



ENZYMATIC MODIFICATION OF BAMBARA GROUNDNUT PROTEIN FOR THE PRODUCTION OF HYDROGELS

By

Faith Matiza Ruzengwe

**Submitted in fulfillment of the academic requirements for the degree Doctor of Food
Science and Technology**

Department of Biotechnology and Food Science

Faculty of Applied Sciences

Durban University of Technology, Durban, South Africa

Supervisor: Professor Tukayi Kudanga

Co-Supervisor: Professor Eric Oscar Amonsou

2021

DECLARATION

I hereby declare that the work reported in this thesis and submitted at the Department of Biotechnology and Food Science at Durban University of Technology for a Doctoral degree is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

Faith Matiza Ruzengwe

Date

Student

As the candidate's supervisors we agree to the submission of this thesis

Prof Tukayi Kudanga

Date

As the candidate's supervisors we agree to the submission of this thesis

Prof Eric O. Amonsou

Date

DEDICATION

This doctoral thesis is dedicated to our son Timukudzeishe Matiza Ruzengwe you left us on 13.01.2021 after only being with us for 13 months. We will always carry the pain and broken hearts of burying our little one.

ACKNOWLEDGEMENTS

I am forever grateful to God Almighty for the strength and seeing me through this journey. Special appreciation to Professor Tukayi Kudanga and Professor Eric Amonsou for the support, patience and guidance throughout the research. Thank you very much for managing to instill in me values of excellence and a keen eye to detail in every step of the way. Through your remarkable work ethic, I have learnt that hard-work and passion is of paramount importance in achieving any set goals and dreams. I acknowledge the financial support from the Durban University of Technology (DUT), Agricultural Research Council and the National Research Foundation for waiving the fees and funding the research programme and my upkeep. I also wish to express my gratitude to the entire staff of the Department of Biotechnology and Food Science especially Melvin Makolomakwa for all the assistance. I appreciate my friends and colleagues Stanley Dula, Brianmax Takundwa, Laurel Kasumbwe, Sandile Ngubane, Nyasha Busu, Ruth Mwakanjumba, Opeyemi Alabi, Blessing Nemadziva, Sithembile Shongwe, Sharmista Gajadhar and others who assisted me in any way. Dr. Vimbainashe Manhivi, Faith Seke, and Langelihle Mpofu thank you very much for being wonderful sisters, for the support and being shoulders to lean on. I would not have achieved this without you. I also want to greatly appreciate my husband, Taurai Chaminuka for all the support, believing in me, baby sitting and allowing me to pursue my dreams. Tennille and Tanisha, thank you so much for being wonderful daughters. To my parents and mother-in-law, I am so grateful. Thank you very much for being the vision carriers, supporting me all the way and helping in taking care of my daughters. Many thanks to my siblings Joseph and my sister-in-law Patricia for the financial support, Kudzai and Takunda for encouraging me. I so much appreciate pastor Gwenzi for the motivations and prayers.

ABSTRACT

Natural polymer-based, especially plant protein hydrogels have recently been gaining attention because of their biodegradability and biocompatibility. Bambara groundnut is a potential source of protein in hydrogel production. However, the use of Bambara groundnut protein in such applications is limited because it is associated with the formation of inadequate crosslinks between polymer chains. Enzymatic treatment can potentially be used for improving the strength of Bambara groundnut protein hydrogel. In this study, the effect of laccase and transglutaminase (separately and in combination) on the microstructural, structural, rheological and mechanical properties of Bambara groundnut protein hydrogels was investigated for potential application in encapsulation and release of bioactive compounds.

In the first part of this study, the effect of pH and NaCl concentration on the rheological and microstructural properties of Bambara groundnut protein gels were optimised using response surface methodology (RSM) to determine ideal starting conditions before enzymatic treatment. The effect of using crosslinking enzymes (transglutaminase and laccase) on the textural, rheological, structural and microstructural properties of Bambara groundnut protein hydrogels were then investigated. Since the effectiveness of enzymatic processes may be limited by using single enzymes, the use of a combination of enzymes was also investigated for the first time in gelation and optimised using RSM. Subsequently, encapsulation efficiency and release properties of the enzymatically crosslinked Bambara groundnut protein hydrogel were investigated using riboflavin as a model bioactive compound.

The heat induced Bambara groundnut protein isolate (BPI) gels optimised for pH and NaCl prior to enzymatic treatment showed $G' > G''$ over a frequency range of 0-100 rad s⁻¹. Although BPI gels displayed the characteristics of weak gels, slightly acidic conditions (pH 6) coupled

with low NaCl concentration (0.5 M) promoted the formation of more rigid gels. These gels had the lowest water holding capacity and thiol content, suggesting the participation of disulphide linkages during network formation. Their microscopy images showed that the network was composed of porous homogeneous aggregates. Amino acid analysis showed that Bambara groundnut protein contains substantial amounts of amino acids including lysine, glutamic acid, cysteine and tyrosine with potential active sites for transglutaminase and laccase action. Laccase modification of Bambara groundnut protein caused a decrease in the gelation point temperature from approximately 85°C in the absence of laccase to 29°C at an activity of 3 U/g protein. Laccase treated samples showed a sharp increase in the G' and G'' values during the heating ramp as well as a wider gap between the moduli suggesting the formation of a more established network structure. The difference between G' and G'' increased to approximately 1 log and the dependency on angular frequency reduced suggesting improvement in the strength of the formed gels. Bambara groundnut protein crosslinking by laccase, was demonstrated by the decrease in thiol and phenolic content and crosslinking of amino acids (glutathione, cysteine and lysine) in model reactions. Microscopy images of the gel showed an increase in homogeneity and compactness of the lath sheet-like structure with increase in laccase activity up to 2 U/g protein.

Transglutaminase crosslinking at 15 U/g protein resulted in the formation of hydrogels with well-organised network structures and small pores. Gel strength improved as observed from the highest G' (6947 Pa) and hardness (5.60 N) recorded upon use of this activity. Transglutaminase-mediated crosslinking of BPI hydrogel was demonstrated by the reduction in amine and thiol groups and the formation of a new protein band (56 kDa) in crosslinked hydrogels. The combined use of transglutaminase and laccase showed a $G' > 10G''$ over a frequency range of 0 – 100 rad/s suggesting the dominance of the elastic behaviour. BPI

hydrogel with the highest hardness (15.96 N) and encapsulation efficiency (98.8%) was formed at 15 and 0.5 U/g protein of transglutaminase and laccase activities, respectively. The lowest swelling capacity recorded in this hydrogel contributed to the lowest release kinetic constants in both simulated gastric fluid (0.51) and simulated intestinal fluid (0.73) in the presence of digestive enzymes which indicated that riboflavin release was due to diffusion and swelling.

Overall, modification of Bambara groundnut protein using a combination of crosslinking enzymes increased the crosslinking density and promoted the formation of strong hydrogels. The hydrogels effectively encapsulated and prevented the early release of a heat sensitive compound (riboflavin) in the stomach while making it available in the small intestines. Therefore, the optimised enzyme combination of laccase and transglutaminase is a potential strategy for application in Bambara groundnut protein gelation.

PREFACE

This thesis is organised into eight chapters and the experimental work is presented in manuscript format. Chapter one gives the general introduction to the thesis. Chapter two provides a critical review of literature on hydrogels as delivery systems, their characteristic features, mechanism of network formation, physical, chemical and enzymatic crosslinking and the main hydrogel preparation (heat set induced and cold set induced) methods used to obtain these crosslinks. A brief review on the use of Bambara groundnut protein as a potential alternative source of protein in hydrogel formation is provided. Chapter three reports on the optimisation of the process parameters (pH and NaCl concentration) of heat induced Bambara groundnut protein gels using response surface methodology. The optimal process parameters were then used in subsequent chapters. Chapter four presents the improvement of the rheological properties of heat induced gels using laccase catalysed modification of Bambara groundnut protein. Chapter five reports on transglutaminase-mediated crosslinking of Bambara groundnut protein hydrogels using the cold set induced method. Chapter six covers the optimisation of the two enzymes (laccase and transglutaminase) using response surface methodology and the encapsulation and release of riboflavin from the produced hydrogels. 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
ACKNOWLEDGEMENTS	iii
ABSTRACT.....	iv
PREFACE.....	vii
TABLE OF CONTENTS	viii
LIST OF FIGURES	xvi
LIST OF TABLES	xx
ABBREVIATIONS	xxii
PUBLICATIONS AND CONFERENCES	xxiv
CHAPTER ONE	1
Introduction.....	1
CHAPTER TWO	4
Literature review	4
2.1. Introduction	4
2.2. Hydrogels	4
2.2.1. Definition and brief description of hydrogels	4
2.2.2. Characteristics of hydrogels	5
2.2.3. Mechanism of network formation in a hydrogel	7
2.2.4. Classification of hydrogels	7

2.2.5. Crosslinking in hydrogels	8
2.3. Enzymatic crosslinking	13
2.3.1. Transglutaminase	14
2.3.2. Laccase.....	17
2.4. Hydrogels preparation methods	21
2.4.1. Heat set induced gelation	21
2.4.2. Cold set induced gelation	27
2.5. Application of hydrogels as delivery systems	28
2.6. Bioactive compounds	29
2.7. Bambara groundnut.....	31
2.8. Conclusion.....	33
2.9. Hypothesis, Aim and Objectives	33
2.9.1. Hypothesis	33
2.9.2. Aim.....	34
2.9.3. Objectives	34
CHAPTER THREE.....	36
Rheological and microstructural properties of Bambara groundnut protein gels.....	36
Abstract	36
3.1. Introduction	36
3.2. Materials and methods	38
3.2.1. Materials.....	38

3.2.2. Preparation of defatted Bambara groundnut flour.....	39
3.2.3. Extraction of storage proteins	39
3.2.4. Least gelation concentration (LGC)	40
3.2.5. Experimental design	40
3.2.6. Bambara groundnut protein gels preparation.....	41
3.2.7. Rheological properties of BPI gels	42
3.2.8. Verification and selection for further analysis.....	42
3.2.9. Thermal properties of different BPI suspensions.....	42
3.2.10. Water holding capacity	43
3.2.11. Thiol quantification	43
3.2.12. Fourier transform infrared (FT-IR) spectroscopy	43
3.2.13. Scanning electron microscope	44
3.2.14. Statistical analysis.....	44
3.3. Results and Discussion	44
3.3.1. Least gelation concentration.....	44
3.3.2. Effect of pH and ionic strength (NaCl concentration) on the rheological properties of BPI gels	45
3.3.3. Model analysis.....	48
3.3.4. Thermal properties of BPI suspensions at different pH and NaCl concentration.....	50
3.3.5. Water holding capacity	51
3.3.6. Thiol quantification	52

3.3.7. FTIR.....	53
3.3.8. Scanning electron microscopy	56
3.4. Conclusion.....	58
CHAPTER FOUR.....	59
Heat-induced gelation profile of Bambara groundnut protein isolate crosslinked with laccase.....	59
Abstract.....	59
4.1. Introduction	60
4.2. Materials and methods	62
4.2.1. Materials.....	62
4.2.2. Extraction of Bambara groundnut protein isolate	62
4.2.3. BPI gel preparation	62
4.2.4. Rheological properties.....	63
4.2.5. Scanning electron microscopy (SEM).....	63
4.2.6. Thiol quantification	63
4.2.7. Phenolic quantification.....	64
4.2.8. Surface hydrophobicity	64
4.2.9. Non-covalent bonds	64
4.2.10. Model reactions.....	65
4.2.11. LC-MS analysis of reaction products	65
4.2.12. Statistical analysis.....	66
4.3. Results and discussion.....	66

4.3.1. Effect of laccase modification on the rheological properties of heat induced BPI gels produced <i>in situ</i>	66
4.3.2. Scanning electron microscopy	73
4.3.3. Effect of laccase on free thiol content of the gels	75
4.3.4. Phenolic quantification.....	75
4.3.5. Surface hydrophobicity (H _o).....	76
4.3.6. Non- covalent interactions	77
4.3.7. Model reactions.....	79
4.4. Conclusion.....	81
CHAPTER FIVE	82
Transglutaminase-mediated crosslinking of Bambara groundnut protein hydrogels: Implications on rheological, textural and microstructural properties	82
Abstract.....	82
5.1. Introduction	82
5.2. Materials and methods	85
5.2.1. Materials.....	85
5.2.2. Extraction of Bambara groundnut protein isolate	86
5.2.3. Bambara groundnut protein isolate amino acid analysis	86
5.2.4. Preparation of Bambara groundnut protein isolate hydrogels.....	86
5.2.5. Free amino quantification.....	87
5.2.6. Thiol quantification	87
5.2.7. SDS-PAGE	87

5.2.8. Fourier transform infrared (FT-IR) spectroscopy	88
5.2.9. Rheological properties of Bambara groundnut protein hydrogels.....	88
5.2.10. Texture analyses	88
5.2.11. Water holding capacity	89
5.2.12. Syneresis	89
5.2.13. Scanning electron microscopy (SEM).....	89
5.2.14. Amino acid model reactions.....	89
5.2.15. LC-MS analysis of reaction products	90
5.2.16. Statistical analysis.....	90
5.3. Results and discussion.....	90
5.3.1. Amino acid composition	90
5.3.2. Free amino groups.....	92
5.3.3. Thiol content	93
5.3.3. SDS-PAGE	94
5.3.4. FTIR.....	96
5.3.5. Effect of Transglutaminase activity on rheological properties of BPI hydrogels.....	97
5.3.6. Texture analysis	100
5.3.7. Scanning electron microscopy	102
5.3.8. Model reactions.....	103
5.4. Conclusion.....	104
CHAPTER SIX	106

Effect of combining laccase and transglutaminase on rheological, textural, encapsulation and delivery properties of Bambara groundnut protein hydrogels	106
Abstract.....	106
6.1. Introduction.....	106
6.2. Materials and methods	108
6.2.1. Materials.....	108
6.2.2. Extraction of Bambara groundnut protein isolate	109
6.2.3. Experimental design	109
6.2.4. Preparation of Bambara groundnut protein isolate hydrogels.....	110
6.2.5. Rheological properties of enzyme-induced Bambara groundnut protein hydrogels.....	110
6.2.6. Verification and selection for further analysis.....	111
6.2.7. Textural properties.....	111
6.2.8. Swelling properties	111
6.2.9. Encapsulation of riboflavin into enzyme-induced Bambara groundnut protein isolate hydrogels.....	112
6.2.10. Determination of riboflavin loading of enzyme-induced Bambara groundnut protein isolate hydrogels	112
6.2.11. In-vitro release of riboflavin from enzyme-induced Bambara groundnut protein isolate hydrogels	113
6.2.12. Statistical analysis.....	114
6.3. Results and Discussion	114

6.3.1. Effect of enzymes on the rheological properties of Bambara groundnut protein isolate hydrogels	114
6.3.2. Model analysis.....	118
6.3.3. Textural properties.....	120
6.3.4. Swelling properties	121
6.3.5. Encapsulation efficiency.....	124
6.3.6. In vitro release properties	125
6.4. Conclusion.....	131
CHAPTER SEVEN.....	132
General discussion	132
CHAPTER EIGHT	137
Conclusions and recommendations	137
8.1. General conclusion	137
8.2. Recommendations	140
References.....	141
Appendices.....	165

LIST OF FIGURES

Figure 2.1: A. Hydrogel morphology as viewed by the naked eye; B. Hydrogel morphology as viewed under an electron microscope.....	5
Figure 2.2: Chemical and physical hydrogels.....	8
Figure 2.3: Schematic of methods for the formation of physically cross-linked hydrogels via a. Ionic interactions b. hydrophobic interactions c. self-assembling of stereo complex formation, d. coiled-coil interactions, e. specific molecular recognition.	10
Figure 2.4: Mechanisms of filamentous (10 mM) and particulate (20 mM) soy protein cold-set gel formation induced by the addition of calcium ions.....	11
Figure 2.5: Schematic of methods for the formation of chemically crosslinked hydrogels by radical polymerisation of a) vinyl monomers and b) macromonomers c) reaction of pendant functional groups, and d) high-energy radiation.....	13
Figure 2.6. Reactions catalysed by transglutaminase. a) acyl transfer reaction; b) crosslinking reaction between glutamine and lysine residues of proteins or peptides. c) deamination	15
Figure 2.7: Copper centers of the laccase (CotA) from <i>Bacillus subtilis</i>	18
Figure 2.8: Oxidation of phenolic compounds catalysed by laccase	19
Figure 2.9: Schematic representation of laccase-catalysed redox cycles for substrates oxidation in the absence (a) and the presence (b) of redox mediators.	20
Figure 2.10: Two main methods used in hydrogel preparation: heat-induced or cold gelation procedure.....	21
Figure 2.11: Frequency dependence of the storage modulus (G') of 18% (w/v) soy protein isolate gels at varying NaCl concentrations.....	23
Figure 2. 12: Storage modulus (G') after a complete temperature cycle of 12% soy protein dispersions as a function of pH at added salt concentrations.....	23

Figure 2.13: SEM images (3000× magnification, bar =10 μm) of microbial transglutaminase induced apricot kernel protein concentrate gels at different concentrations.....	25
Figure 3.1. Effect of pH and NaCl on (a) storage modulus (b) loss modulus (c) Tan δ of Bambara groundnut protein gels.....	47
Figure 3. 2: 3-D surface plots showing interaction effects of pH and NaCl on (a) storage modulus (G') and (b) loss modulus (G'') of Bambara groundnut protein isolate gels.....	49
Figure 3.3: Water holding capacity of Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations.....	52
Figure 3.4: Thiol content of Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations.....	53
Figure 3.5: FTIR spectra of Bambara protein isolate gels made at at different pHs and NaCl concentrations.....	54
Figure 3.6: SEM micrographs of Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations	57
Figure 4. 1: Effect of laccase activity on storage modulus (G') and loss modulus (G'') vs time during the heating process (a) 0 U/g protein, (b) 1 U/g protein, (c) 2 U/g protein & (d) 3 U/g protein of Bambara groundnut protein isolate.	69
Figure 4. 2: Effect of laccase activity on (a) storage modulus (G') and loss modulus (G'') (b) Tan δ (c) complex modulus as a function of angular frequency of Bambara groundnut protein isolate gels.....	71
Figure 4.3: SEM micrographs of Bambara groundnut protein isolate gels made at different laccase activities; (a) 0 U/g (b) 1 U/g (c) 2 U/g and (d) 3 U/g protein.	74

Figure 4.4: Effect of laccase activity on the free thiol content of Bambara groundnut protein isolate gels.....	75
Figure 4.5: Effect of laccase activity on the phenolic content of Bambara groundnut protein isolate gels.....	76
Figure 4.6: Surface hydrophobicity (H_o) of Bambara groundnut protein isolate gels made at different laccase activities.....	77
Figure 4.7: Mass spectra and proposed structures of (A) cysteine-cysteine (B) glutathione-cysteine (C) glutathione-glutathione (D) tyrosine-tyrosine (E) tyrosine-cysteine (F) tyrosine-glutathione conjugates formed through laccase treatment.....	80
Figure 5.1: Effect of transglutaminase activity on the free amine content of Bambara groundnut protein isolate hydrogels	93
Figure 5.2: Effect of transglutaminase activity on the thiol content of Bambara groundnut protein isolate hydrogels	94
Figure 5.3: SDS-PAGE of (a) Bambara groundnut protein isolate hydrogels made at varying transglutaminase activity and (b) transglutaminase enzyme, Lane M: marker	95
Figure 5.4: FTIR spectra of Bambara groundnut protein isolate hydrogels made at varying transglutaminase activities.	96
Figure 5.5: Effect of transglutaminase activity on (A) storage modulus (G'), (B) loss modulus (G'') and (C) $\tan \delta$ as a function of angular frequency of Bambara groundnut protein isolate hydrogels.....	100
Figure 5.6: SEM micrographs of Bambara groundnut protein isolate hydrogels made at varying transglutaminase activities; (a) 0 U/g (b) 5 U/g (c) 10 U/g (d) 15 U/g (e) 20 U/g and (f) 25 U/g protein.	103

Figure 5.7: Mass spectra and proposed structures of glutamine-lysine crosslinked peptides formed through transglutaminase treatment.	104
Figure 6.1: Frequency sweep properties of Bambara groundnut protein isolate hydrogels made with varying activities of transglutaminase and laccase (a) Storage modulus (G') (b) Loss modulus (G'') and (c) $\tan \delta$	117
Figure 6.2: 3-D surface plots showing the interaction effects of transglutaminase and laccase on the storage modulus (G') of Bambara groundnut protein isolate hydrogels.....	119
Figure 6.3: Swelling of Bambara groundnut protein isolate hydrogels made with varying activities of transglutaminase and laccase in a) SGF (pH 1.2) and b) SIF (pH 7.5).....	123
Figure 6.4: Effect of pH and digestive enzymes on the release of riboflavin from enzyme-induced Bambara groundnut protein isolate hydrogels in a) SGF (pH 1.2) b) SIF (pH 7.5) c) SGF (pH 1.2) with pepsin and d) SIF (pH 7.5) with pancreatin.....	128
Figure 6.5: In vitro release of riboflavin from enzyme-induced Bambara groundnut protein isolate hydrogels in simulated gastrointestinal conditions.....	129

LIST OF TABLES

Table 3.1: Process variables used in the central composite design for Bambara groundnut protein isolate gel preparation.....	41
Table 3.2. Quadratic model for G' , G'' and linear model regression coefficients for rheological properties of Bambara groundnut protein isolate gels.	48
Table 3.3: Thermal properties of Bambara groundnut protein isolate dispersions made at different pHs and ionic strengths.	50
Table 3.4: Percentage of secondary structures determined by FT-IR spectroscopy in Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations.....	55
Table 4.1: The effect of laccase activity on the power law parameters (A_n and n^*) from the frequency sweep (G^*) of Bambara groundnut protein isolate gels.....	73
Table 4.2: Non- covalent interactions in Bambara groundnut protein isolate gels made at different laccase activities.....	78
Table 5.1: Amino acid composition of Bambara groundnut protein isolate (g/100g).....	91
Table 5.2: Effect of transglutaminase activity on the percentage secondary structures of Bambara groundnut protein isolate hydrogels	97
Table 5.3: Effect of transglutaminase activity on hardness and cohesiveness of Bambara groundnut protein isolate hydrogels.....	101
Table 5. 4: Effect of varying transglutaminase activity on the water holding capacity and syneresis of Bambara groundnut protein isolate hydrogels.	101

Table 6.1: Process variables used in the central composite design for Bambara groundnut protein isolate hydrogel preparation.	110
Table 6.2: Quadratic model for G' and linear model regression coefficients for rheological properties of BPI hydrogels.	118
Table 6.3: Effect of enzyme activities on the hardness of Bambara groundnut protein isolate hydrogels crosslinked with transglutaminase and laccase	121
Table 6.4: Encapsulation efficiency of riboflavin in Bambara groundnut protein isolate hydrogels crosslinked with transglutaminase and laccase at varying activities	125
Table 6.5: Release kinetic constants of riboflavin from Bambara groundnut protein isolate hydrogels crosslinked with transglutaminase and laccase at varying activities	131

ABBREVIATIONS

ANOVA: Analysis of variance

BPI: Bambara groundnut Protein Isolate

CCD: Central composite design

EE: Encapsulation efficiency

FTIR: Fourier Transform Infra-Red

GRAS: Generally Recognised As Safe

GuHCl: Guanidium Hydrochloride

G'': Loss modulus

G': Storage modulus

G*: Complex modulus

HCl: Hydrochloric acid

ΔH : Protein denaturation enthalpy

LGC: least gelation concentration

kDa: kilo-Dalton

NCDs: Non- communicable diseases

PAGE: Polyacrylamide Gel Electrophoresis

RSM: Response surface methodology

SD: Standard deviation

SDGs: Sustainable Development Goals

SDS: Sodium dodecyl sulfate

SEM: Scanning Electron Microscopy

SGF: Simulated gastric fluid

SH: Sulfhydryl

SIF: Simulated intestinal fluid

SPI: Soy Protein Isolate

TGase: Transglutaminase

T_d: denaturation temperature

T_m: onset temperature of denaturation

WHO: World Health Organisation

PUBLICATIONS AND CONFERENCES

Publications

- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. (2020). Transglutaminase-mediated crosslinking of Bambara groundnut protein hydrogels: Implications on rheological, textural and microstructural properties. *Food Research International*, 137, 109734.
- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. (2020). Rheological and microstructural properties of Bambara groundnut protein gels. *LWT*, 123, 109070.

