation concentration (LGC)) whilst vicilin had the lowest gelling capacity though both are comparable in terms of composition and structure. The low LGC of globulin could be attributed to the combined effect of legumin and vicilin in terms of amino acid composition, and degree of interaction in the heat-induced process. The high LGC of Bambara vicilin was attributed to the dominance of repulsive force in its dispersion which corroborated the observed highest solubility compared to globulin and legumin. The frequency sweep data revealed that an elastic characteristic of the gels prepared at the least gelation concentration (LGC) was dominant as the G′ values were greater than G″ irrespective of their LGC. Bambara protein gels prepared at a concentration of 22.5%, to minimize the protein concentration effects, showed frequency-dependent behaviours of both the storage and loss moduli. It was observed that weakly structured gels were formed as the difference between the G′ and G″ was smaller than 1 log cycle. The scanning electron microstructural display of Bambara protein gels showed that vicilin gel had a more defined laths sheet-like structure with many interdependent micropores. This was consistent with the frequency sweep data of the gels where the vicilin gel appeared more rigid (high G′) than globulin and legumin. The disparity in the gel points of Bambara protein fractions was ascribed to differences in amino acid and subunit composition together with the thermal unfolding properties of each fraction, and the presence of disulfide linkages in globulin and legumin. The dominancy of the β-sheet structure and the higher crosslink density of vicilin gel were linked to the stiffness observed in the gel as shown by the complex modulus data.

The poor functional properties of plant proteins which impede their applications as food ingredients in the food system may be enhanced by modifying their structure. The effect of plasma-activated water (PAW) on the structure, physicochemical and functional properties of Bambara globulin was investigated to gain knowledge on how the reactive species in the

plasma-activated water interacted with Bambara proteins to cause structural modification that influenced its functional properties in food systems. SDS-PAGE data showed that treatment of the Bambara protein with plasma-activated water did not seem to change the molecular weight distribution of the protein. Plasma-activated water had no significant effect on the amino acid profile of Bambara globulin, except for glutamic acid. A redshift in the wavelength of the fluorescence spectrum was noted for the plasma-treated Bambara globulin. The hydrophobicity value recorded for plasma-treated globulin was higher than the untreated. These properties were accompanied by the unfolding and dissociation of the reversible protein aggregates including the protein subunits by the etching process of plasma treatment. The FTIR data reflected the degradation of α-helix secondary structures and rearrangement of β-structures as evident by the hydrophobicity and fluorescence data. Plasma treatment did not change the isoelectric point of Bambara globulin, and the solubility profile was not significantly altered. The formation of aggregates, as influenced by the attachment of water micelles to the protein molecules, by the reaction of the reactive species from plasma-activated water was suggested to have caused a significant increase in the polydispersity index (PDI) value. Bambara globulin had reduced emulsifying capacity after treatment with PAW whilst the emulsion stability was significantly increased. The foaming properties of Bambara globulin were enhanced by its unfolded structure as confirmed by the fluorescence and hydrophobicity data.

Food-derived bioactive peptides with relatively short residue lengths have been indicated to replace or complement synthetic angiotensin I-converting enzyme (ACE) and renin inhibitors in the control of hypertension. The inhibition properties of angiotensin-I-converting and renin enzymes by Bambara globulin and vicilin hydrolysate fractions and their amino acid sequencing profile were investigated to contribute to identification and development of their potent peptide inhibitors. Hydrolysates from both globulin and vicilin had similar protein content. The protein content of the membrane fractions (<1 and <3 kDa) increased with increasing peptide size for both Bambara globulin and vicilin hydrolysates. The amino acid composition of Bambara globulin hydrolysate is similar to that of the hydrolysate from vicilin. Bambara proteins hydrolysates contained a high amount of arginine. The presence of arginine in substantial quantity served as a precursor to producing nitric oxide (NO) which promotes vasodilation through the enzyme endothelial nitric oxide synthase. The rate of hydrolysis with pepsin and pancreatin showed an upward trend throughout the hydrolysis reaction. Surface hydrophobicity increased significantly ($p<0.05$) with an increase in peptide size from <1 to <3 kDa. The strongest ACE inhibitory activity was observed at 1 mg/mL concentration for low

molecular weight peptide fractions of globulin and vicilin, respectively. Unlike ACE inhibition, high molecular peptide fraction favoured renin inhibitory activity at the same concentration. ACE and renin inhibitory activity was attributed to the high hydrophobic and aromatic amino acids content of pepsin and pancreatin hydrolysate.