Submitted manuscript

- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. (2021). Heat-induced gelation profile of Bambara protein isolate crosslinked with laccase. *Submitted to RSC Advances*.

Conferences

- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. Effect of transglutaminase on rheological, textural and microstructural properties of Bambara protein hydrogels, 34th EFFoST International Conference, 10- 12 November 2020.
- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. Enzymatic modification of Bambara groundnut protein for the production of hydrogel, Faculty Research Day, 11 November 2019.
- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. Optimisation of Bambara groundnut protein gelation process, 23rd SAAFoST Biennial International Congress and Exhibition, Birchwood hotel and conference centre, Johannesburg, 1-4 September 2019.
- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. Rheological properties of Bambara groundnut gels as influenced by pH, NaCl and Laccase, 1 st World of Rheology workshop, Anton Paar Building, Midrand, 28-29 August 2019.
- Ruzengwe, F. M., Amonsou, E. O., & Kudanga, T. Enzymatic modification of Bambara groundnut protein for the production of hydrogels, South Africa – Germany Bilateral Second Joint Workshop, 13 – 15 February 2019.

CHAPTER ONE

Introduction

Hydrogels are hydrophilic, three-dimensional network polymers with the ability to absorb large amounts of water and biological fluids while maintaining their structure (Bahram *et al.*, 2016). Their resistance to dissolution arises from the crosslinks between the network chains (Ahmed, 2015). Natural polymer-based, especially plant protein hydrogels, have recently been gaining attention because of their biodegradability and biocompatibility (Wen *et al.*, 2018). The most used source of plant protein in hydrogels has been soybean. However, the high demand for soy protein in various applications has led to the search for alternatives with similar or even better functional properties. Bambara groundnut (*Vigna subterranea*) can be an alternative source of protein (19- 25%) (Arise *et al.*, 2017). However, studies on gelation of proteins from Bambara groundnut have only been limited to the least gelation concentration determination (Adebowale *et al.*, 2011) and the formation of gels from vicilin, which is the major storage protein of the legume (Diedericks *et al.*, 2019). Diedericks *et al.* (2019) reported that the formed gels were composed of weak links as concluded from the percolation and fractal scaling model and the heterogeneous aggregated structure. Thus, Bambara groundnut protein use in gelation is associated with the formation of inadequate crosslinks between the polymers limiting the applications of the formed hydrogels as encapsulation materials for delivery purposes.

Researchers have studied the production of hydrogels through physico-chemical crosslinking facilitated by heating, salt addition and pH alterations. However, the produced hydrogels lack pH and salt stability (Tao *et al.*, 2016), and have a poor mechanical strength (Tang *et al.*, 2006). In an effort to improve these properties, the addition of chemicals such as glyceraldehyde and glutaraldehyde was investigated. Of these, glutaraldehyde-formed hydrogels show enhanced

mechanical strength but have limited application due to the toxicity of the chemical (Caillard *et al.*, 2008; Song & Zhang, 2008). Thus, studies have successively shifted to investigating enzymatic crosslinking since enzymes are generally regarded as safe (GRAS).

Crosslinking enzymes such as transglutaminase and laccase can form covalent bonds between molecules resulting in the formation of firm hydrogels. The most widely used enzyme in plant protein hydrogels is transglutaminase. Transglutaminase (TGase) catalyses crosslinking reactions of protein molecules. This is achieved through an acyl transfer reaction between a carboxamide group of a protein or peptide bound glutamine and an amino group of a lysine residue, forming a relatively protease-resistant inter or intramolecular (glutamyl) lysine isopeptide bond (Heck *et al.*, 2013; Hu *et al.*, 2015). TGase crosslinking has been reported to significantly improve the strength and release properties of soy protein hydrogels (Song & Zhang, 2008; Hu *et al.*, 2015). However, TGase is highly specific, thus there is scope for investigation of other crosslinking enzymes in gelation.

Laccase is a potential alternative crosslinking enzyme in plant protein gelation. Laccase is a multi-copper containing oxidative enzyme which performs one-electron oxidation of a wide range of substrates such as diphenols, methoxy-substituted monophenols, and aromatic and aliphatic amines, and simultaneously reduces molecular oxygen to water (Kudanga *et al.*, 2011). Laccase-catalysed crosslinking has been reported to increase the firmness of α -lactalbumin gels (Ma *et al.*, 2020) and whey protein isolate gels (Quan *et al.*, 2019) as well as viscoelastic properties of skim milk gels (Struch *et al.*, 2015). Laccase has mainly been used in crosslinking animal protein-based gels; however, because of the shift in consumer preferences towards using plant proteins, there is need to explore its use in other protein sources such as Bambara groundnut protein.

Bambara groundnut protein has substantial amounts of amino acids; glutamic acid (15.91%), aspartic acid (9.32%), lysine (5.95%), tyrosine (2.99%) and histidine (2.41%) (Arise *et al.*, 2017), with potential active sites for transglutaminase and laccase action. Therefore, this study investigated the effect of transglutaminase and laccase on rheological, mechanical, microstructural and release properties of Bambara groundnut protein hydrogels. Since the effectiveness of enzymatic processes may be limited by using single enzymes, the use of a combination of enzymes was also investigated.

CHAPTER TWO

Literature review

2.1. Introduction

The chapter presents the literature relevant to this study. The areas covered include an introduction of hydrogels, their characteristic features, mechanism of network formation, physical, chemical and enzymatic crosslinking and the main hydrogel preparation (heat set induced and cold set induced) methods. This is followed by challenges in the delivery of bioactive compounds. Also reviewed is the use of the Bambara groundnut protein as an alternative source of protein in hydrogel formation.

2.2. Hydrogels

2.2.1. Definition and brief description of hydrogels

Hydrogels are three dimensional, crosslinked and tissue-like polymer chains capable of retaining water and biological fluids in large amounts in their swollen state (Varaprasad *et al.*, 2017). They can also be defined as water-swollen polymeric materials that have a three-dimensional network structure depending on the covalent and non-covalent interactions between the macromolecules with water being the dispersion medium (Kopecek, 2007; Clark & Ross-Murphy, 2009). Peppas *et al.* (2000) define hydrogels as a hydrophilic polymeric network of three-dimensional crosslinked structures which is obtained from either natural or synthetic polymers and have the capability of absorbing considerable amounts of water. Thus, the simple reaction of one or more monomer units/ polymer chains and crosslinker units results in water swelled and crosslinked polymeric network known as a hydrogel. Their ability to retain huge quantities of water and biological fluids arises from the hydrophilic functional groups (such as $-\text{NH}_2$, COOH , $-\text{OH}$, $-\text{CONH}_2$, $-\text{CONH}-$, and $-\text{SO}_3\text{H}$) which are attached to the

polymeric backbone. In contrast, the dissolution resistance arises from the network chains' crosslinks (Figure 2.1) (Ahmed, 2015). Figure 2.1 shows the hydrogel morphology as viewed by the naked eye and under an electron microscope.

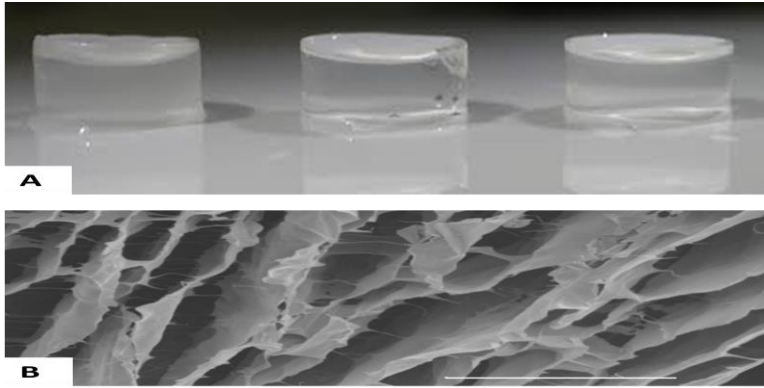


Figure 2.1: A. Hydrogel morphology as viewed by the naked eye; B. Hydrogel morphology as viewed under an electron microscope (Ferreira *et al.*, 2011).

Polymer chain and water or biological fluids interactions occur through counterbalanced capillary, osmotic and hydration forces causing chain network expansion. This hydrogel equilibrium state is dependent on the magnitude of opposing effects determining some of the characteristic properties such as internal transport, diffusion and strength (Buwalda *et al.*, 2014). The water inside the hydrogel allows solute molecules to spread freely, while the water on its own is held together by the polymer (Okay, 2009).

2.2.2. Characteristics of hydrogels

Water holding capacity and permeability of the hydrogel are its most vital characteristics. The moment the hydrogel comes into contact with water, the polar hydrophilic groups become hydrated forming primary bound water, and the network starts to swell. The hydrophobic groups are then exposed, and they interact with the water molecules forming hydrophobically-bound water known as secondary bound water (Gulrez *et al.*, 2011). In some instances, the primary and secondary bound water combine to give the total bound water. The network will

absorb more water referred to as the free or bulk water because of the network chains' osmotic driving force towards infinite dilution. The extra water fills up gaps between the network or chains and the larger pore centres (Ullah *et al.*, 2015). According to Ganji *et al.* (2010), the absorbed water quantity is dependent on temperature, the water molecules and polymer chains' specific interactions. However, covalent or physical crosslinks resist this further swelling resulting in an elastic retraction force of the network such that the hydrogel reaches a point of swelling equilibrium. The hydrogel then disintegrates or dissolves if the network chain or crosslinks are degradable. Thus, the production of biodegradable hydrogels containing labile bonds is beneficial, especially for delivery. These bonds can be broken, either enzymatically or chemically, under physiological conditions (Hennink & Nostrum, 2002; Hoffman, 2002). Also, a hydrogel should be slow to release the encapsulated material.

The third property of a hydrogel is biocompatibility. Biocompatibility refers to the compatibility within the immune system and the degradation of the produced compounds that must not be harmful. Ideally, the hydrogels are supposed to be metabolised into harmless products, which the renal filtration process can excrete (Gulrez *et al.*, 2011). Apart from this, because of the hydrogels' soft and rubbery nature, irritation around the surrounding tissue is minimised (Smetana, 1993; Anderson & Langone 1999).

Another characteristic feature of a hydrogel is hardness, which results from the crosslinks between the various polymer chains, further resulting in viscoelastic and, at times, pure elastic behavior (Gulrez *et al.*, 2011). Additionally, hydrogels are often relatively deformable and can conform to the shape of the surface they are applied (Hoare & Kohane, 2008).

2.2.3. Mechanism of network formation in a hydrogel

Hydrogels are formed from joining macromolecular chains. Depending on the structure and conformation of the starting material, the linking initially results in larger branched soluble polymers called a sol. Progression in the linking process causes an increase in the size of the branched polymer and a decrease in solubility. The infinite formed polymer, permeated with finite branched polymers, has a network and is called the gel (Gulrez *et al.*, 2011). Gelation also is known as sol-gel transition, which is the conversion of the finite branched polymer system to infinite molecules. During the process, a critical point referred to as the gelation point is reached when the gel first appears (Rubinstein & Colby, 2003).

2.2.4. Classification of hydrogels

There are two broad categories of hydrogels: permanent and reversible or physical gels (Figure 2.2). Permanent hydrogels are sometimes referred to as chemical hydrogels; these are composed of covalent crosslinks that form a strong and stable network (Hennick & Nostrum, 2002). The equilibrium swelling state of these hydrogels is dependent on the polymer-water interaction parameter and crosslink density (Rosiak & Yoshii, 1999). Reversible/physical hydrogels are connected molecular entanglements and/or secondary forces such as the ionic, hydrogen bonding or hydrophobic interactions. The physical interactions existing between the various polymer chains prevent dissolution in these hydrogels (Hennick & Nostrum, 2002). However, these physical interactions are reversible and can be disrupted by stress application and fluctuations in physical conditions (Rosiak & Yoshii, 1999).

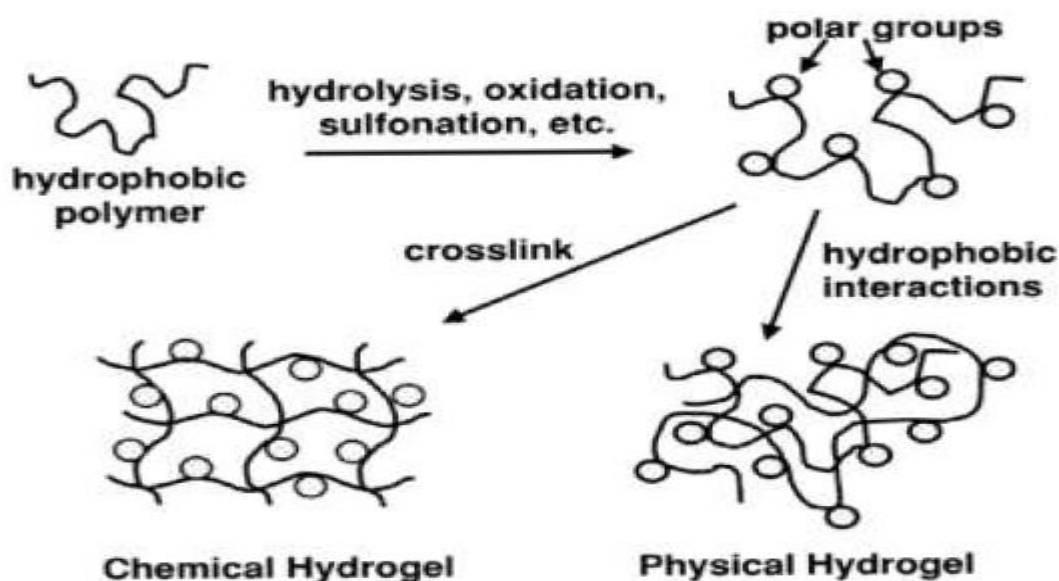


Figure 2.2: Chemical and physical hydrogels (Hoffman, 2002).

2.2.5. Crosslinking in hydrogels

Physical hydrogels are made up of physical crosslinks, whereas chemical hydrogels are held together by chemical crosslinks, as highlighted earlier. Crosslinking of hydrogels takes place both in-vitro (during the gel preparation) and in-vivo (after the application at an exact location in the human body). A low molecular weight crosslinking agent must be introduced into a non-mixture polymer to initiate crosslinking (Ullah *et al.*, 2015).

2.2.5.1. Physical crosslinking

Physically crosslinked hydrogels have physical domain junctions, hydrogen bonding, hydrophobic interaction and ionic complexation. This allows solvent casting, post-process bulk modification, and ease of fabrication, reshaping, biodegradation and non-toxicity, which is usually lacking in chemically crosslinked hydrogels (Park *et al.*, 2002). Further suggesting that since it is devoid of chemical reactions, there is no potential harm that is posed to the integrity of incorporated bioactive agents (Teixeira *et al.*, 2012). Interactions between polymer chains in an amphiphilic block and graft copolymer in a physically crosslinked gel are established by ionic and/or hydrophobic interactions (Kopecek & Yang, 2009; Zhao *et al.*, 2010). Once

crosslinks are introduced between polymers, the networks that are produced show viscoelastic behaviour and in some cases pure elastic behaviour (Hennick & Nostrum, 2012). Apart from the above mentioned, the other advantage of this type of crosslinking is that it is reversible (Teixeira *et al.*, 2012).

In contrast to these advantages, interactions with body functions, both physiological and mechanical, may seriously affect their in vivo stability, causing the gel to collapse (Teixeira *et al.*, 2012). Physical crosslinking also has the drawback of being difficult to decouple variables like gelation time, network pore size, chemical functionalization, and degradation. As a result, because the hydrogel's strength is directly linked to the chemical properties of the constituent gelators, its design flexibility is limited.

Physical crosslinking can be induced by heating, acidification and salt (calcium chloride, magnesium chloride and sodium chloride) addition (Figure 2.3). The addition of the salt screens out the repulsive force by neutralising electrostatic repulsion, thereby leading to the aggregation of the protein molecules hence gel formation (Hongsprabhas *et al.*, 1999). Although both the divalent and monovalent salt ions screen out the electrostatic interactions between the charged protein molecules, divalent cations such as Ca^{2+} have an extra advantage of crosslinking negatively charged carboxylic acid groups (Hongsprabhas & Barbut, 1997b). Combined with the greater screening power, this ability allows divalent cations to be able to induce gelation at much lower concentration (Hongsprabhas & Barbut, 1997a). Salt bridges are formed between carboxyl groups of neighboring protein molecules, forming a space-filling network (Bryant & McClements, 1998; Marangoni *et al.*, 2000). The network formed is a three-dimensional network stabilised by van der Waals or hydrophobic interactions (Remondetto & Subirade, 2003).

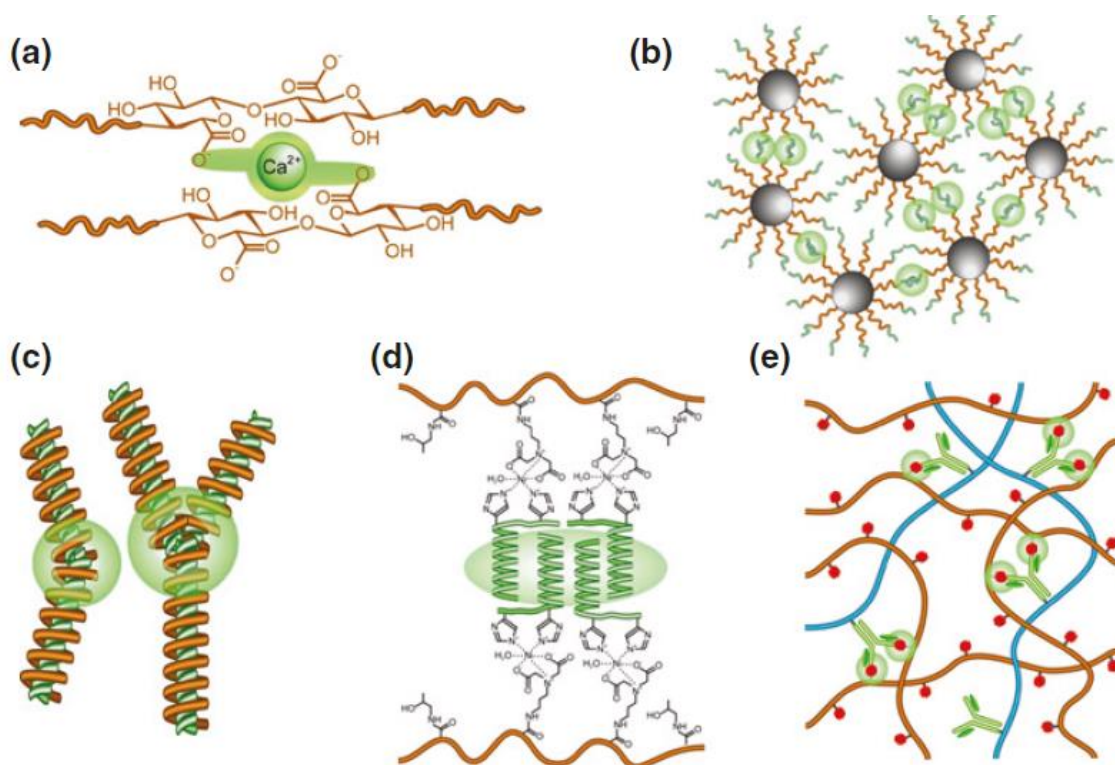


Figure 2.3: Schematic of methods for the formation of physically cross-linked hydrogels via a. Ionic interactions b. hydrophobic interactions c. self-assembling of stereo complex formation, d. coiled-coil interactions, e. specific molecular recognition (Ebara *et al.*, 2014).

Calcium chloride has been successfully used in cold set induced gelation to modify protein hydrogels by inducing crosslinks between neighboring proteins. It has been used in lactoglobulin (Remondetto *et al.*, 2002, Remondetto & Subirade, 2003) and soy protein (Maltais *et al.*, 2008) cold set induced hydrogels. The calcium chloride is added to the protein solution after preheating and cooling. Calcium ions neutralise the negative charges on the surface of the structural units, and a three-dimensional network is formed through two mechanisms, with each occurring at a different calcium concentration. Studies done by Maltais *et al.* (2008) have shown that varying the calcium chloride concentration from 10 mM to 20 mM results in two different forms of gels a filamentous and a particulate gel, respectively (Figure 2.4).