CHAPTER EIGHT

8. Conclusion and recommendations

8.1. Conclusion

Bambara globulin contains about 70% vicilin fraction with a limited proportion of legumin protein stabilized by a disulfide bond. Bambara globulin and fractions were good sources of lysine (9.2%). The protein fractions contain higher methionine content than the values recommended by the FAO/WHO (1991). The β-sheet structure was the dominant secondary structure in Bambara globulin and vicilin, unlike the legumin fraction, which has a helical structure.

The least gelation capacity (LGCs) of Bambara proteins fractions at pH 7 significantly differed among the protein fractions. The sol-gel transition temperatures followed the order vicilin > legumin > globulin. The dependency of the storage modulus (G′) and loss modulus (G″) of Bambara globulin on the heating time suggested a rapid establishment of the gel network. The G′ of Bambara vicilin and legumin increased over the heating time, indicating a progressive establishment of their gel network. The observed sol-gel transition temperatures and gel structure development in Bambara proteins were attributed to the presence of disulfide linkage in globulin and legumin and the variation in amino acid and subunit composition including the thermal unfolding properties of the protein fractions. Bambara vicilin produced a more stable structured gel compared to globulin and legumin fractions. Bambara globulin and vicilin gel displayed greater gel firmness in terms of elastic resistance to deformation compared to legumin gel.

Bambara groundnut globulin treated with plasma-activated water, for the protein to be included in the food matrices such as emulsion and foams, significantly impacted the structural properties of the protein as evident in the loss of helical structure and over a 3-folds increase in β-turns. The treatment changed the protein from a folded state to an unfolded state. The Low molecular weight peptides (<1 kDa) fractions from both globulin and vicilin hydrolysate favoured ACE inhibitory activity. Whilst high molecular weight peptide fractions showed greater inhibition of renin activity.

Bambara proteins could be applied as food gels. The high solubility of Bambara vicilin in the acidic region makes it a potential ingredient in the production of acidified beverages. Plasma-

activated water-treated modified Bambara globulin can be incorporated in food formulations such as stabilization of food foams such as ice cream, and marshmallows.

8.2. Recommendation

- There should be industrial applications of the beneficial features of Bambara proteins in food product development and human health. This can include the utilization of these proteins in actual food formulations such as the stabilization of food foams (ice cream, marshmallows) and food emulsions (salad dressings and mayonnaise) compared to commercial protein emulsifier and foaming agents. Bambara vicilin may be used in the formulation of acidic beverages. It is, also, important to evaluate how the application of Bambara protein would influence the sensory characteristics including texture, flavour, and aroma of the products.
- Research may be conducted further to determine how plasma-activated water treatment will impact the gelation and rheological properties of Bambara globulin.
- There should be further studies to identify and purify the most active antihypertensive peptides from globulin and vicilin hydrolysates reported in this study. The *in vivo* activities of the blood pressure-lowering effects of these peptides should be determined using animal models such as spontaneously hypertensive rats and human intervention trials.