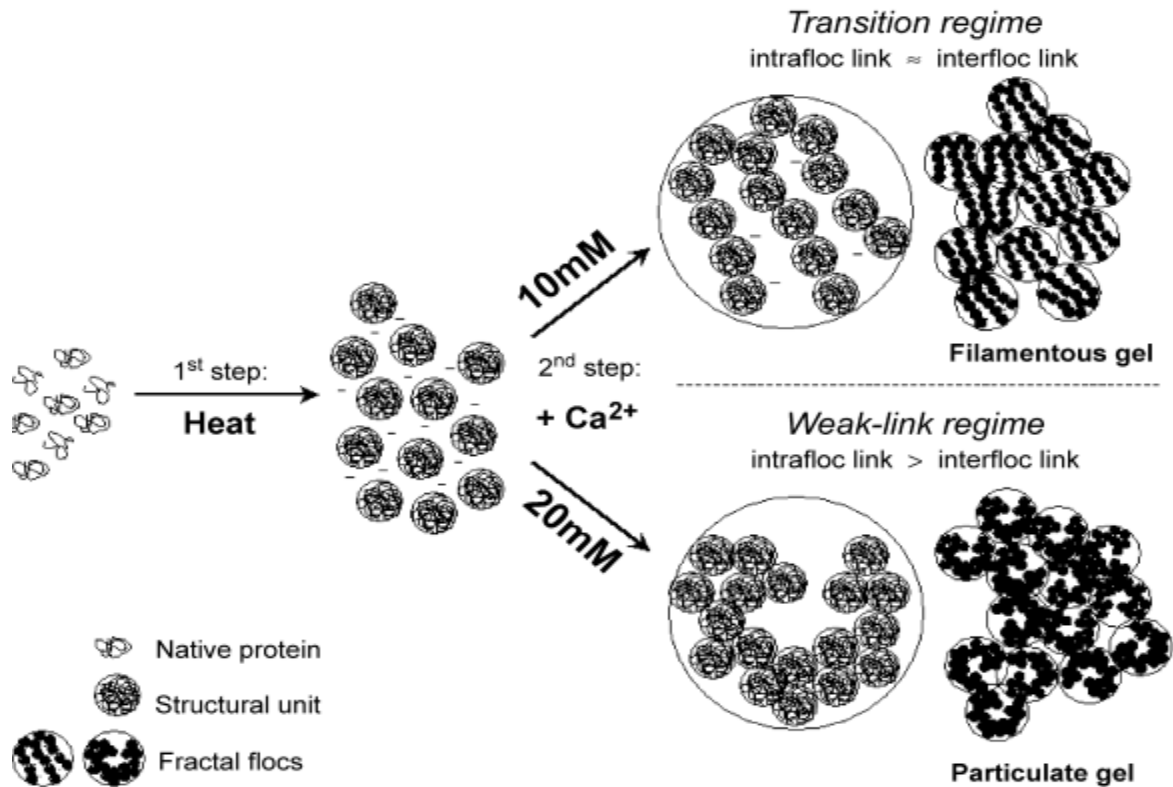


Figure 2.4: Mechanisms of filamentous (10 mM) and particulate (20 mM) soy protein cold-set gel formation induced by the addition of calcium ions (Maltais *et al.*, 2008).

The filamentous gel has a fine homogeneous and even protein network that consists of very fine strands in a dense arrangement with relatively even-sized pores. The surface charge in a filamentous gel is not screened totally, hence keeping the energy barrier between two units limiting the interaction. This is more appropriate for the delivery of riboflavin near the junction of the colon (Maltais *et al.*, 2009). The structure of a particulate gel is non-homogeneous and has large aggregates and pores, resulting in a random shaped matrix without any pattern. Quasi total repulsive force screening occurs between structural units, resulting in random aggregation. The gel is characterised by micro and macro syneresis leading to shrinkage and weak bond regime composed of weak interactions among flocs (Maltais *et al.*, 2008). According to Remondetto & Subirade (2003), at high salt concentration, there is a rapid reduction in repulsive forces on the structural unit surfaces, thereby lowering the energy barrier. The

particulate gel was shown to be effective in the delivery of riboflavin in the upper portion of the small intestines (Maltais *et al.*, 2009).

2.2.5.2. Chemical crosslinking

Chemical crosslinking is achieved through covalent bond formation between the polymer chains by using a crosslinking agent. Hence, it is a highly versatile method of creating hydrogels with excellent mechanical stability. The crosslinks are formed from either polymerisation (e.g. acryloyl group), radiation (e.g. γ -ray), small-molecule crosslinking (e.g. glutaraldehyde) and polymer-polymer crosslinking (e.g. condensation reaction) (Figure 2.5). The mechanical strength of the network is relatively high, depending on the nature of the formed covalent bonds that are in the building blocks and crosslinks. However, crosslinking agents used in chemical crosslinking are able to undergo undesirable reactions with the bioactive compounds encapsulated in the hydrogel matrix (Lindblad *et al.*, 2007). Covalently crosslinked hydrogels also show low swelling and lack of pH-controlled release under basic conditions (Berger *et al.*, 2004).

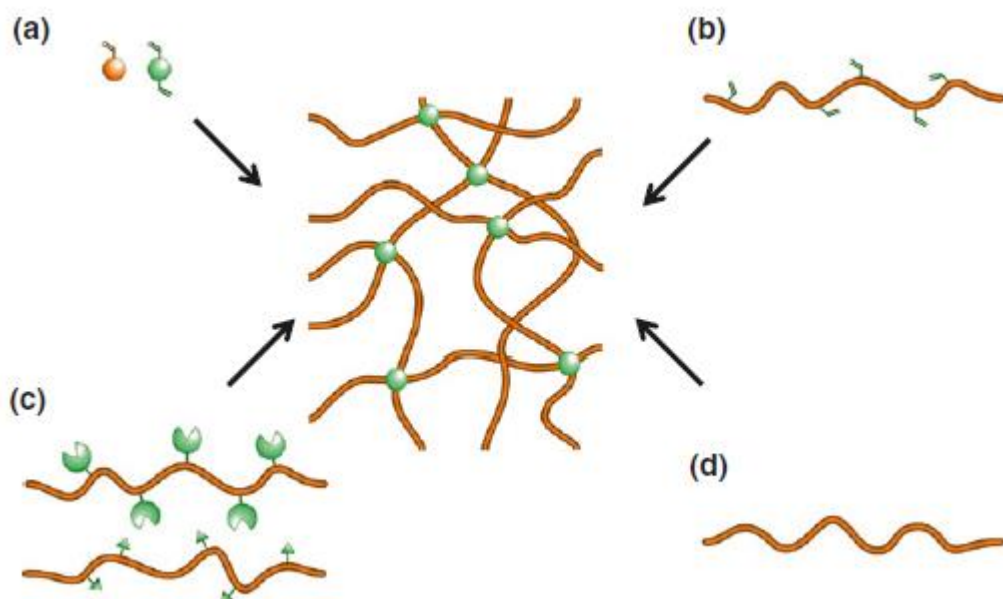


Figure 2.5: Schematic of methods for the formation of chemically crosslinked hydrogels by radical polymerisation of a) vinyl monomers and b) macromonomers c) reaction of pendant functional groups, and d) high-energy radiation (Ebara *et al.*, 2014).

A wide range of chemicals have been used to induce chemical crosslinks, and amongst them are glutaraldehyde and glyceraldehyde. According to Caillard *et al.* (2008), the effect of glyceraldehyde crosslinking in soy protein hydrogels was less pronounced. On the other hand, although glutaraldehyde formed soy protein hydrogels with enhanced mechanical strength, its application is limited due to the toxicity of the chemical (Caillard *et al.*, 2008; Song & Zhang, 2008), but it is generally recognised as safe (GRAS). Apart from chemicals, crosslinking enzymes can be used in hydrogel production. Hence, this study focuses on the use of enzymes in initiating crosslinking in protein hydrogel production since it introduces stronger bonds and enzymes are GRAS.

2.3. Enzymatic crosslinking

Enzymes are defined as key biological catalysts that have a key role in many biological and chemical processes. Their catalysed reactions are highly specific and selective towards

substrates. The use of enzymes introduces covalent bonds between polymers, thus producing firmer hydrogels compared to using chemicals. Besides, due to the substrate specificity, the use of enzymes has the potential to eliminate the unwanted side reactions or toxicity occurring in photo-initiators or organic solvents mediated reactions. Also, bioactivity loss is avoided due to the use of enzymes (Teixeira, 2012). Enzyme catalysed reactions are mild at normal physiological conditions; hence they can be used for crosslinking natural polymers that fail to withstand harsh chemical conditions.

2.3.1. Transglutaminase

Transglutaminase (TGase) (EC 2.3.2.13) is a protein-glutamine γ -glutamyl transferase belonging to the transferase class of enzymes (Trespalacios & Pla, 2007; Marx *et al.*, 2008). The enzyme catalyses the formation of an isopeptide bond that is protease resistant. This is achieved through the acyl transfer reaction between γ -carboxamide group of a protein or peptide bound glutamine and the first order ϵ -amino groups of various compounds for instance protein acceptors of an acyl residue (Figure 2.6a) (Ozer *et al.*, 2007; Abd-Rabo *et al.*, 2010; Buettner *et al.*, 2012). In the presence of lysine as the acceptor of the acyl residue bound in a polypeptide chain the process of crosslinking is induced resulting in the formation of inter or intramolecular crosslinks (Figure 2.6b) (Kashiwagi *et al.*, 2002; Heck *et al.*, 2013; Hu *et al.*, 2015). Additionally, the enzyme catalyses the deamination reaction in the absence of free amine groups (Figure 2.6c) (Kuraishi *et al.*, 2001).

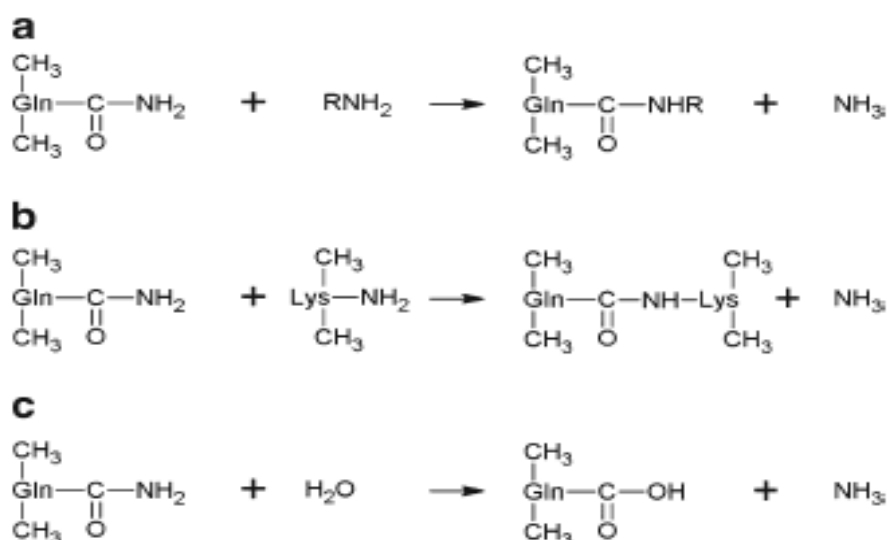


Figure 2.6. Reactions catalysed by transglutaminase. a) acyl transfer reaction; b) crosslinking reaction between glutamine and lysine residues of proteins or peptides. c) deamination (Kieliszek & Misiewicz, 2013).

Microbial TGase (mTGase) has a low molecular weight of approximately 38 kDa (Yokoyama *et al.*, 2004) and is the most widely used in gelation studies. It does not require calcium ions to be activated like TGase from the animal origin. The catalytic activity of TGase is high at 40°C and pH 5.5 (Ho *et al.*, 2000). TGase has been reported to be unstable at 50°C, and 50% of its activity is lost when incubated for 30 min.

During TGase crosslinking, the primary amine group is covalently attached to the other peptide bond, thereby modifying the chemical and physical properties like viscosity, elasticity, thermal stability, and resilience of proteins (Gan *et al.*, 2009). Apart from the presence of glutamine and lysine residues in abundance, accessibility of these residues is vital for TGase crosslinking. Generally, the primary and three-dimensional protein structures determine whether a glutamine residue reacts as an acyl donor substrate of the enzyme, thereby restricting the number of proteins acting as glutaminyl substrates (Villalonga *et al.*, 2003). The use of transglutaminase has therefore been studied in conjunction with different physical methods such as microwave,

homogenising (Guo *et al.*, 2013), high-intensity ultrasound (Hu *et al.*, 2015; Zhang *et al.*, 2016), and heating (Song & Zhang, 2008; Wen *et al.*, 2018).

Studies have shown that TGase catalyses crosslinking of proteins such as whey proteins, soy proteins and wheat proteins. Tang *et al.* (2006) used TGase in the formation of cold-set tofu and found that most of the conglycinin and the acidic subunits of glycinin in the soy protein isolate were crosslinked. According to Gan *et al.* (2009) treatment of soy protein with microbial transglutaminase led to reduced solubility, improved mechanical properties such as tensile strength and elongation at break and increased surface hydrophobicity of the soy protein gel. In another study, increasing the concentration of mTGase resulted in shorter gelation time and a stronger soy protein gel formation (Jin & Zhong, 2013). Song & Zhang (2008) pointed out the possibility of producing TGase-induced cold set soy protein gel for releasing the 5-aminosalicylic drug. The authors reported that by increasing temperature and changing TGase/Soy Protein Isolate mass ratio and gelation time, the hydrogel strength could be modulated. A relatively low amount of the drug 5-aminosalicylic acid was released from the hydrogel. This was related to the stronger interactions between the hydrogel matrix and 5-aminosalicylic acid resulting from the formation of hydrogen bonds (Song & Zhang, 2008). In a study by Hu *et al.* (2015), soy proteins pretreated with high-intensity ultrasound (HIU) were cross-linked using TGase. The release of riboflavin from non-HIU and HIU in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) in the absence of digestive enzymes showed that compared to non-HIU riboflavin hydrogels, the 40 min HIU pretreatment decreased the amount of riboflavin released after 6 h incubation from 92.77 to 41.99% in SGF, and from 60.68 to 40.53% in SIF, respectively. Thus, TGase can be successfully used for protein crosslinking in hydrogel formation for encapsulation purposes.

The main disadvantages of using TGase are that it can remain in the food component, and that crosslinking results in protein polymers and networks with very high molecular weight. Also, uncontrolled reactions can occur with reactive groups present in foodstuffs from proteins and other ingredients resulting in complex molecules of unknown dimensions and composition (Boumans & Ijngaards, 2001). This can reduce the amount of the released encapsulated material. Therefore, there is a need to explore other enzymes for crosslinking in protein hydrogel production, where it is mainly used and even trying a combination of enzymes at low enzyme activities.

2.3.2. Laccase

Laccases (benzenediol: oxygen oxidoreductase EC 1.10.3.2) are multi-copper containing enzymes that perform mono-electron oxidation of a broad range of organic substrates such as diphenols, methoxy-substituted monophenols, aromatic and aliphatic amines and simultaneously reduce molecular oxygen to water (Kudanga *et al.*, 2011). They belong to the blue copper family of oxidases. Laccase molecule as an active holoenzyme form is a dimeric or tetrameric glycoprotein containing four copper atoms per monomer bound to three redox sites (Type 1, Type 2, and Type 3 Cu pair). This monomer has a molecular mass that ranges from about 50-100 kDa with acidic isoelectric point around pH 4.0. Laccase has a high level of glycosylation, contributing to the high stability of the enzyme. The four copper atoms are distributed as follows: one mononuclear termed T1 where the reducing substrate place is and the one trinuclear cluster T2/T3 where oxygen binds and is reduced to water (Mayer & Staples, 2002). Electrons transfer from the substrate molecules happens through the T1 copper to the trinuclear T2/T3 center: two and six histidines, respectively, coordinate T2 and T3 copper atoms. The strong anti-ferromagnetic coupling between the two T3 copper atoms is maintained

by the hydroxyl bridge (Figure 2.7). Two molecules of water are, therefore, formed after four electrons have been transferred to the dioxygen in the trinuclear center (Bertrand *et al.*, 2002).

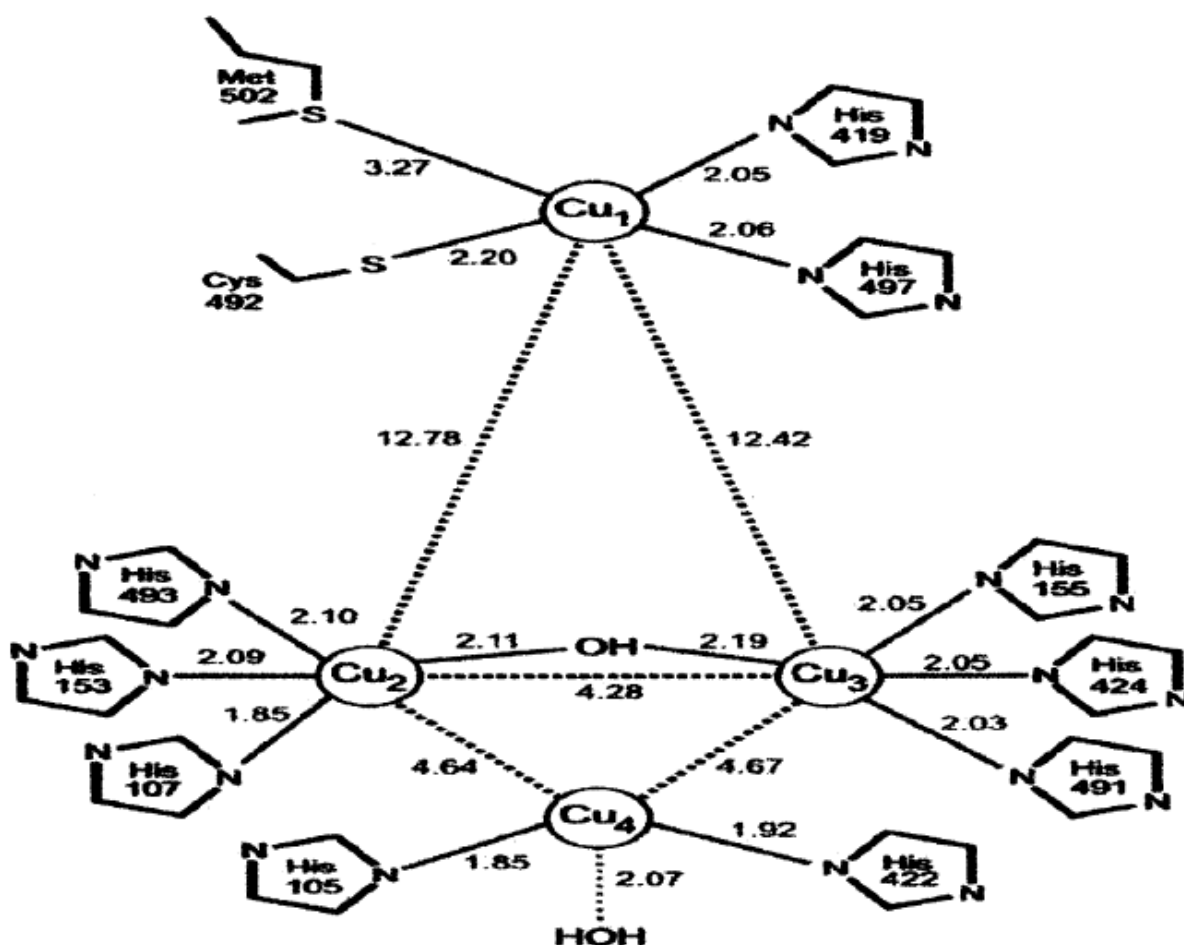


Figure 2.7: Copper centers of the laccase (CotA) from *Bacillus subtilis* (Enguita *et al.*, 2003).

Laccase catalyses protein crosslinking. During the crosslinking the enzyme abstracts a hydrogen atom from phenolic compounds (Figure 2.8) and aromatic amines to produce radicals (Gerrard, 2002). The radicals can be rearranged to form quinones, or they can go through a variety of coupling reactions. Similarly, amino, sulfhydryl, thioether, phenolic, indole, and imidazole groups of proteins can react with o -quinones produced by laccases from low molecular compounds, resulting in crosslinking products. Laccases can, therefore, directly oxidise tyrosine and cysteine residues of proteins generating radicals that will undergo

crosslinking (Buchert *et al.*, 2004; Mattinen *et al.*, 2006; Selinheimo *et al.*, 2008). Laccase crosslinks tyrosine-containing peptides through tyrosyl radicals forming isodityrosine and a small amount of dityrosine bonds (Mattinen *et al.*, 2005). In cysteine, disulfide bonds are formed by the oxidation of cysteines into cysteine (c-c) (Labat *et al.*, 2000; Mattinen *et al.*, 2005).

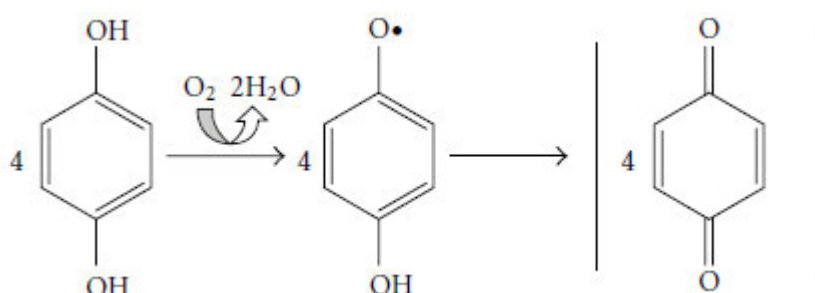


Figure 2.8: Oxidation of phenolic compounds catalysed by laccase (Selinheimo, 2008).

The mechanism of the laccase catalysed reactions can be represented by the schemes shown in Figure 2.9. The simplest case in which the substrate molecules are oxidised to the corresponding radicals by direct interaction with the copper cluster is shown in Figure 2.9a. Nevertheless, usually, the substrates of interest cannot be directly oxidised by laccases because they are either too large to penetrate active site of the enzyme, or have a particularly high redox potential (Struch *et al.*, 2015). The addition of redox mediators can overcome this drawback. Redox mediators are low molecular weight compounds that can act as intermediate substrates for laccase action. The oxidised radicals of the intermediates can interact with the bulky or high redox potential substrate targets (Figure 2.9b) (Osma *et al.*, 2010). These low molecular weight molecules are found in many plants. They include chlorogenic (CHA), caffeic (CA), ferulic acid (FA) and flavonoid catechin hydrate (CAT), whose structures are polyphenolic making them potential substrates for laccases (Cushnie & Lamb, 2005; Ratnam *et al.*, 2006; Jus *et al.*,

2008). Their presence improves the crosslinking efficiency of laccases. Hence, the addition of these phenolic compounds helps overcome the inaccessibility of functional groups (Matheis & Whitaker, 1984; Thalmann & Lötzbeyer, 2002; Strauss & Gibson, 2004; Selinheimo *et al.*, 2008; Fairhead & Thony-Meyer, 2010).

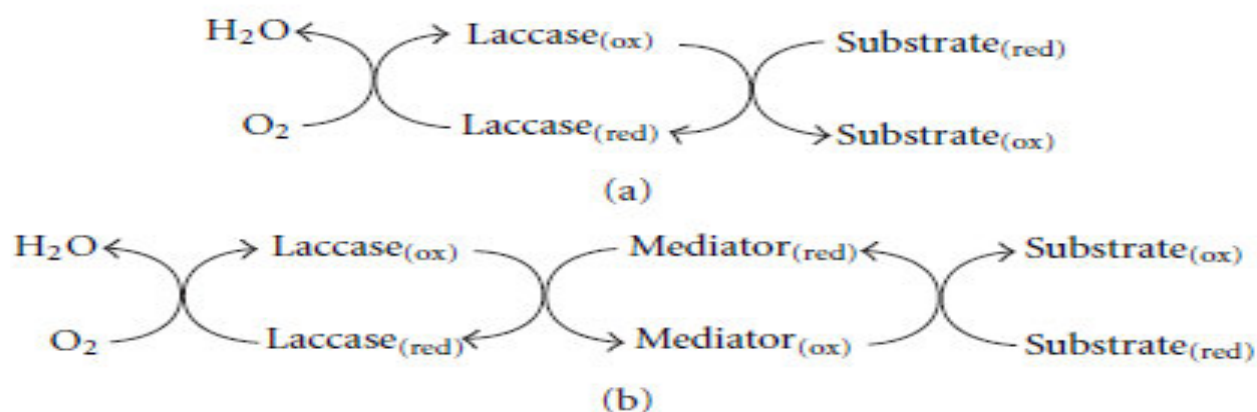


Figure 2.9: Schematic representation of laccase-catalysed redox cycles for substrates oxidation in the absence (a) and the presence (b) of redox mediators (Riva, 2006).

Laccase crosslinking has been widely used in the formation of gels from animal proteins. Laccase crosslinking, in combination with a mediator ferulic acid, was shown to increase the firmness of the sodium caseinate gels (Ercili-Cura *et al.*, 2009) and improve the viscoelastic properties of skim milk gels (Struch *et al.*, 2015). Only one study has reported the use of laccase in the gelation of plant proteins. In the study, laccase use in combination with ferulic acid resulted in a decrease in storage modulus and weaker potato protein gel strength, and this was attributed to the irregular and heterogeneous network structures (Gui *et al.*, 2020). Thus, in this case, the use of laccase in combination with a phenolic compound decreased the gel properties. Alternatively, laccase crosslinking can be achieved without combining it with phenolic compounds. Laccase crosslinking in the absence of phenolic acids improved the water holding capacity and strength of α -Lactalbumin gels (Ma *et al.*, 2020) and whey protein isolate gels

(Quan *et al.*, 2019). Hence, it might be interesting to demonstrate whether laccase crosslinking without phenolic acids has the potential to improve the strength of plant protein gels since they contain substantial amounts of tyrosine, which can act as an intermediary.

2.4. Hydrogels preparation methods

There are two main methods used in the preparation of hydrogels, especially from protein, and these are heat-induced gelation and cold set induced gelation (Figure 2.10).

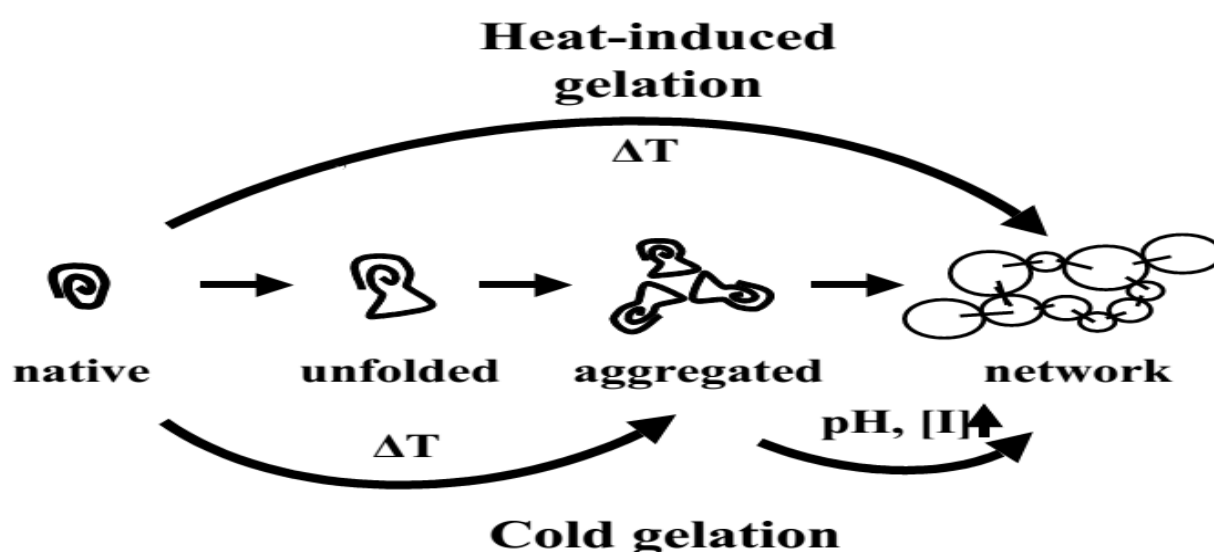


Figure 2.10: Two main methods used in hydrogel preparation: heat-induced or cold gelation procedure (Alting, 2003).

2.4.1. Heat set induced gelation

Protein hydrogels can be made by heating the protein solution, and this process is mainly known as heat-set induced. This has been widely used on most globular proteins, milk protein and soy protein hydrogels. When a protein solution containing a minimum concentration is heated above denaturation temperature, molecular unfolding and exposure of hydrophobic structures of the protein occurs, thereby resulting in the first aggregation of unfolded protein molecules and then gel formation (Caillard *et al.*, 2008; Maltais *et al.*, 2008). Intermolecular β -sheet structures are formed, and they appear to participate in gel formation (Nagano *et al.*,

1994a; Nagano *et al.*, 1994b). Only when the amount of the aggregated protein exceeds the critical protein concentration does gelation occur (Renkema *et al.*, 2001; Renkema *et al.*, 2002).

The gelling properties (such as strength, opacity, and water-holding capacity) of the heat-set gels depends on the pH, heating time, protein concentration, ionic strength, the extent of denaturation and the presence of specific ions (Renkema *et al.*, 2002; Tripalo, 2004). Chen *et al.* (2017), reported that the size and density of fractal aggregates made from soy protein were shown to increase with decreasing pH and increasing ionic strength. In contrast, the gel stiffness did not depend on these parameters. The aggregate and gelation rate significantly increased with increasing temperature but also did not influence the gel stiffness. The gelation rate has, therefore, been shown to be strongly dependent on temperature. Hence, it takes a longer time to form gels below 60°C than at higher temperatures. However, salt addition or pH alteration, for instance, in soy protein suspensions, was shown to cause gel formation even at lower temperatures (Maltais *et al.*, 2008; Campbell *et al.*, 2009; Lu *et al.*, 2010). According to Hua *et al.* (2005), the stiffness of gels at a concentration of 80 g/L and heat treatment at 95°C for 15 min increased with increasing NaCl concentration until 0.2 M. Above this a further increase in the salt concentration led to a slight decrease of the gel stiffness. Wu *et al.* (2017), reported that when soy protein gels were made at neutral pH, their elastic property (G') increased with the increase in the NaCl concentration up to 0.2 M, further increase resulted in a decrease in the G' (Figure 2.11). These changes were attributed to more proteins being incorporated in the network (salting in and salting-out effect) and the size of the aggregates building the gel networks (Renkema *et al.*, 2002; Wu *et al.*, 2017). Stiffer gels have also been reported at pH <6 than at pH > 6 in the presence of NaCl up to 0.5 M (Figure 2.12) (Renkema *et al.*, 2002).

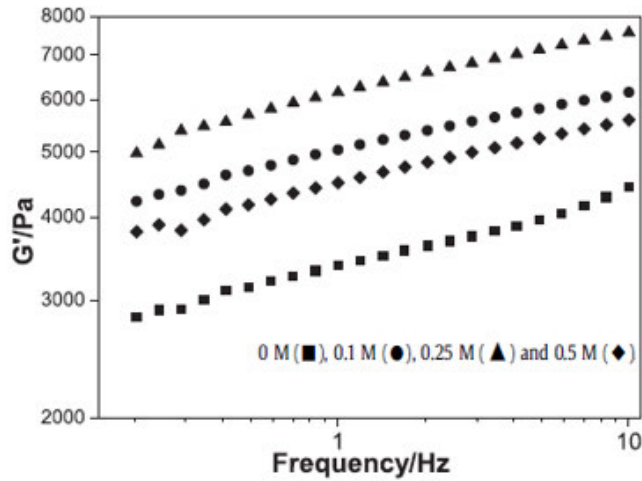


Figure 2.11: Frequency dependence of the storage modulus (G') of 18% (w/v) soy protein isolate gels at varying NaCl concentrations (Wu *et al.*, 2017).

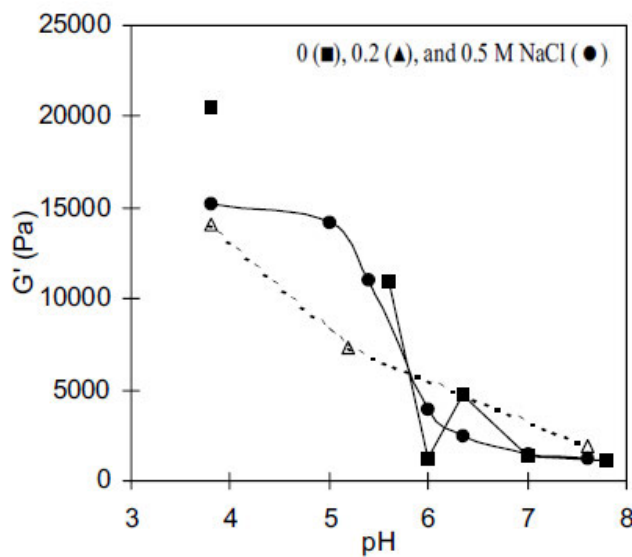


Figure 2.12: Storage modulus (G') after a complete temperature cycle of 12% soy protein dispersions as a function of pH at added salt concentrations (Renkema *et al.*, 2002).

Denaturation temperature of soy protein has been shown to increase with increasing NaCl concentration implying that soy proteins have higher thermal stability in the presence of salt (Bikbov *et al.*, 1983; Jiang *et al.*, 2010, Renkema *et al.*, 2002). Nagano *et al.* (1994) reported an increase in the gelation time and a reduction in the gelation rate for globulin protein with an

increase in NaCl concentration, and this might be due to the shift in the denaturation temperature to higher values upon increasing the ionic strength. NaCl has a shielding effect on the protein molecules resulting in a reduction in the electrostatic repulsion between the protein molecules; this influences the protein structure (Li *et al.*, 2018) and aggregate size (Wu *et al.*, 2017).

Heat-induced gels have also been formed in the presence of crosslinking enzymes such as transglutaminase and laccase. Different processing conditions (enzyme activity, temperature and time) have been shown to yield gels with varying properties for various applications. The crosslinking enzyme is added to the protein solution and incubated at the required temperature for a specified period. For instance, the soy protein solution was incubated after TGase addition at 40°C for 30 min to allow the enzyme to react, and after that, the samples were heated at 95°C for 10 min (Sun & Arntfield, 2011). An increase in the TGase activity led to the rise in the storage modulus, meaning an increase in the gel strength (Sun & Arntfield, 2011). In another study, TGase use was observed to improve the elasticity as well as a positive linear relationship between the increase in enzyme activity (0-0.7% w/w) and shear stress and strain of heat-induced pea protein gels (Shand *et al.*, 2008). Hence, the strength and elasticity of TGase treated pea protein gels were enhanced, and the resulting shear stress and strain at failure was similar to that of homologous soy protein gels and the commercial meat bologna (Shand *et al.*, 2008).

Apart from the increase in the strength, the homogeneity of the formed networks has also been reported to improve upon enzymatic crosslinking and with the increase in the enzyme activity. Wen *et al.* (2018), reported a denser and more homogeneous gel network with more compaction, fewer pores and smoother surface (Figure 2.13) of protein gels made from bitter

apricot kernel upon an increase in the TGase activity (0 – 20 U/g). Similar microstructures have also been reported for various crosslinked protein gels, such as those from wheat gluten (Wang *et al.*, 2016).

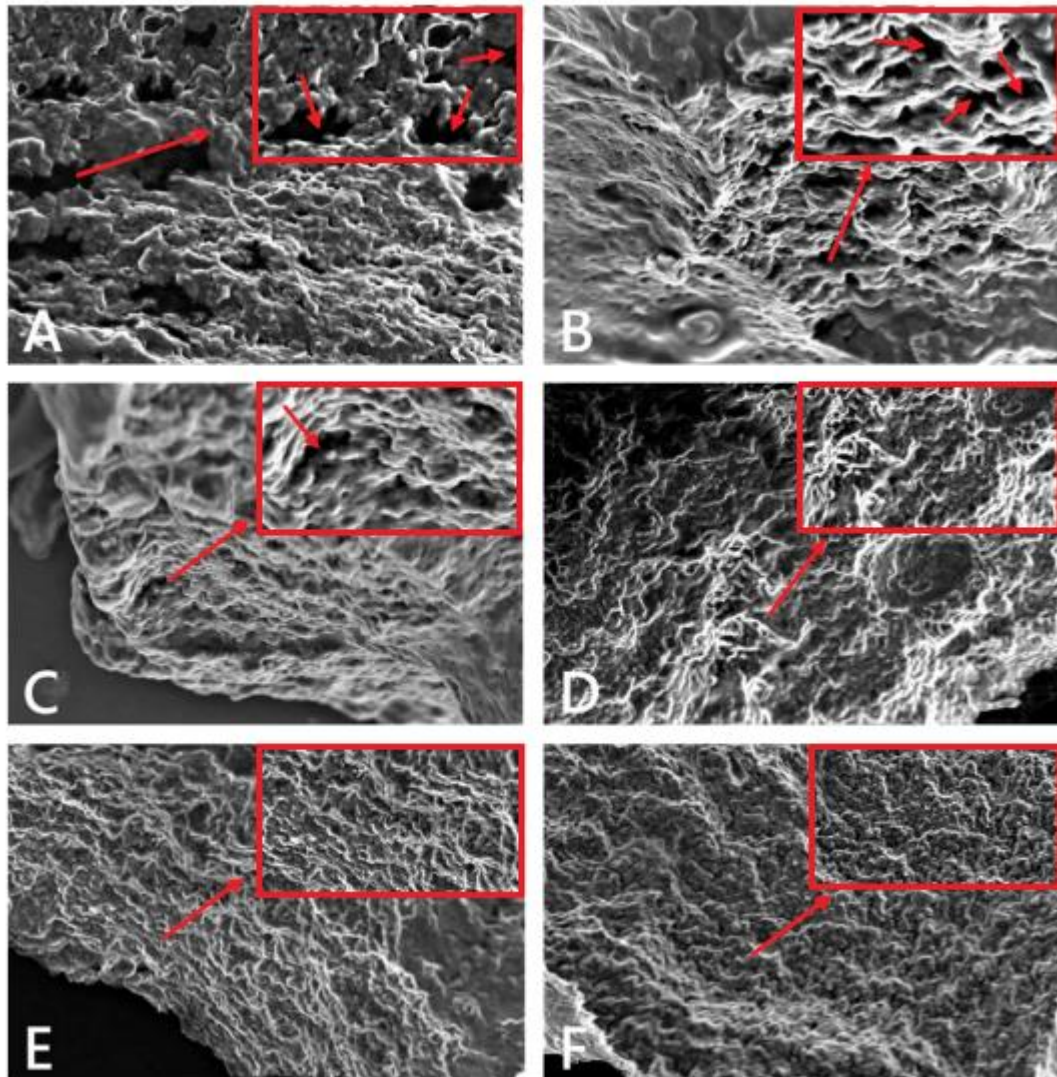


Figure 2.13: SEM images (3000× magnification, bar=10 μm) of microbial transglutaminase induced apricot kernel protein concentrate gels at different concentrations. (A) 0 U/g (B) 4 U/g (C) 8 U/g (D) 12 U/g (E) 16 U/g (F) 20 U/g (Wen *et al.*, 2018).

From the above-discussed studies, TGase crosslinking has successfully been used for the improvement of the strength of protein hydrogels. However, studies on the application of these gels in encapsulation and delivery purposes is limited. Although the TGase treated protein gels

from apricot kernel had higher resistance to simulated gastric fluid, their encapsulation efficiency was shown to decrease with the increase in TGase activity from 12 to 20 U/g protein. This was attributed to the surface hydrophobicity, which limited protein-riboflavin interaction and resulted in a lower encapsulation efficiency (Wen *et al.*, 2018). Hence, gel formation has remained an area of continued research to form gels with the desired properties.

Laccase crosslinking in heat-induced protein gel formation has been mainly studied in milk proteins. Lactalbumin was incubated at different time intervals (2, 4, 6, 8, 12, and 24 h), with laccase (80 U/g; U/w protein) at 40°C. After that, the samples were heated at 95°C to form a heat-induced gel. The water holding capacity and gel strength were observed to increase with the increase in incubation time (2 to 6 h) (Jiang *et al.*, 2017). Rheological properties (storage modulus and gelation time) of laccase induced whey protein gels increased after heating at 60°C for 10 min (Quan *et al.*, 2019). These studies, therefore, highlight the potential use of laccase in the formation of heat-induced gels. The adjustment of processing conditions, including laccase activity, provides new opportunities to extend the range of gel properties from plant proteins for application in food systems that have not been exploited before.

Significant drawbacks of heat-induced gels include that they are often coarse and have poor water holding capacity (Tang *et al.*, 2006). Heat-induced hydrogels are also only used for the incorporation of those molecules that are heat resistant because the gel is formed during heating. If the heat-sensitive molecule were to be incorporated in heat-induced gels, they would not serve their function, or the heat will deactivate the molecule. To overcome these limitations, non-thermal gelation treatments have, therefore, been investigated.

2.4.2. Cold set induced gelation

Cold set gelation is a two-step process, preheating of the protein solution and addition of the crosslinker). The preheating step causes the protein molecules to unfold, thereby opening their globular structure and exposing the reactive amino acid groups (Ju & Kilara, 1998; Maltais *et al.*, 2005). Previous studies have shown that upon heat treatment at temperatures higher than 70°C, the proteins denature exposing hydrophobic parts of the polypeptide and reactive thiol groups (Dannenberg & Kessler, 1988; Maltais *et al.*, 2008). These unfolded proteins tend to remain separate at pH values far from the isoelectric point of the proteins and at low ion concentrations, due to the electrostatic repulsive forces between them (Bryant & McClements 1998). Hence, it is important to keep the protein concentration way below a critical value and the ionic strength low enough to prevent immediate over aggregation of protein molecules and reduce the possibilities of forming heat-induced gels (Maltais *et al.*, 2005). After heating, the protein solution is cooled, and the following step is the addition of the cross-linker (e.g. calcium chloride) or reducing pH to the isoelectric point of the protein so that the gel forms. The advantage of this method is that the bioactive compound is added to the cooled protein suspension before crosslinker addition; this is highly desirable when using heat-sensitive compounds (Maltais *et al.*, 2008).

Crosslinking enzymes can also be added as crosslinkers. Only one study has reported the improvement of the strength of pea globulin hydrogels through thermal pretreatment and subsequently, TGase (20 U/g protein) crosslinking (Djoullah *et al.*, 2018). In the stated study, the release properties were not investigated. Hence, in this study, the cold set gelation method will be used to investigate whether the hydrogels are capable of encapsulating the bioactive compound and delivering it at the appropriate targeted site as well as having a higher encapsulation efficiency.

2.5. Application of hydrogels as delivery systems

Edible delivery system can be referred to as an efficient carrier if it possesses two crucial characteristics. It should be able to maintain the active structural form of a bioactive compound until the time of delivery and provide this preserved form effectively to the physiological target (Janaswamy & Youngren, 2012). The food industry is, therefore, facing some constraints since few factors need to be considered when developing these delivery systems. For example, delivery systems must be made from ingredients that are food-grade, and must be able to withstand the food processing, storage, transport and utilisation conditions while maintaining the bioavailability of the bioactive compounds. Subsequently, the quality attributes like appearance, texture, flavor and mouthfeel of the foods they are in should not be altered (McClements, 2014).

Food-grade ingredients such as proteins, polysaccharides, lipids and minerals can be used to produce edible systems. The polymers (proteins, polysaccharides and lipids) can form different structures suitable for bioactive compound/nutraceutical encapsulation (McClements, 2014). Additionally, there has been a rise in developing food-grade delivery systems for bioactive compounds/nutraceuticals encapsulation, protection and release at the appropriate site of action within the human body (Kimpel & Schmit, 2015; McClements, 2015; Shin *et al.*, 2015). Also, proteins are generally recognised as safe and, therefore, do not pose any harm to humans.