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Appendices

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Composition and some functional properties of Bambara groundnuts vicilin fraction

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ABSTRACT

Bambara groundnut is a drought tolerant pulse grain and an alternative source of protein. In this study, Bambara vicilin (7S) protein fraction was extracted and characterised in comparison to the storage protein. The proline content of vicilin fraction (4.3 g/100 g protein) was almost four times that of the storage protein. Fluorescence and hydrophobicity data suggested a less folded structure for the vicilin fraction when compared to the storage protein. Gel electrophoresis indicated three major polypeptides (50, 70 and 80 kDa) in the vicilin fraction with the presence of disulfide bond(s) in one of the strands. In contrast to the storage protein, which had < 40% protein solubility at pH 2–9, vicilin had a significantly ($p < 0.05$) higher solubility with a minimum of 50% at pH 5 and maximum of 82% at pH 3. Emulsifying stability of the proteins significantly differed ($p < 0.05$) at pH 7. Foaming capacity of the vicilin fraction was significantly ($p < 0.05$) higher than that of the storage protein at pH 3, 7, and 9. It could be concluded that Bambara vicilin fraction could serve as a potential ingredient in the formulation of food products at acidic pH.

1. Introduction

In recent decades, there is a growing interest in the utilization of leguminous grain storage proteins for food and industrial applications (Nadathur, Wanasundara, & Scanlin, 2017; Tavano, Inacio da Silva, Demonte, & Neves, 2008). Among legume proteins, soybean protein has gained popularity in the food industry as a trusted food ingredient due to its nutritional value and functionality (Makeri et al., 2017). However, the inability of soybean to yield better on low fertility poor soil and under extreme drought conditions coupled with the growing demand for plant proteins have resulted in the search for alternative legume protein sources, especially from pulses.

The protein composition of legumes may vary remarkably with the variety or legume type. The major storage proteins in legumes are albumins and globulins (Shen & Tang, 2014), with the latter further subdivided into vicilins (7S) and legumins (11S) in pea (Stone, glycosylated (Mendoza et al., 2001). Vicilins have been found to possess a more hydrophilic surface, which makes them more soluble in aqueous solutions and have potentially better functionality when compared to the legumins (Stone et al., 2015). For example, purified kidney bean vicilin showed better emulsifying properties than the protein isolates (Shen & Tang, 2014). Knowledge of the composition and functionality of major storage proteins and corresponding fractions (e.g. vicilin or legumin) will be critical for the development of protein ingredients with excellent functional attributes such as emulsifying and foaming properties.

Bambara groundnut, a pulse grain of African origin (Mazahib, Nuha, Salawa, & Babiker, 2013) has been of interest due to its ability to grow under extreme drought conditions (Thammarat, Leena, Punnanee, & Soottawat, 2015). It is also a good source of protein.. (15–27%) (Arise, Amonsou, & Ijabadeniyi, 2014). In our previous study, we investigated the composition of the major storage proteins in Bambara grain (Arise,

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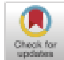

Effect of cold plasma-activated water on the physicochemical and functional properties of Bambara groundnut globulin

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ABSTRACT

There is a growing interest in sustainable and green technology for the improvement of functional properties of grain proteins by altering their composition and structure. This study investigated the structure, physicochemical and functional properties of Bambara groundnut globulin after hydration with plasma-activated water (PAW). Bambara groundnut globulin was dispersed in PAW and hydrated at 4 ℃ for about 12 h. The exposure of Bambara groundnut globulin to plasma resulted in a significant loss of helical structure and over 3-fold increase in β-turns in comparison with the untreated Bambara groundnut protein. Amino acid data for the plasma-treated globulin showed 20 % reduction in glutamic acid content. A slight redshift was observed in fluorescence intensity data of the plasma-treated Bambara groundnut protein. This suggested an unfolding of the protein structure, which also correlated with the observed increased hydrophobicity. However, protein profiles by gel electrophoresis, surface charge, and pH-solubility patterns appeared similar for both plasma-treated and untreated Bambara groundnut globulin samples. Bambara groundnut globulin had reduced emulsifying ability after exposure to plasma as indicated by an increase in the average oil droplet sizes. However, foaming capacities were significantly better and stable at up to 15 mg protein/mL. The hydration of Bambara groundnut globulin with plasma-activated water modifies the structural conformation, reduces the proportion of acidic amino acids of the protein, and improves the foaming properties. Cold plasma treatment by hydration does not seem to improve the emulsifying properties of Bambara groundnut globulin.