Protein macromolecules have peculiar and diversified properties associated with their molecular weights, distributions, composition and the presence of reactive groups (Alhaique *et al.*, 2012). Such features, therefore, require several chemical modifications tailored explicitly to these multitasking materials according to the particular use assigned. Biocompatible carriers thus continue being developed for administering bioactive compounds orally. For example,

modification of gel-forming ability has led to the formation of gels of various mechanical and microstructural properties achieved through controlling the assembly of protein and polysaccharide molecular chains (Chen *et al.*, 2006). Hydrogels have proven to be the best and very efficient for the delivery of bioactive compounds (Davis & Leach, 2011; Zhang *et al.*, 2011). Hence this research focuses on the production of hydrogels with improved properties for appropriate and efficient delivery of bioactive compounds.

2.6. Bioactive compounds

There has been a rise in diet-based prevention of chronic disease based on the fact that the prevention of diseases is always more effective than curing it (Velikov & Pelan, 2008; McClements *et al.*, 2009; Aditya *et al.*, 2017). Food fortification with some food components has the capability of inhibiting or preventing chronic diseases (Laparra & Sanz, 2010; Rubio *et al.*, 2013). Studies have been done to identify, isolate and purify health-promoting components found in natural edible materials such as microorganisms, plants and animals, resulting in a group of compounds referred to as bioactive components (Gupta *et al.*, 2010).

Bioactive compounds, mainly identified as nutraceuticals, offer an excellent opportunity to improve public health (Maltais *et al.*, 2008). Nutraceuticals are substances that have a physiological benefit and have the potential to protect against chronic diseases. Hence, they improve health, delay the ageing process, prevent chronic diseases, increase life expectancy and also provide support to the structure or functioning of the body (Aditya *et al.*, 2017).

The effectiveness of nutraceuticals/ bioactive compounds in disease prevention is dependent on the preservation of their bioavailability. This has posed a difficult challenge since, after oral administration, only a small proportion of molecules will be remaining, mainly due to

insufficient gastric residence time, low permeability and/or solubility within the gut. Apart from that, bioactive compounds are unstable under food processing conditions (such as temperature, oxygen, light) and in the gastro-intestinal (GI) tract (that is the pH, enzymes, presence of other nutrients). As a result of these conditions, the bioactive compound's activity and potential health benefits are therefore limited (Bell *et al.*, 2001). Therefore, poor solubility, low chemical stability and low bioavailability reduces the efficacy of many bioactive compounds (Velikov & Pelan 2008, McClements *et al.*, 2009; Huang *et al.*, 2010). According to McClements (2012), though some bioactive compounds are being used as functional ingredients for the fortification of food and beverage products, many of those reported to have biological activity are highly hydrophobic molecules making their incorporation into foods by simple mixing difficult. Thus, they should be encapsulated within edible delivery systems such as natural polymer-based hydrogels specifically designed for their integration into food products.

Riboflavin is an example of a bioactive compound which protects against cardiovascular diseases and cancers (Powers, 2003). Deficiency of riboflavin may lead to impaired handling of iron and night blindness. Though riboflavin has been shown to be involved in a number of metabolic reactions humans are not able to synthesise it. Hence, it is supposed to be obtained as a nutrient via intestinal absorption (Yoshimatsu *et al.*, 2014). In addition, previous studies have reported that the absorption of riboflavin might be enhanced if riboflavin is retained in the gastrointestinal system for a longer period of time (Kagan *et al.*, 2006 ; Hu *et al.*, 2015). Hence, the creation of a vehicle for riboflavin delivery with potential to cause longer retention in the gastrointestinal system is beneficial for supplementation of riboflavin.

2.7. Bambara groundnut

The most commonly used source of plant protein in hydrogel formation has been soybean. Bambara groundnut (*Vigna subterranea*) can be a potential alternative source of protein (19-25%) (Arise *et al.*, 2017). It is an indigenous African pulse that is grown under subsistence farming for food across the continent (Atiku *et al.*, 2004). In South Africa, Bambara groundnut is mainly produced in KwaZulu Natal, Eastern Cape, Mpumalanga, Limpopo and Northern Province. Bambara groundnut is tolerant of drought, pests and diseases with the ability to produce a reasonable crop when grown on poor soils (Collision *et al.*, 2000; Eltayeb *et al.*, 2011; Murevanhema & Jideani, 2013).

The pulse also contains 30-40% carbohydrates (Adegbola & Bamshaiye, 2011; Hillock *et al.*, 2012; Murevanhema & Jideani, 2013). Although Bambara groundnut is composed of healthy nutritional components, it remains underutilised. Underutilisation of Bambara groundnut may be due to lack of sufficient research to unlock its potential. Bambara groundnut is primarily used for human consumption either when fully ripe or immature. Immature seeds can be consumed fresh after boiling or grilling, as a meal and even mixed with immature groundnuts or green maize (Adegbola & Bamshaiye, 2011). When fully ripe, the seeds are dried and roasted, broken into pieces, boiled, crushed and eaten as a relish. The ripe seeds can also be milled into a flour that can be used to make biscuits and/or otherwise mixed with cereals and boiled to make porridge (Barimalaa *et al.*, 2005, Adegbola & Bamshaiye, 2011). The flour has also shown the potential as a composite in bread production (Alozie *et al.*, 2009).

The major storage proteins in legumes are the 11S- legumin family or the 7S vicilin family. The major storage protein in Bambara groundnut protein has been hypothesised to be vicilin after the observation of two major bands 52 kDa and 62 kDa under both reducing and non-

reducing conditions (Kudre *et al.*, 2013, Arise *et al.*, 2017) and bands at 55 kDa and 62 kDa (Boye *et al.*, 2010; Rui *et al.*, 2011; Shevkani *et al.*, 2015). Variations of the 7S and 11S protein fractions have been found to affect the functional properties of the legume proteins (Barac & Stanojevic, 2005). Functional properties are the intrinsic physicochemical characteristics affecting the behaviour of food commodities during different processing methods (Aremu *et al.*, 2007).

The functional properties of legume proteins are essential for the successful utilisation of the proteins. One of the main functional properties necessary in protein ingredients is gelation (Eltayeb *et al.*, 2011), among others such as emulsion, protein solubility, water and oil absorption capacity (Aremu *et al.*, 2007). Studies on Bambara groundnut protein gelation have only been limited to the least gelation concentration (LGC) determination (Adebowale *et al.*, 2011) and the formation of gels from vicilin, which is the major storage protein of the legume (Diedericks *et al.*, 2019). The LGC that is the lowest protein concentration at which the gel remains in an inverted tube, is used as an index of gelation capacity (Eltayeb *et al.*, 2011). The lower the LGC, the better the gelation ability of the protein ingredient (Akintayo *et al.*, 1999). In a study by Eltayeb *et al.* (2011), increasing the protein isolate concentration from 2 to 20% w/v has been reported to result in firmer Bambara groundnut protein gels due to increased protein interactions. This is because of the decrease in the thermodynamic affinity of proteins for the aqueous solution, which increases the interaction between proteins and hence improves the gelation capacity (Eltayeb *et al.*, 2011). Diedericks *et al.* (2019) reported that the gels formed from the vicilin of Bambara groundnut were composed of weak links. This was concluded after fitting the rheological data into the percolation and fractal scaling model and the observed heterogeneous aggregated structure. Thus, there is a need to improve the formed

gel properties for their applications as encapsulation materials for delivery purposes, which can be achieved through enzymatic crosslinking.

Bambara groundnut protein contains large amounts of amino acids such as aspartic acid (9.32%), glutamic acid (15.91%), histidine (2.41%) and lysine (5.95%) (Arise *et al.*, 2017) which are also high in soy protein (Adebowlae *et al.*, 2011, Feyzi *et al.*, 2015) with potential active sites for TGase crosslinking. Additionally, the protein has substantial amounts of amino acids; tyrosine, cysteine and glutathione (Arise *et al.*, 2017) with potential active sites for laccase action. The presence of additional subunits besides the vicilin fraction, which may be associated with some legumin subunits in Bambara groundnut protein isolate (Arise *et al.*, 2017), may impact the gelation behaviours of the protein differently.

2.8. Conclusion

The production of hydrogels with improved properties continues to be an area of concern. Enzymatic crosslinking of proteins significantly improves the hydrogel matrix and strength hence improving their release properties. However, there is limited use of laccase in plant protein hydrogel formation. Research into the Bambara groundnut protein use as an alternative to soy protein in gelation is necessary to reduce the dependency on soy protein in various applications.

2.9. Hypothesis, Aim and Objectives

2.9.1. Hypothesis

The addition of transglutaminase and laccase to the Bambara groundnut protein will result in crosslinking leading to covalent bond formation subsequently enhancing the protein hydrogel strength which will further improve the hydrogel release properties.

Laccase will directly oxidise cysteine and tyrosine leading to formation of radicals which undergo crosslinking leading to the formation of disulphide, and isodityrosine or dityrosine bonds (Buchert *et al.*, 2004). Transglutaminase will form protease-resistant inter or intra molecular (glutamyl) lysine isopeptide bond (Heck *et al.*, 2013; Hu *et al.*, 2015). Hence, enzymatic crosslinking will result in structural modification and the formation of BPI gels with higher strength as well as an organised, dense and stable matrix (Wen *et al.*, 2018). Increase in crosslinks between interacting molecules and formation of a dense network structure is associated with an increase in the encapsulation efficiency and prolonged gastric resident time (Maltais *et al.*, 2010; Liu *et al.*, 2017; Wen *et al.*, 2018).

2.9.2. Aim

To improve the microstructural, structural, rheological and mechanical properties of Bambara groundnut protein hydrogels using enzymes, for potential application in encapsulation and release of bioactive compounds.

2.9.3. Objectives

1. To determine the rheological and microstructural properties of Bambara groundnut protein gels as influenced by reaction conditions (pH and ionic strength) using response surface methodology.
2. To determine the effect of crosslinking enzymes (transglutaminase and laccase) on the textural, rheological, structural and microstructural properties of Bambara groundnut protein hydrogels

3. To determine the encapsulation efficiency and release properties of the enzyme (transglutaminase and laccase) crosslinked Bambara groundnut protein hydrogel using riboflavin as a model bioactive compound.

CHAPTER THREE

Rheological and microstructural properties of Bambara groundnut protein gels

Abstract

The increasing demand for plant proteins has stimulated the search for alternative and novel protein sources for various food applications. Rheological and microstructural properties of Bambara groundnut protein isolate (BPI) gels, prepared under varying pH and salt conditions, were investigated and optimised using response surface methodology. BPI gels showed $G' > G''$ over a frequency range of 0-100 rad s^{-1} . Although BPI gels displayed the characteristics of weak gels, slightly acidic conditions (pH 5.59-6) coupled with low NaCl concentration (0.5 M) promoted the formation of more rigid gels. At slightly acidic conditions, NaCl had a stabilising effect on the protein structure, as demonstrated by the increase in denaturation temperature and enthalpy of denaturation with increasing NaCl concentration. In addition, an increase in α helix and β turn contents and disappearance of random coils were observed when 0.5 M NaCl was added at pH 6. Gels with the highest strength had the lowest water holding capacity and thiol content, suggesting the participation of more disulphide linkages during network formation. Microscopy images of gels prepared at the slightly acidic pH in the presence of NaCl showed porous homogeneous aggregates. The BPI gels have potential applications for the improvement of food texture.

3.1. Introduction

Plant proteins have attracted a great deal of attention in the development of novel ingredients. This may be attributed to the preference and shift in consumer trends towards dietary choices that are based on health, religious and/or ethical perspectives (vegetarian, allergies and genetic modification) (Ben-Harb *et al.*, 2018). Proteins from wheat and soybean are the most

commonly used plant proteins in food applications (Hu *et al.*, 2015; Wang *et al.*, 2017). However, the high demand for proteins in various applications and anticipated rise in world population from 7.4 billion in 2017 to 9.8 billion in 2050 (United Nations, 2017) would result in increased protein demand. Thus, the need to search for alternative protein ingredients with similar or even better functional and nutritional properties. Hence, the promotion of research and utilisation of proteins from underutilised legumes as substitutes (Sun & Arntfield, 2010). Bambara groundnut (*Vigna subterranea*) is an alternative source of protein (19-25 g/100 g), similar to pea. Bambara groundnut protein isolate (BPI) is cheap and has low allergenicity (Adebowale *et al.*, 2011; Arise *et al.*, 2017).

The ability of legume proteins to form gels enables their application in texture improvement and encapsulation for delivery purposes (Sun & Arntfield, 2010; Hu *et al.*, 2015). However, protein gelation is affected by an interplay of factors such as; protein concentration, pH, ionic species, and heating temperature and time (Renkema *et al.*, 2002). The effect of the mentioned factors on protein gelation properties as well as gel strength can be evaluated using small strain oscillatory (dynamic) testing because of the sensitivity of the method to changes in protein physical structure (Westphalen *et al.*, 2005). For instance, soy protein gels prepared at neutral pH showed an increase in the elastic property (G') with increase in NaCl concentration due to more proteins being incorporated into the network (Renkema *et al.*, 2002). The changes in storage modulus were also attributed to the size of aggregates building the gel networks (Wu *et al.*, 2017). Salt (NaCl) has a shielding effect on the protein molecules thereby reducing the electrostatic repulsion within the protein chains, thus influencing the protein structure (Li *et al.*, 2018) and size of protein aggregates formed during gelation (Wu *et al.*, 2017).

Furthermore, protein sources and variation in compositional subunits may significantly influence gel formation and properties. For instance, protein gels prepared from soy, were observed to be stronger than those from pea and lupin (Batista *et al.*, 2005). Although the gelation mechanism of plant proteins such as soy and pea has received considerable attention, insight into Bambara groundnut gelation needs to be elucidated. Studies on Bambara groundnut protein isolate (BPI) gelation have been limited to the determination of least gelation concentration (Eltayeb *et al.*, 2011; Adebowale *et al.*, 2011) and only the gelation of vicilin (Diedericks *et al.*, 2019), the major storage protein fraction of the pulse (Arise *et al.*, 2017). However, Bambara groundnut protein isolate contains additional subunits besides the vicilin fraction, which may be associated with some legumin subunits (Arise *et al.*, 20017). The presence of these subunits may impact the gelation behaviours of the protein isolate differently. In addition, varying pH and ionic strength (NaCl concentration) is likely to affect the Bambara groundnut protein structure, and thus the strength and microstructure of the resultant gels. The influence of process parameters such as pH and NaCl also depends on the protein source, composition and conformational structure. There is limited information on relationship between the structural and gelation properties of Bambara groundnut protein as influenced by variations in pH and NaCl. Hence, the aim of this study was to investigate the effect of pH and NaCl concentration on the rheological and microstructural properties of heat induced BPI gels. Furthermore, the process parameters were optimised using response surface methodology.

3.2. Materials and methods

3.2.1. Materials

Bambara groundnuts were purchased from Jozini KwaZulu-Natal (KZN) province South Africa. Guanidine hydrochloride, *Ellman's reagent* (5,5' dithio-bis-[2-nitronenzoic acid]), Tris, glycine, sodium hydroxide, hydrochloric acid, sodium phosphate monobasic, sodium

phosphate dibasic, Bradford reagent, BSA standard and potassium bromide were purchased from Sigma-Aldrich, South Africa.

3.2.2. Preparation of defatted Bambara groundnut flour

Defatted Bambara groundnut flour was prepared as previously described (Busu & Amonsou, 2019). Bambara groundnuts were soaked in water (250 g in 1 L) for 6 h to soften the seed coat and manually dehulled. Dehulled Bambara groundnuts were then oven-dried at 40°C overnight and ground using a BL487 Kenwood food blender (Kenwood, South Africa). Fine flour was obtained through sieving using a screen mesh of 355 µm. Bambara groundnut flour was defatted three times by suspending in *n*-hexane at a ratio of 1:10 (flour: solvent) and stirring for 2 h using a MS7-H550-Pro magnetic stirrer (DLAB Scientific, USA) at a speed of 500 rpm. After each oil extraction, the *n*-hexane was decanted. Defatted flour was air dried at room temperature overnight to remove the remaining hexane. The defatted flour was kept in air tight plastic bags at 4°C in a cold room.

3.2.3. Extraction of storage proteins

BPI was extracted from the flours using the modified isoelectric precipitation procedure (Boye *et al.*, 2010). Defatted flour was suspended in water at 1:10 (flour to water ratio) and pH adjusted to 9.0 with 1 M NaOH, to facilitate protein solubilisation. The suspension was stirred for 1 h at 1000 rpm and centrifugation carried out thereafter. The centrifuge was set at 10 000 x g for 15 min at 4°C. The supernatant was collected, and pH adjusted to 4.6 with 1 M HCl to precipitate the protein, which were then recovered by centrifugation at 10 000 x g for 15 min at 4°C. The recovered protein isolate was washed with 25 ml of water, and pH adjusted to 7.0 using NaOH. The water was removed and the protein isolates were frozen at -80°C in an Evosafe series VF720-86 Bio freezer (Snijders labs, Netherlands), freeze-dried (Christ freeze

drier, Germany) and kept at 4°C in a cold room until required. The BPI had a protein content of 89 g/100 g as determined by the Bradford method (Bradford, 1976) using BSA standard. BSA standard was dissolved in phosphate buffer, pH 8 and absorbance read at 595 nm using a Cary 100 CONC UV-Vis spectrophotometer (Varian Inc, USA). The BSA standard curve was used to calculate the protein content.

3.2.4. Least gelation concentration (LGC)

LGC was determined before assessing the effect of pH and NaCl so that further investigations could be done using a constant concentration. LGC was determined as previously described (Adebowale *et al.*, 2011), with minor modifications. BPI suspensions of 20 mg/ml to 200 mg/ml protein concentration were prepared by rehydrating in 5 ml distilled water for 2 h and centrifuged at $4000 \times g$ for 15 min using an Eppendorf 5810R Centrifuge (Eppendorf, Germany). The supernatant was collected and heated at 95°C for 30 min in a water bath (Julabo TW2, Germany). The samples were rapidly cooled with cold running water and further cooled overnight at 4°C in the cold room. LGC was considered as the lowest concentration when the sample did not slip from the inverted test tube. The LGC determined was used for gel preparations.

3.2.5. Experimental design

The effect of pH and NaCl concentration on the gelation properties were optimised by response surface methodology (RSM) using central composite design (CCD). The independent variables were pH (6-8) and NaCl concentration (0 - 0.5 M). The levels of pH and NaCl concentrations used were determined from literature. The dependent variables were G' and G'' derived from rheological measurement data. Table 3.1 summarises the experimental design (13 runs) with coded levels. These were generated by statistical software Design Expert version 11 (StatEase

Inc., USA). Each experimental run was prepared in a randomised order. A quadratic polynomial model regression (Equation 1) was used to describe system behaviour.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i X_j + \varepsilon \dots\dots\dots \text{Equation (1)}$$

where Y is the response variable, β_0 is a constant; β_i is the linear and β_{ij} the interactive coefficient; X_i is the level of the independent variable and ε is the random error. The optimisation objective was to maximise G' and G'' of the gels. Design Expert 11 (StatEase Inc., USA) was used for numerical optimisation and this was found at a point that maximises the desirability function.

Table 3.1: Process variables used in the central composite design for Bambara **groundnut** protein isolate gel preparation.

Factors	Coded (Xi)				
	$-\alpha$	-1	0	+1	$+\alpha$
pH	5.59	6	7	8	8.41
NaCl	0	0	0.25	0.5	0.60

3.2.6. Bambara groundnut protein gels preparation

BPI gels were prepared as previously described (Wu *et al.*, 2017), with some modifications. BPI (120 mg/ml) was rehydrated for 2 h in distilled water at the desired pH and NaCl concentration according to the experimental design. The pH was adjusted using 1 M NaOH or HCl and measured on a HI 2211 Hannah pH meter (Hanna Instruments, United Kingdom). The BPI suspensions were then adjusted to 100 mg/ml concentration. After centrifugation at 4000 \times g for 15 min, the supernatant was collected and carefully placed in Teflon vessels. The supernatant was then heated at 95°C for 30 min in a water bath to induce gelation. Gels formed were cooled using running water and further cooled in ice water for 30 min before analysis.

3.2.7. Rheological properties of BPI gels

Rheological properties of BPI gels were determined as previously described (Wu *et al.*, 2017) using a rheometer (Anton Paar MCR501, RHEOPLUS/32 V3.41 software) (TA Instruments, New Castle, Delaware). The measuring system consisted of parallel plate geometry (25 mm diameter and 1.15 mm gap). A constant temperature of 25°C was used. To prevent evaporation, a thin layer of paraffin oil was used to coat the edge of the sample. Strain (0.5%), within the linear viscoelastic region (LVR) was used for analysis. Frequency sweep tests were performed from 0.1 to 100 rad/s to determine the time scale dependent viscoelastic response parameters. Storage modulus (G') and loss modulus (G'') values obtained at an angular frequency of 63.1 rad/s when the gel structures had a lower relaxation time, were used for the determination of the effect of pH and NaCl concentration on BPI gelation.

3.2.8. Verification and selection for further analysis

The optimum gel, G' and G'' actual values were compared to the predicted values for verification purposes at 95% confidence interval. Five experimental conditions were then chosen from the RSM data for comparison with the optimum.

3.2.9. Thermal properties of different BPI suspensions

Thermal properties of the BPI suspensions prepared at varying pHs and NaCl concentrations were determined by a differential scanning calorimeter Q2000, V24.10 (TA Instruments, USA) as previously described (Arntfield & Murray, 1981). Measurements were performed using a heating rate of 10 °C/min, within a temperature range (30-150°C) in a nitrogen atmosphere (25 ml/min). Thermograms were used to compute the temperature for the onset of denaturation, denaturation temperature and enthalpy of denaturation.

3.2.10. Water holding capacity

BPI gel water holding capacity was measured as previously described (Salvador *et al.*, 2009) with minor modifications. BPI gel (3 g) was weighed into a 50 ml falcon tube and centrifuged at $8000 \times g$ for 30 min. The tubes were inverted for 15 min to drain water. Water holding capacity was expressed as the ratio of mass of gel after centrifugation to the mass of gel before centrifugation.

3.2.11. Thiol quantification

Changes in the thiols were quantified using a modified method of Beveridge *et al.* (1974). BPI gel 100 mg/ml was prepared using 5 M GuHCl/Tris-Glycine and centrifuged at $16000 \times g$ for 5 min after vortexing at 1500 rpm for 10 min using an SI-100 vortex mixer (MRC, United Kingdom). To 100 μ l of the supernatant, 150 μ l of GuHCl/Tris-Glycine and 50 μ l of Ellman's reagent were added. Samples were incubated at room temperature for 5 min and absorbance read at 412 nm using a Cary 100 CONC UV-Vis spectrophotometer (Varian Inc, USA). A cysteine standard curve was used for calculating the results.

3.2.12. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was used to estimate the changes in the secondary structure of the BPI gels prepared at different pHs and NaCl concentrations. FTIR was carried out as previously described (Farjami *et al.*, 2015) using a Perkin Elmer 2000 FTIR spectrometer (Perkin Elmer, USA). Freeze dried (as previously described, section 3.2.3) BPI gel samples were finely crushed together with ground salt (potassium bromide) and pressed using a 10 MPa mechanical compressor system for 8 min into a tablet. Scanning was done from 4000 cm^{-1} to 400 cm^{-1} at 4 cm^{-1} resolution.

3.2.13. Scanning electron microscopy

BPI gel surface morphologies were observed using scanning electron microscopy (SEM) (Zeiss Ultra Plus FEG SEM, Zeiss, Germany) following a previously reported method (Hu *et al.*, 2013). Six freeze dried gel samples were cut using a single side stainless steel blade into slices of 5 mm length, 5 mm width and 1 mm height, coated with gold (layer thickness of 2 nm) and viewed at 5 kV. Several images were obtained at magnifications of 1 000 × and 5 000 ×.

3.2.14. Statistical analysis

Unless otherwise stated, all experiments were done in triplicate. Analysis of variance (ANOVA) was used to analyse data and Fischer's Least Significant Differences Test was used to compare means ($p < 0.05$ and $p < 0.01$).

3.3. Results and Discussion

3.3.1. Least gelation concentration

BPI had a least gelation concentration of 100 mg/ml. This was slightly higher than the 80 mg/ml reported by Adebawale *et al.* (2011). The minor variation could be attributed to a longer duration of heat treatment, which favours unfolding and network formation. An increase in protein concentration from 100 mg/ml to 200 mg/ml, resulted in firmer BPI gels due to increased protein interactions (Eltayeb *et al.*, 2011). Protein interactions are increased by the thermodynamic affinity of proteins for the aqueous solution, thus improving gelation capacity (Eltayeb *et al.*, 2011).

3.3.2. Effect of pH and ionic strength (NaCl concentration) on the rheological properties of BPI gels

Frequency sweep data showed G' (Figure 3.1a) greater than G'' (Figure 3.1b) irrespective of pH and NaCl concentration used in BPI gel preparation. G' and G'' values were highly dependent on angular frequency and the difference between these two parameters was less than 1 log, suggesting that the gels formed were weak. Higher dependency of G' and G'' on angular frequency could be due to more protein bonds becoming stress free at lower frequencies (Renkema *et al.*, 2002). In addition, G' values were low and $\tan \delta$ values high (Figure 3.1c), further confirming that the gels formed were weak (Sun & Arntfield, 2010). For instance, at an angular frequency of 10 rad/s, G' and $\tan \delta$ values for BPI gel prepared at pH 5.59 and 0.25 M NaCl were 3500 Pa and 0.22, respectively. This was close to the low G' of 4000 Pa and high $\tan \delta$ of 0.2 observed by Sun & Arntfield (2010) for pea protein gels made at 0.3 M NaCl, pH 5.65 to 5.7.

G' values of the BPI gels were higher at slightly acidic pH as compared to the neutral and slightly basic pH, within the NaCl concentration range studied. Renkema *et al.* (2002), also reported stiffer soy protein gels at pH <6 than at pH >6 in the presence of NaCl (up to 0.5 M). In this study, the highest G' value was observed at 0.25 M NaCl. This was in agreement with results from G' values of soy protein isolate gels in studies done by Wu *et al.* (2017) at pH 7 and at pH 6 and 7 (Renkema *et al.*, 2002). The high G' values recorded could be attributed to the more compact aggregated network of the gels as shown from the SEM images (section 3.3.8) and more disulphide bond formation as shown by the low free thiol content (section 3.3.6).

Tan δ values of the BPI gels showed a monotonical decrease at low frequencies (Figure 3.1c), highlighting a more viscous flow. However, at high frequencies a more elastic behaviour, which appeared to be independent of NaCl concentration and pH, was observed from the plateau on the graph. Thus, the gels had a characteristic of a viscoelastic material.

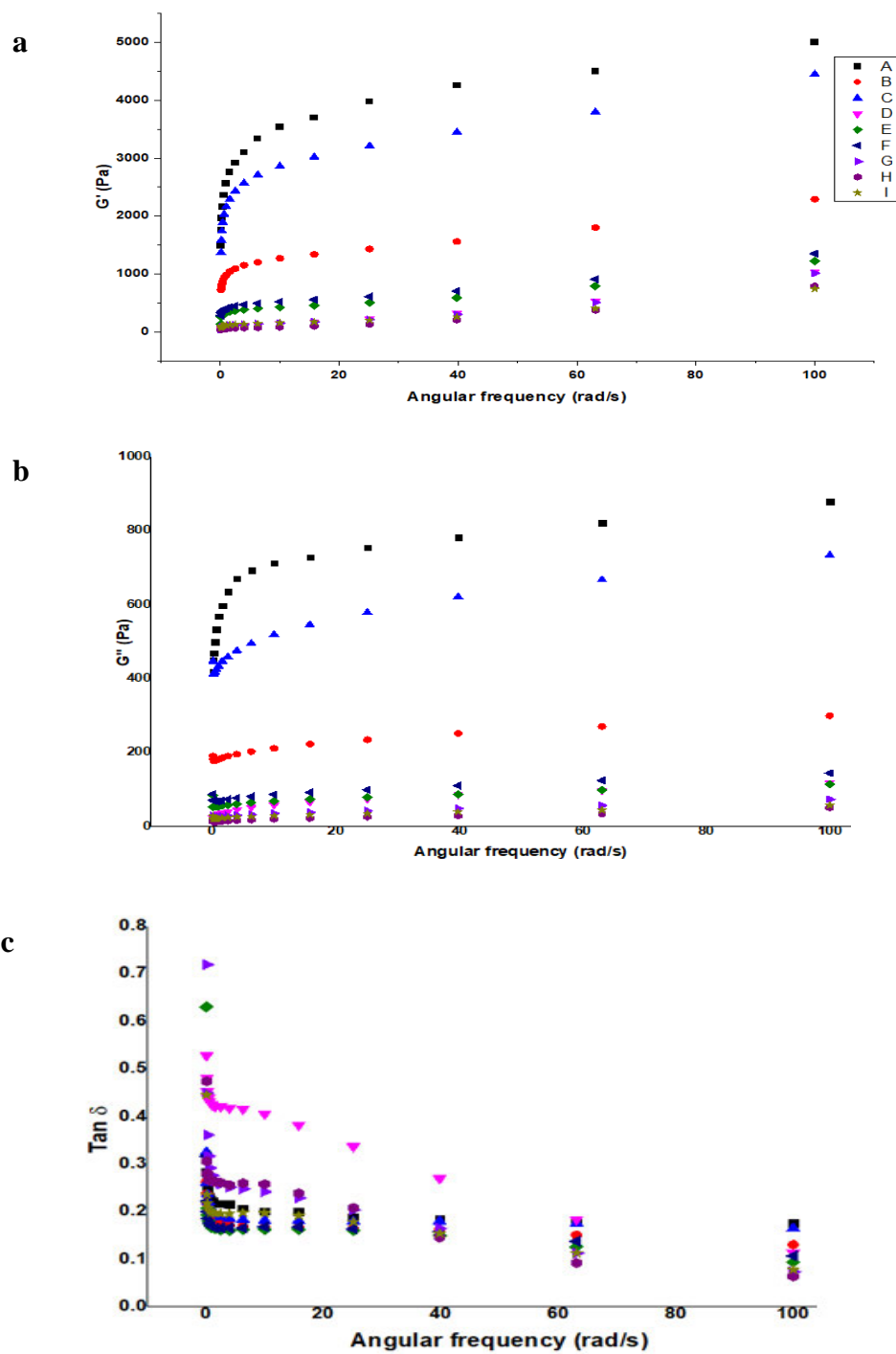


Figure 3.1. Effect of pH and NaCl on (a) storage modulus (b) loss modulus (c) $\tan \delta$ of Bambara groundnut protein gels. A. pH 5.59 & 0.25 M NaCl B. pH 6 & 0 M NaCl C. pH 6 & 0.5 M NaCl D. pH 7 & 0 M NaCl E. pH 7 & 0.25 M NaCl F. pH 7 & 0.6 M NaCl G. pH 8 & 0 M NaCl H. pH 8 & 0.5 M NaCl I. pH 8.41 & 0.25 M NaCl.

3.3.3. Model analysis

The quadratic model p-values for G' and G'' were both <0.0001 indicating that the models were significant ($p < 0.05$). The lack of fit p-values were 0.2142 and 0.0831 for G' and G'' , respectively, thus the model fitted the experimental data well ($p > 0.05$). Furthermore, the model could adequately describe the relationship between the investigated factors and responses. The factors; pH and NaCl concentration and their interaction had significant effects on G' and G'' (Table 3.2). The adjusted R^2 and predicted R^2 were very close and the difference with the actual R^2 was less than 0.2. This indicates a good correlation between actual and predicted values and the experiment has a high probability of representing the whole sample.

Table 3.2. Quadratic model for G' , G'' and linear model regression coefficients for rheological properties of Bambara groundnut protein isolate gels.

Coefficients	G'	p-value (G')	G''	p-value (G'')
Intercept	672.10		90.38	
A- pH	-1314.91	< 0.0001	-242.23	< 0.0001
B- Ionic strength (NaCl)	+337.90	0.0147	+60.59	0.0351
AB	-533.75	0.0053	-104.75	0.0095
A^2	+914.24	< 0.0001	+171.09	0.0001
B^2	-17.16	0.8916	-5.52	0.8430
R^2	0.98		0.97	
Adjusted R^2	0.96		0.95	
Predicted R^2	0.88		0.81	

The 3-D surface plots showed that slightly acidic pH coupled with 0.5 M NaCl had a pronounced effect on G' (Figure 3.2a) and G'' (Figure 3.2b). The optimisation goal was to maximise G' and G'' of the gels. The predicted values were pH 6 and 0.5 M NaCl (ionic strength) with a desirability of 0.81. The model predicted a maximum G' of 3755.73 Pa and

G'' of 663.516 Pa which were close to the actual G' of 3800 Pa (Figure 2a) and G'' of 667 Pa (Figure 3.2b), confirming the adequacy of the model.

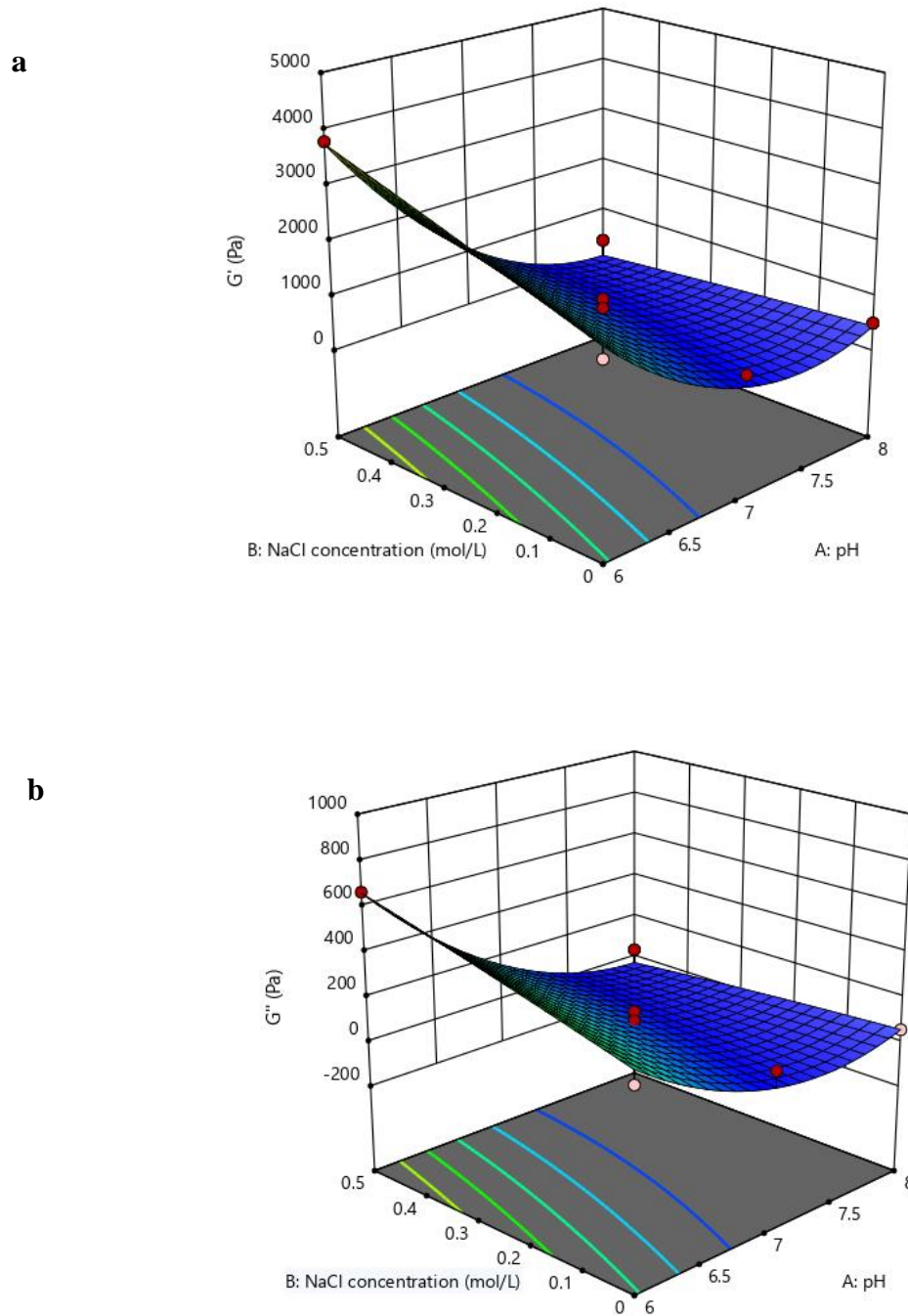


Figure 3.2: 3-D surface plots showing interaction effects of pH and NaCl on (a) storage modulus (G') and (b) loss modulus (G'') of Bambara groundnut protein isolate gels.

3.3.4. Thermal properties of BPI suspensions at different pH and NaCl concentration

Thermal properties of the BPI dispersions were influenced by pH and NaCl concentration (Table 3.3). The protein showed one endothermic peak at each investigated pH or NaCl concentration. The lowest denaturation temperature was observed at slightly acidic pH (pH 6), in the absence of NaCl. However, the incorporation of NaCl up to 0.5 M led to an increase in the denaturation temperature. This is possibly due to the nonspecific ion effects acting on electrostatic interactions between protein charged groups (Sun & Arntfield, 2010). NaCl neutralised amino acid charged side chains, thereby reducing intra and inter chain repulsion and stabilising water structure. More stable conformation like increased β -sheets or α helix and the decrease in random coils were observed from ion specific interactions (Kwon & Kim, 1994) as confirmed by the FTIR data (section 3.3.7). These results were in agreement with the study done by Sun & Arntfield (2010) who observed a higher denaturation temperature and ΔH of 94.28°C and 17.84 J/g, respectively upon addition of 0.3 M NaCl compared to 86.21°C and 15.81 J/g, respectively when no salt was added. Heat denaturation is a prerequisite for protein gel formation which usually starts at the onset of denaturation (Zhou *et al.*, 2015). The temperature (95°C) used in this study was higher than the onset temperatures of denaturation for all conditions tested.

Table 3.3: Thermal properties of Bambara groundnut protein isolate dispersions made at different pHs and ionic strengths.

pH	NaCl (M)	T _m (°C)	T _d (°C)	ΔH (J/g)
7	0	60.65 ^c ± 5.18	84.06 ^b ± 1.13	28.68 ^a ± 2.01
5.59	0.25	48.74 ^b ± 2.85	94.90 ^c ± 0.86	57.06 ^d ± 1.21
6	0	41.57 ^a ± 3.63	71.39 ^a ± 3.46	28.67 ^a ± 1.12
6	0.5	48.24 ^b ± 2.76	111.61 ^e ± 5.00	29.34 ^a ± 1.82
7	0.25	68.75 ^d ± 3.92	105.89 ^d ± 2.32	36.42 ^c ± 2.40
8.41	0.25	47.80 ^b ± 1.34	104.12 ^d ± 0.40	33.01 ^b ± 1.29

Mean \pm SD (n = 3). Values along the column followed by different superscripts are significantly different (p<0.05). T_m: onset temperature of denaturation T_d: denaturation temperature Δ H: Protein denaturation enthalpy

3.3.5. Water holding capacity

Lower water holding capacity was observed in the BPI gels made at slightly acidic pH than at neutral and slightly basic pH (Figure 3.3). Addition of NaCl at pH 6 increased water holding capacity of the formed gels while the opposite was observed at neutral pH. For instance, addition of 0.25 M NaCl at pH 7 led to significant (p<0.01) decrease in the water holding capacity of the BPI gels compared to the water holding capacity observed at pH 7 in the absence of NaCl. This is possibly due to the open structure (observed from the SEM micrographs) from which water could be easily expelled. The increase in NaCl concentration reduces electrostatic repulsion between proteins through charge screening, thus more protein-protein interactions are favoured instead of protein-water binding (Bryant & McClements, 1998). Although BPI gels made at slightly acidic pH in the presence of 0.25 M or 0.5 M NaCl had the highest gel strength, they showed the lowest water holding capacity (Figure 3.3). This could be attributed to the higher expulsion of water from the large aggregated network (Remondetto *et al.*, 2002) and the reduced protein-water binding.

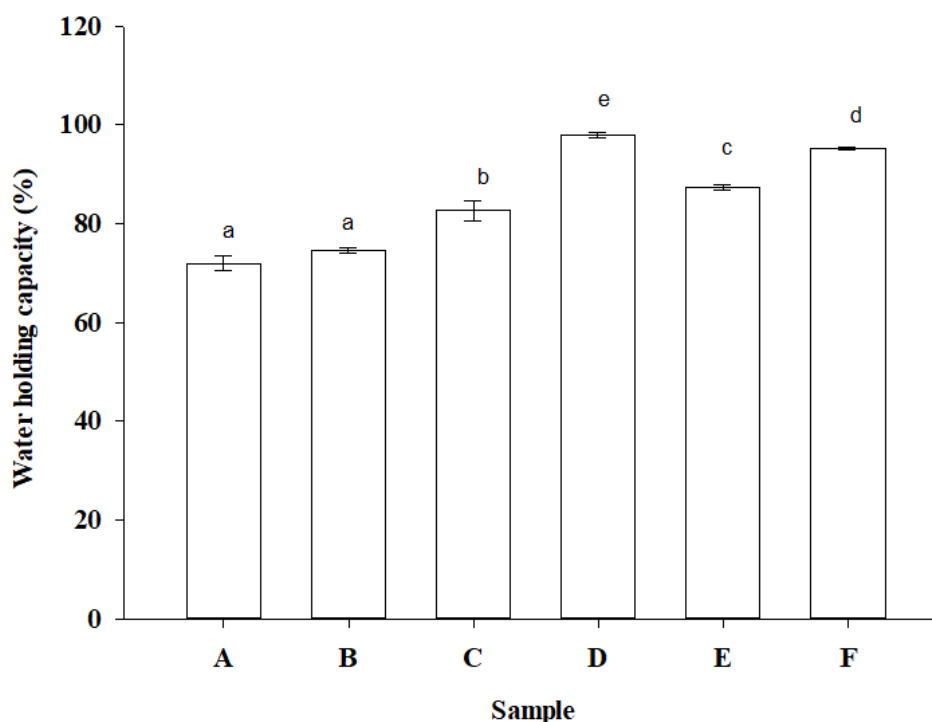


Figure 3.3: Water holding capacity of Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations. A. pH 5.59 & 0.25 M NaCl B. pH 6 & 0 M NaCl C. pH 6 & 0.5 M NaCl D. pH 7 & 0 M NaCl E. pH 7 & 0.25 M NaCl F. pH 8.41 & 0.25 M NaCl. Values are means \pm SD (n=3). Bars with different small letters differ significantly at $p < 0.05$.

3.3.6. Thiol quantification

The thiol content of the BPI gels varied with changes in pH and NaCl concentration (Figure 3.4). BPI gels made at slightly acidic pH had the lowest thiol content compared to those produced at slightly basic pH. NaCl incorporation at any pH led to a decrease in the thiol groups of the BPI gels. For example, at pH 6, adding 0.5 M NaCl led to a significant ($p < 0.01$) reduction in thiol groups. The addition of salt facilitates protein-protein interactions due to the screening of electrostatic repulsion between polypeptides (Bryant & McClements, 1998; Renkema *et al.*, 2002). Thus, with more proteins participating in the gelation process there is a marked reduction in the free thiol groups due to more disulphide bond formation which strengthens the gels formed (Wang & Damodaran 1990, Wang *et al.*, 2017). Gels with the lowest thiol content had the highest G' values which implies that disulphide bond formation

improved the relative strength of the BPI gels. The highest thiol content was observed at pH 7 in the absence of NaCl. This is because in the absence of NaCl, proteins carry their native charges hence greater electrostatic repulsion leading to reduced protein-protein interactions. It was therefore not surprising that low G' values were observed at neutral pH and in the absence of NaCl.

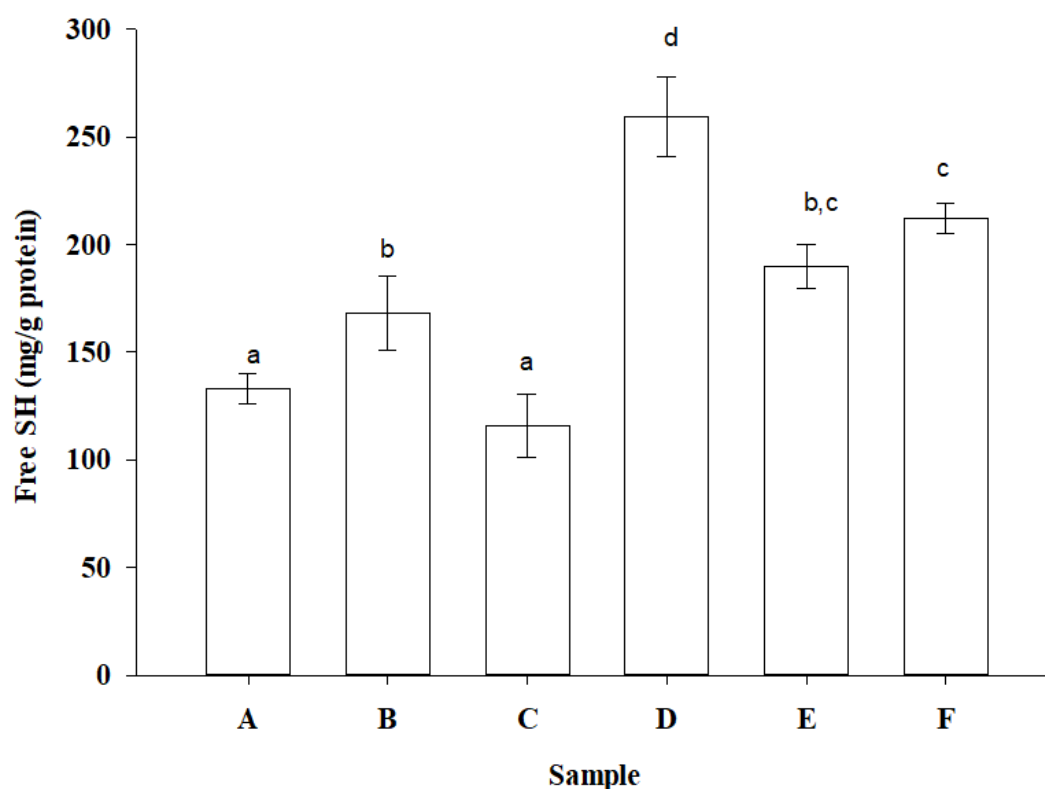


Figure 3.4: Thiol content of Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations. A. pH 5.59 & 0.25 M NaCl B. pH 6 & 0 M NaCl C. pH 6 & 0.5 M NaCl D. pH 7 & 0 M NaCl E. pH 7 & 0.25 M NaCl F. pH 8.41 & 0.25 M NaCl. Values are means \pm SD (n=3). Bars with different small letters differ significantly at $p < 0.05$.

3.3.7. FTIR

The FTIR of the BPI gels made at different pHs and NaCl concentrations showed broader peaks corresponding to the amide I (1700: 1600 cm^{-1}) and amide II (1600: 1500 cm^{-1}) regions (Figure 3.5).

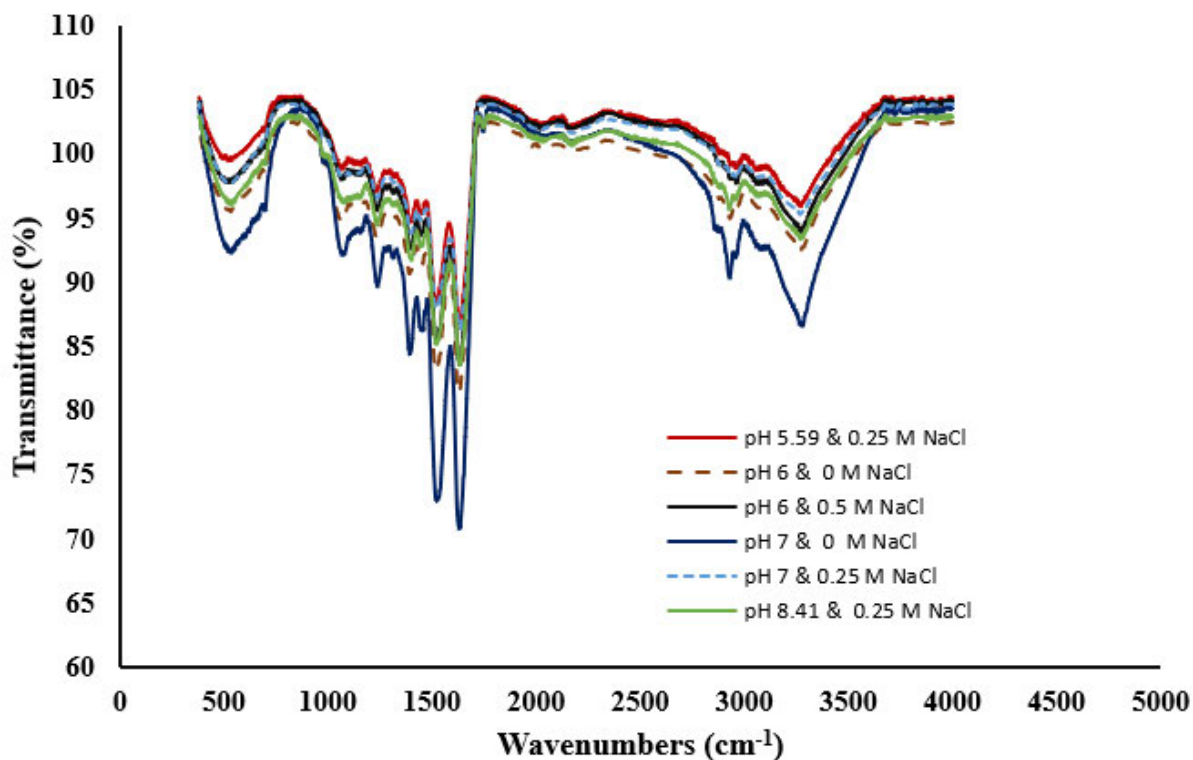


Figure 3.5: FTIR spectra of Bambara protein isolate gels made at different pHs and NaCl concentrations.

Secondary structural changes of the BPI gels caused by changes in pH and NaCl were quantified using the amide 1 region ($1700\text{--}1600\text{ cm}^{-1}$) (Table 3.4). Relative proportions attributed to the intermolecular β -sheets due to protein aggregation ($1612\text{--}1614\text{ cm}^{-1}$), antiparallel β -sheets ($1629\text{--}1632\text{ cm}^{-1}$ and $1670\text{--}1688\text{ cm}^{-1}$), random coils ($1640\text{--}1646\text{ cm}^{-1}$), α -helixes ($1648\text{--}1660\text{ cm}^{-1}$), and β -turns ($1660\text{--}1670\text{ cm}^{-1}$) were observed in the deconvoluted data (Carbonaro & Nucara, 2010; Wang *et al.*, 2017). The highest contents of β -sheets and turns were observed at slightly acidic pH in the presence of 0.25 M NaCl. This could have contributed to the highest G' value recorded for the gel produced at these conditions. However, the addition of 0.5 M NaCl at slightly acidic pH 6 resulted in an increase in the α -helix and β -turns, a slight decrease in β sheets and disappearance of the random coil, when compared to a gel produced at the same pH but without adding NaCl. This suggests the conversion of random coils into ordered structures. NaCl decreases the stability of ordered secondary structures like

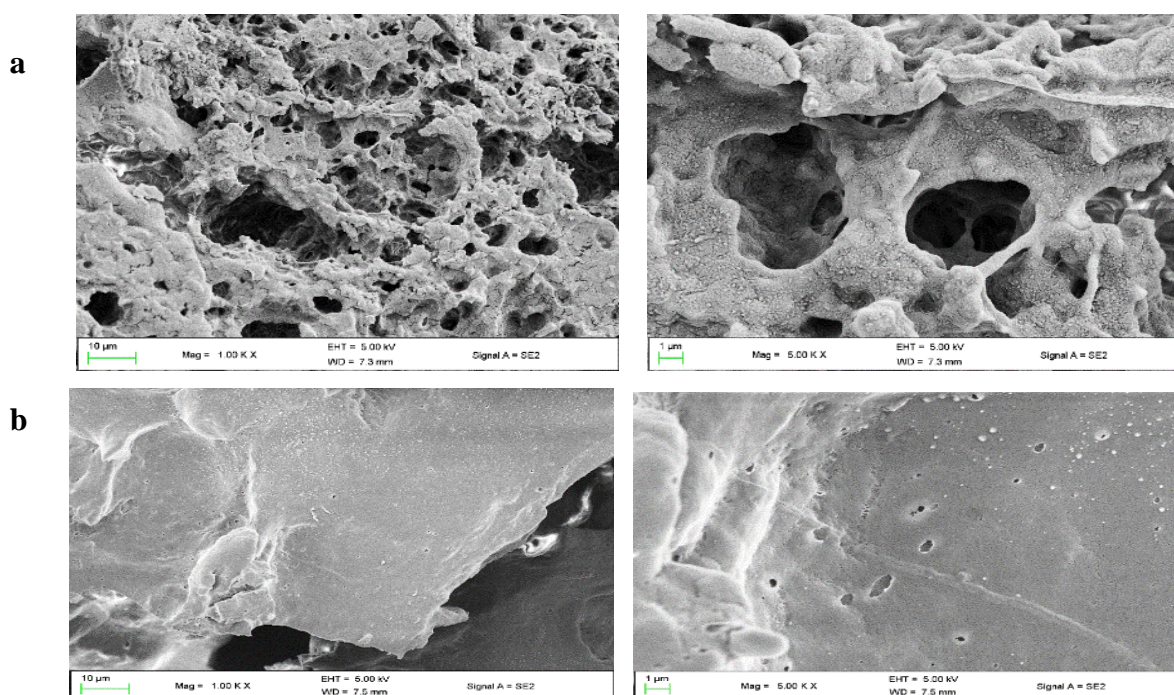
β sheets by breaking hydrogen bonds (Levy-Moonshire *et al.*, 2009). At pH 7 it was observed that the addition of 0.25 M NaCl, led to an increase in intermolecular β -sheets and turns and a reduction in α -helix compared to the gel produced at pH 7 and no NaCl. In a similar study, egg protein gels revealed a decrease in α -helix structures with increase in NaCl concentration (Li *et al.*, 2018). The increase in β -sheets indicates unfolding of the compact structure and β -aggregate formation (Ngarize *et al.*, 2004). The reduction in α -helix structures can be attributed to the protein unfolding, dissociation and rearrangement of Bambara groundnut protein molecules (Wang *et al.*, 2017).

Table 3.4: Percentage of secondary structures determined by FTIR spectroscopy in Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations.

Secondary structures	Peak range (cm ⁻¹)	pH 5.59 & 0.25 M NaCl (%)	pH 6 & 0 M NaCl (%)	pH 6 & 0.5 M NaCl (%)	pH 7 & 0 M NaCl (%)	pH 7 & 0.25 M NaCl (%)	pH 8.41 & 0.25 M NaCl (%)
Intermolecular β -sheets	1612-1614	22.66	37.24	33.27	10.55	20.68	13.47
Antiparallel β -sheets	1629-1632 1670-1694	43.28	5.39	4.78	30.52	30.65	40.07
β -turns	1662-1684	33.52	12.18	16.98	11.46	30.48	10.75
α -helix	1630 1648-1660	0.33	22.98	44.97	47.47	18.20	35.71
Random coil	1640-1650	0.21	22.22	0	0	0	0

3.3.8. Scanning electron microscopy

Scanning electron microscopy images showed BPI gels of different homogeneous networks and aggregate sizes (Figure 3.5). At slightly acidic pH, BPI gels made with 0.25 M or 0.5 M NaCl were composed of a dense network structure with smaller and larger aggregates, respectively. This may have contributed to the higher G' values and the lower water holding capacity observed. The results are in agreement with Wu *et al.* (2017), who observed higher G' values for soy protein isolate gels with a higher network ratio, larger and more compact aggregates compared to those with lower G' values. During heat treatment, protein unfolds exposing amino acid side chains and free sulfhydryl groups become accessible for intermolecular interactions; hence a more compact protein network is formed via aggregation (Wang *et al.*, 2014). At pH 7, porosity of the BPI gels increased with increase in NaCl concentration thus contributing to the lower water holding capacity. At slightly basic pH the BPI gels were less porous.



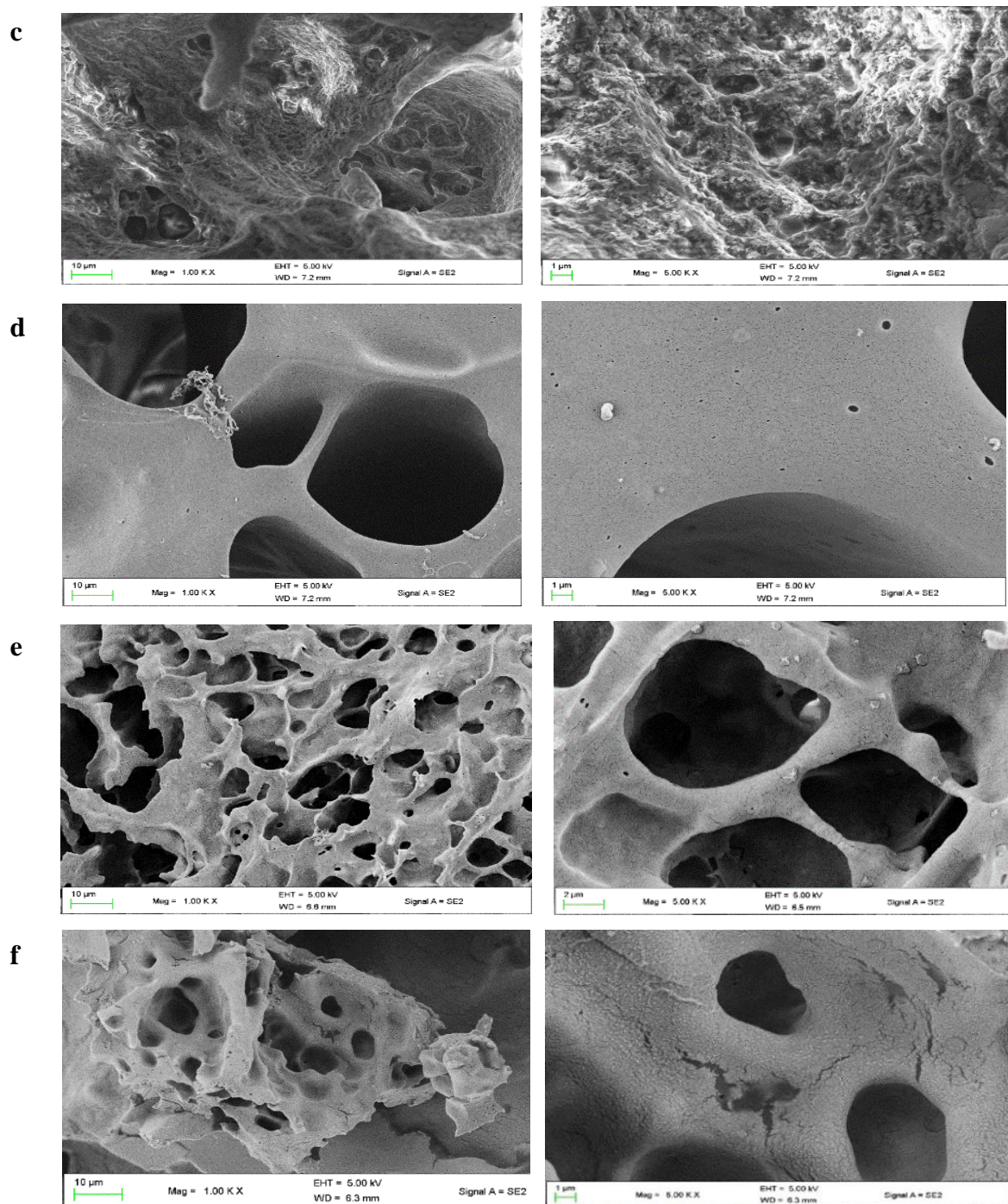


Figure 3.6: SEM micrographs of Bambara groundnut protein isolate gels made at different pHs and NaCl concentrations. (a) pH 5.59 and 0.25 M NaCl (b) pH 6 and 0 M NaCl (c) pH 6 and 0.5 M NaCl (d) pH 7 and 0 M NaCl (e) pH 7 and 0.25 M NaCl (f) pH 8.41 and 0.25 M NaCl.

3.4. Conclusion

Variations in pH and NaCl concentration influenced the frequency sweep (G' and G'') values of the BPI gels. High dependence of G' and G'' on angular frequency indicated weak gels. BPI gel with the highest G' and G'' was formed at optimum conditions of pH 6 and 0.5 M NaCl. At optimum conditions, the water holding capacity and thiol content of the BPI gel were 82.6% and 115.77 $\mu\text{g/g}$ protein, respectively. The gel had a higher content of α helix conformation, followed by β sheets and β turns. Scanning electron microscopy images of the BPI gel showed a large aggregated compact network structure. The BPI gels have potential application for the improvement of food texture.

CHAPTER FOUR

Heat-induced gelation profile of Bambara groundnut protein isolate crosslinked with laccase

Abstract

Enzymatic modification of protein can introduce crosslinks, which can potentially improve gel network and strength. In this study, the rheological properties of laccase modified (1 – 3 U/g protein) Bambara groundnut protein isolate (BPI) heat-induced gels formed *in situ* were investigated. Throughout incubation, Bambara groundnut protein dispersions without laccase showed G' greater than G'' , suggesting the presence of structure. Laccase addition showed an initial breakdown in structure ($G'' > G'$) at an enzyme dependent dose, followed by structure build-up during the heating and cooling process, resulting in increased gel strength compared to the untreated protein gel. The highest gel strength was recorded at laccase activity of 2 U/g protein. An improvement in gel structure and strength was also demonstrated by the reduction in dependency of G' on angular frequency with a difference of approximately 1 log between G' and G'' . Furthermore, the gel point temperatures of Bambara groundnut protein were significantly decreased with increasing laccase activity. At laccase activity of 3 U/g protein, the gel point temperature of Bambara groundnut protein (29°C), was approximately 3 folds lower than that of the protein without laccase (85°C). The crosslinking effects of laccase on Bambara groundnut protein gels was corroborated by the decrease in thiol and phenolic contents as well as the crosslinking of amino acids (glutathione, cysteine and lysine) in model reactions. Microscopy images showed homogeneous and compact lath sheet-like structure of the BPI gels at 2 U/g protein. Overall, the results indicated that laccase can be used in the improvement of texture of Bambara groundnut protein-based foods.

4.1. Introduction

Gelation of globular proteins is important in the formation of foods with desirable texture, structure and stability. Globular protein gels can also bind to other food components such as water, fats, flavours and stabilise them in the dispersed phase (Shand *et al.*, 2007). As such, globular protein gels are important in the preservation of metastable food structures, increasing shelf-life, fat replacement (Cao & Mezzenga, 2020) and as delivery systems (Hu *et al.*, 2015). Soybean has been the most used source of globular proteins. However, Bambara groundnuts have recently been attracting attention as alternative protein sources due to the need to meet the anticipated rise in demand especially in the African tropics. As a result, more research has been carried out on structure, composition and functionality of Bambara groundnut protein to facilitate its utilisation. Among these works, information on gelation and the subsequent gel properties of Bambara groundnut protein are very recent and limited in literature.

The study by Diedericks *et al.* (2020) reported the formation of a Bambara groundnut protein gel with a weak-link regime as concluded from the percolation and fractal scaling model and the heterogeneous structures. In the previous study (chapter 3), the gel strength of heat induced Bambara groundnut protein isolate was improved by focusing on the optimisation of process parameters such as pH and NaCl concentration. Although homogeneous aggregate structures were observed at pH 6 and 0.5 M NaCl concentration, the formed gels were weak, as indicated by the higher dependence of storage and loss modulus on frequency sweep (chapter 3). Like all other plant protein heat-induced gels, they were composed of weak network structures.

Enzymatic crosslinking of proteins has gained attention in improving the network structures of plant protein gels since enzymes are generally regarded as safe (GRAS) (Shand *et al.*, 2007; Sun & Arntfield, 2011). Crosslinking enzymes such as transglutaminase (TGase) and laccase

catalyse protein crosslinking, thereby introducing inter or intra covalent bonds within the network structures (Heck *et al.*, 2013). Recently, we reported the formation of cold set-induced Bambara groundnut protein gels in the presence of TGase; their strength increased with increase in enzyme activity up to 15 U/g protein (chapter 5). This suggests that crosslinking enzymes can improve Bambara groundnut protein gel strength. Although transglutaminases have predominantly been used in protein crosslinking, interest in laccase has been increasing because of its broad substrate specificity.

Laccase (benzenediol: oxygen oxidoreductase EC 1.10.3.2) is a multi-copper containing enzyme which performs one-electron oxidation of a wide range of substrates such as diphenols, methoxy-substituted monophenols, and aromatic and aliphatic amines (Kudanga *et al.*, 2011). Laccase has been reported to catalyse the crosslinking of peptides and proteins through oxidation of tyrosine, cysteine or glutathione residues (Mattinen *et al.*, 2006). For example, tyrosine residues are oxidised to produce tyrosyl radicals, followed by the coupling of the formed radicals to generate crosslinked proteins through the formation of dityrosine bonds (Ma *et al.*, 2020). Laccase crosslinking has been mostly used in improving the properties of animal proteins such as the firmness of sodium caseinate gels (Cura *et al.*, 2009), viscoelastic properties of skim milk gels (Struch *et al.*, 2015), water holding capacity and strength of α -Lactalbumin gels (Ma *et al.*, 2020) and whey protein isolate gels (Quan *et al.*, 2019). However, a recent study on potato proteins showed the potential of laccase to crosslink plant proteins (Gui *et al.*, 2020). Bambara groundnut protein isolate (BPI) contains substantial amounts of reactive amino acids such as cysteine (1.4 g/100g) and tyrosine (2.9 g/100g) (section 5.3.1), with potential active sites for laccase crosslinking. However, the potential of laccase to improve gelation properties of Bambara groundnut protein gels has not yet been investigated. Therefore, the current study investigated the effect of laccase on the rheological and

microstructural properties of heat-induced Bambara groundnut protein gels. Also, the present study reports for the first time the effect of laccase modification on the thermal gelation profile of BPI dispersions.

4.2. Materials and methods

4.2.1. Materials

Amino acids: cysteine, tyrosine and glutathione, *Trametes versicolor* laccase, *Ellman's reagent* (5,5' dithio-bis-[2-nitronenzoic acid]), and all other chemicals were purchased from Sigma-Aldrich, South Africa. Bambara groundnuts were purchased from Jozini, KwaZulu Natal (KZN) province, South Africa.

4.2.2. Extraction of Bambara groundnut protein isolate

Bambara groundnut protein isolate with a protein content of 90 g/100g, as determined by the Bradford method (Bradford, 1976), was extracted using the modified isoelectric precipitation method (Boye *et al.*, 2010) explained in section 3.2.3.

4.2.3. BPI gel preparation

BPI (10%, w/v) was rehydrated for 2 h in 0.5 M NaCl solution and the pH adjusted to pH 6 using 1 M NaOH. The suspension was then centrifuged (Eppendorf 5810R Centrifuge, Germany) at $4000 \times g$ for 5 min. The supernatant was collected into falcon tubes, and laccase was added at different activities (0, 1, 2, and 3 U/g protein). The mixture was incubated at 25°C for 30 min followed by heating at 95°C for 30 min in a water bath to induce gelation. The gels were cooled using running water and further cooled in ice for 30 min. BPI gels were frozen at -80°C in a Bio freezer (Evosafe series VF720-86, Snijders labs, Netherlands), freeze-dried using a freeze drier (Christ, Germany) and kept in a cold room at 4°C until further analysis.

4.2.4. Rheological properties

The rheological properties of gels were performed using a rheometer (MCR102 Rheometer, Anton Paar, Austria) equipped with parallel plate geometry (diameter of 50 mm and 1 mm gap). Immediately, after adding laccase to the BPI solution prepared, as mentioned above in section (4.2.3.), the solutions were put on the parallel plate. A time-sweep analysis was carried out at a shear frequency of 1 Hz and a strain of 0.5% to ensure measurements are being performed within the linear viscoelastic region (LVR). The temperature was maintained at 25°C for 30 min. Thereafter, the temperature was raised from 25°C to 95°C (2 °C/min), maintained at that temperature for 20 min and cooled from 95°C to 25°C at the same rate as before (2 °C/min). Frequency sweep (0.01-100 Hz) was measured after cooling to 25°C using a 0.5% strain to ensure the measurements are being performed within the LVR. All samples were covered by a thin layer of paraffin oil to minimise evaporation during measurements. The dynamic rheological parameters used to evaluate the gel network were the storage modulus (G'), loss modulus (G''), $\tan \delta$ and complex modulus (G^*).

4.2.5. Scanning electron microscopy (SEM)

BPI gel surface morphologies were observed using scanning electron microscopy (SEM) (ZEISS ULTRA PLUS FEG-SEM, Zeiss, Germany) following the method previously described (Hu *et al.*, 2013). Freeze-dried gel samples were cut into slices of 5 mm length, 5 mm width and 1 mm height, coated (layer thickness of 2 nm) with Quorum Q150R ES Sputter Coater (Quorum Technologies, UK) and viewed at 5 kV. Images were obtained at a magnification of 1 000 \times .

4.2.6. Thiol quantification

The free thiol content of the gels was quantified as previously described in section 3.2.11.

4.2.7. Phenolic quantification

The total phenols in the freeze-dried BPI gels were determined using Folin-Ciocalteu reagent following the method by Nguimbou *et al.* (2014). Folin-Ciocalteu reagent (75 μ L) was added to 50 μ L of BPI gel suspension (50 mg/ml) and allowed to stand for 3 min. Thereafter, 750 μ L of 20% (w/v) Na_2CO_3 solution was added and absorbance was measured at 760 nm using a Genesys 150 UV-Visible spectrophotometer (Thermo Scientific, USA). Differences in the absorbance were used to show the differences in the total phenols.

4.2.8. Surface hydrophobicity

The surface hydrophobicity of the BPI gels was analysed using 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescence probe using a method previously described by Kato and Nakai (1980) with modification. BPI gels were dispersed in deionised water (1 mg/ml). Serial dilutions were carried out to make concentrations of 0.15 to 0.019 mg/ml. ANS (20 μ L) was added to 4 ml of the BPI sample and the fluorescence intensity was measured immediately using a Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, USA) at an excitation wavelength (390 nm) and emission wavelength (470 nm). The initial slope of the fluorescence intensity versus the concentration calculated using linear regression analysis ($R^2 \geq 0.99$) was taken as the H_0 .

4.2.9. Non-covalent bonds

The non-covalent bonds of the BPI gels were determined using the method of Jia *et al.* (2016), with a minor modification. BPI gels (0.3 g) were treated with various reagents (10 ml) so as to cleave different types of bonds: 0.05 M NaCl (LA), 0.6 M NaCl (LB), 0.6 M NaCl +1.5 M urea (LC) and 0.6 M NaCl +8 M urea (LD). The samples were stirred at 25°C for 1 h and the supernatant was collected by centrifugation at $8000 \times g$ for 5 min at 4°C. Soluble protein

content in the supernatant was measured using the Bradford (1976) method. The proteins that were partially solubilised in these various solvents were classified as non-specific associations (soluble protein in LA), ionic bonds (the difference between soluble protein in LB and LA), hydrogen bonds (the difference between soluble protein in LC and LB) and hydrophobic interactions (the difference between soluble protein in LD and LC). The solubility in the different solvents is only an estimate of the amount of each type of bond.

4.2.10. Model reactions

Model reactions were carried out in an aqueous solution using reactive amino acids to predict possible reactions that would occur in Bambara groundnut protein gels when laccase was added. Cysteine (5 mM) or glutathione (5 mM) oxidation was carried out in the presence and absence of tyrosine by incubating at 25°C for 1 h in 50 mM ammonium acetate buffer (pH 5.5). Laccase activity of 2.5 U was added to initiate the reaction. At the end of the reaction laccase was precipitated out by adding an equal volume of ice-cold methanol and keeping on ice for 20 min. The samples were clarified through 0.22 µm Whatman microfilters (Sigma-Aldrich, South Africa) and 1.5 ml aliquots were transferred to clean vials.

4.2.11. LC-MS analysis of reaction products

The LC-MS of reaction products were carried out on a Shimadzu UHPLC system coupled to a Shimadzu ESI mass spectrophotometer (Shimadzu, Kyoto, Japan) as previously described (Manhivi *et al.*, 2018) with minor modifications. A Sunfire C18 reversed-phase column (Waters, Johannesburg, South Africa) was used for separating the reaction products using gradient elution. The elution gradient comprised of 0.1% formic acid (A) and acetonitrile (B) and the setup was as follows: 98% A (initially); 98% A to 0% A (20 min); 0% A to 98% A (20–23 min). Acquired peaks were analysed using Shimadzu LabSolutions software.

4.2.12. Statistical analysis

Unless otherwise stated, all experiments were carried out in triplicate. Analysis of variance (ANOVA) was used to analyse the data and Fischer's Least Significant Differences Test was used to compare means ($p < 0.05$ and $p < 0.01$).

4.3. Results and discussion

4.3.1. Effect of laccase modification on the rheological properties of heat induced BPI gels produced *in situ*

The rheological properties were studied *in situ* so as to investigate the influence of laccase on the structural development of BPI gels and determine the gel point.

4.3.1.1. Temperature gelation profile

Laccase modification substantially influenced the viscoelastic behaviours of Bambara groundnut protein dispersions during incubation at 25°C for 30 min, heating, isothermal point heating and cooling phase (Figure 4.1 A, B, C & D). In the absence and at low enzyme activity (0 – 1 U/g protein), Bambara groundnut protein showed the presence of structure at an early stage of the incubation with $G' > G''$ and with the G' slightly increasing during this period. The observed increase in G' is most likely due to aggregation of denatured protein and the main driving force is the hydrophobic interactions (Zhao *et al.*, 2020). Since $G' > G''$ was observed from the start of the incubation, a sharp increase in G' from the baseline (Xiao *et al.*, 2021) was therefore used as the gel point for the protein dispersions incubated with 0 and 1 U/g protein. The gel point for the Bambara groundnut protein dispersions with 0 and 1 U/g protein of laccase were approximately 85°C and 65°C respectively. Thus, though G' remained greater

than G'' upon the addition of laccase at 1 U/g protein, both the gap between G' and G'' during the incubation period and the gel point reduced.

Increase in laccase activity >1 U/g protein resulted in Bambara groundnut protein dispersions displaying a dominant viscous flow behaviour with $G'' > G'$ during incubation, which suggests that high amounts of laccase resulted in initial structure break down within the protein matrix and which subsequently change flow behaviours from elastic to viscous-like material. Laccase performs the monoelectronic oxidation of amino acids such as tyrosine resulting in the formation of reactive free radicals (Ma *et al.*, 2020). The formation of these radicals is anticipated to have resulted in cleavage of covalent bonds within the protein. $G'-G''$ crossover point was then taken as the gel point for the BPI samples incubated with 2 and 3 U/g protein laccase (Nobel *et al.*, 2020). The gel point of the Bambara groundnut protein with laccase activity of 2 U/g protein was observed to be a broad range (38 - 60°C) becoming more apparent at approximately 60°C. A lower gel point at around 29°C was observed for the Bambara groundnut protein dispersions made with laccase at 3 U/g protein. Based on previous studies, reactive radicals formed from laccase oxidation can cause various chemical modification to proteins such as aggregation, polymerisation and fragmentation (Lantto *et al.*, 2005, Lantto *et al.*, 2007; Steffenson *et al.*, 2008) and the extent of these effects on protein could be dependent on enzyme dosage (Lantto *et al.*, 2005). For instance, at a low enzyme activity of 1 U/g protein fragmentation rate was lower than the rate of polymerisation hence G' values were $> G''$ values at the onset of the period of incubation. In the mixture with laccase activity of 2 U/g protein, the $G'' > G'$ observed at the onset was due to fragmentation, however the increase in G' at faster rate relative to G'' could be attributed to polymerisation exceeding fragmentation hence causing the build-up in the network structure (Lantto *et al.*, 2005). The temperature variations could be associated with the extent of structure build up linked to enzymatic crosslinking.

The observed increase in G' during isothermal heating suggested gel strengthening associated with aggregation and the gradual development in the three-dimensional network structure (Campo-Deano *et al.*, 2009). During the cooling phase all the samples showed a slight increase in both the G' and G'' as observed by the plateau in all the graphs. The slight increase indicated the continuation of crosslinking with slower formation and rearrangement in the network structure (Sun & Arntfield, 2010). It was also observed that the reduced gel point of Bambara groundnut protein dispersions with added laccase (1- 2 U/g protein) was accompanied with a rapid growth in the G' and G'' as well as a wider gap between the moduli. This suggested the formation of a more established network structure.

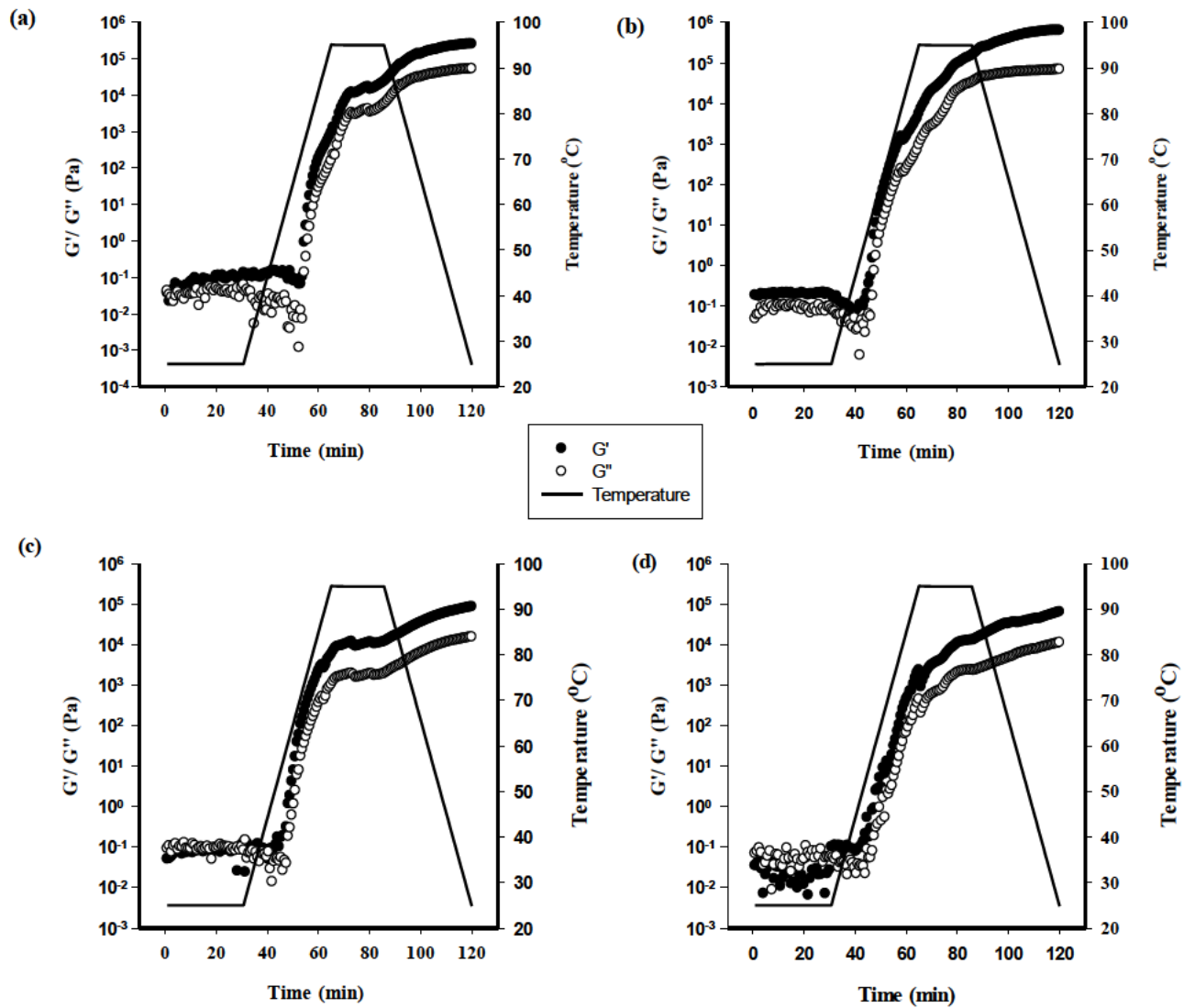


Figure 4.1: Effect of laccase activity on storage modulus (G') and loss modulus (G'') vs time during the heating process (a) 0 U/g protein, (b) 1 U/g protein, (c) 2 U/g protein & (d) 3 U/g protein of Bambara groundnut protein isolate.

4.3.1.2. Frequency sweep

The introduction of laccase influenced the viscoelastic parameters of the BPI gels throughout the angular frequency range of 0.1 – 100 rad/s (Figure 4.2a). G' values of all the formed BPI gels were higher than the G'' values over the angular frequency range of 0.1-100 rad/s. Laccase modification increased the difference between G' and G'' parameters of gels from less than 1 log in the gels made with unmodified BPI to approximately 1 log after enzymatic modification.

Such a difference indicates that laccase modification of BPI resulted in the formation of a stable network (Deng *et al.*, 2018). Also, laccase modification reduced the dependency of G' and G'' on the angular frequency hence highlighting the improvement of BPI gel strength upon enzyme addition.

The highest G' values were observed in the BPI gel modified with laccase activity of 2 U/g protein. These high G' values recorded in the laccase-modified BPI gels can be attributed to the polymerisation of protein molecules and increased disulphide bond formation as shown by the low free thiol groups and supported by model reactions. Further increase in laccase activity (3 U/g protein) caused a decrease in the G' values. The weakening of the structure could be attributed to the fragmentation of the protein molecules at high enzyme dosages (Lantto *et al.*, 2005). Lantto *et al.* (2005), in their study on chicken breast, myofibril proteins, reported that the use of an optimal level of laccase resulted in polymerisation exceeding protein fragmentation hence causing an increase in the gel strength. A further increase in laccase activity possibly caused fragmentation to exceed polymerisation of protein molecules leading to a decrease in G' values.

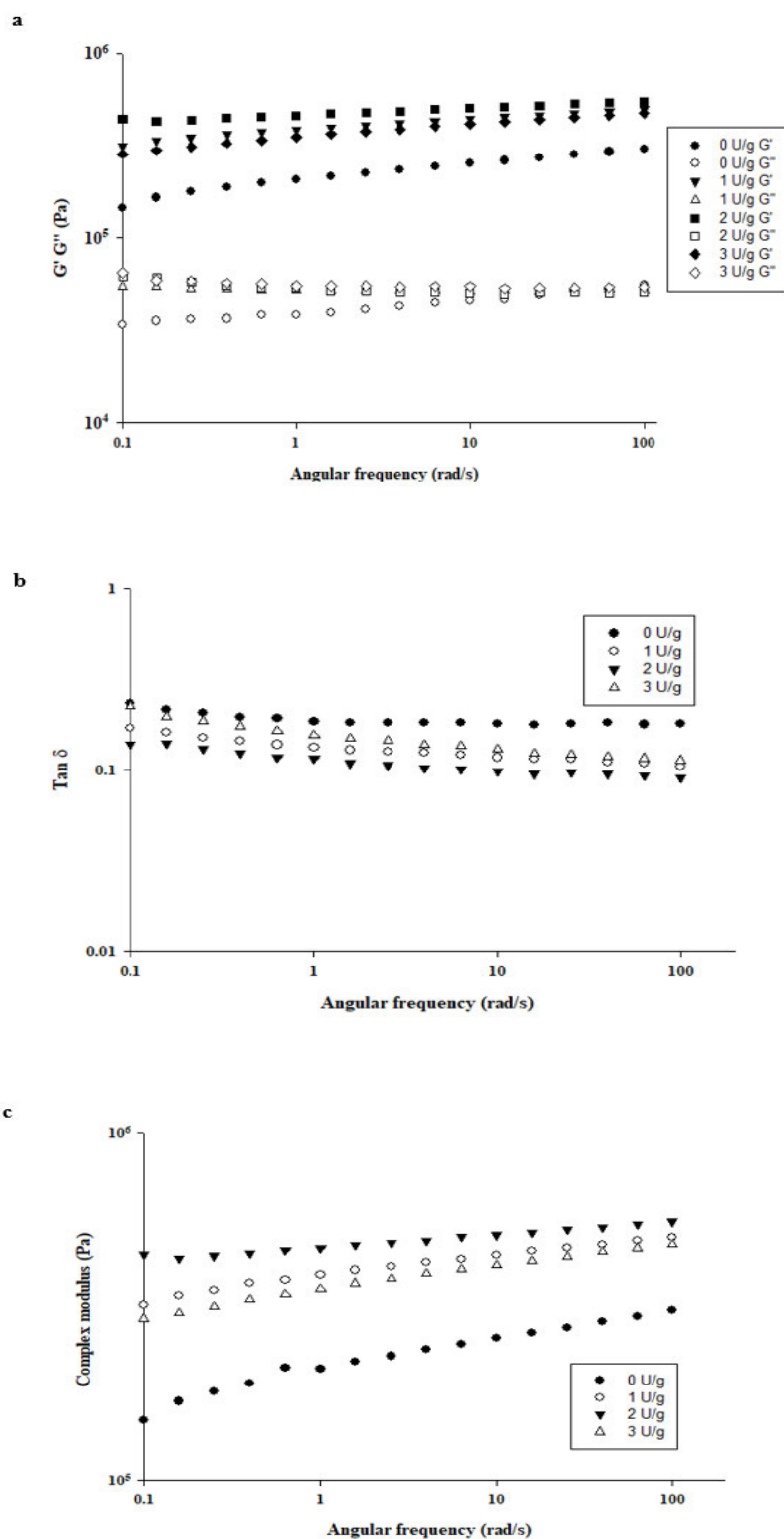


Figure 4.2: Effect of laccase activity on (a) storage modulus (G') and loss modulus (G'') (b) $\tan \delta$ (c) complex modulus as a function of angular frequency of Bambara groundnut protein isolate gels.

Laccase modification of BPI resulted in an increase in the elasticity of the formed gels as observed from the decrease in the $\tan \delta$ values (Figure 4.2b). The gel made with unmodified BPI (laccase activity of 0 U/g protein) had the highest $\tan \delta$ value. Laccase modification resulted in a decrease in the $\tan \delta$ values, thereby suggesting that the gels formed were more elastic (chapter 5). The lowest $\tan \delta$ values of 0.10 and 0.09 were recorded for the BPI gels made with laccase activity of 1 and 2 U/g protein, respectively. Gels are classified as strong if the G' and G'' parameters are independent of the angular frequency and the difference between the two moduli is tenfold ($\tan \delta < 0.1$) (Clark & Ross-Murphy, 1987).

Laccase modification caused an increase in the BPI gel G^* values over the angular frequency range studied (Figure 4.2c). Such an increase further suggests an increase in the strength of the BPI gels. The G^* increased with the increase in the laccase activity up to 2 U/g protein, suggesting stronger gels with no change in the relative elasticity (Sun & Arntfield, 2010).

The slope of the G^* was analysed using the power-law model which states that:

$$G^* = A_n \omega^{n^*} \quad (1)$$

where A_n is a measure of the strength of the crosslinking polymer network (Moresi *et al.*, 2004) and hence it indicates the strength of the gel. The angular frequency range 0.1 – 100 rad/s is represented by ω . The power-law exponent n^* describes the three-dimensional structure characterising a gel; that is, it is a measure of the crosslinks in the protein network (Dileep *et al.*, 2005). The gel strength increase upon laccase modification was further confirmed by the high A_n values recorded in enzymatically modified gel samples (Table 4.1). Laccase activity of 2 U/g protein produced the gel with the highest strength corresponding to the highest A_n value. The decrease in the n^* upon laccase treatment of the BPI gels showed an increase in the

degree of crosslinking (Peyrano *et al.*, 2021). The increase in the degree of crosslinking with the increase in laccase activity up to 2 U/g protein could be attributed to polymerisation of protein molecules exceeding fragmentation. As previously stated, at a higher laccase dosage, the fragmentation of the protein molecules exceeded polymerisation hence causing the observed decrease in the degree of crosslinking. These results agreed with the SEM images, which showed the formation of a dense and homogeneous network with an increase in laccase activity up to 2 U/g protein.

Table 4.1: The effect of laccase activity on the power law parameters (A_n and n^*) from the frequency sweep (G^*) of Bambara groundnut protein isolate gels

Enzyme activity	A_n (Pa)	n^*	R^2
0 U/g	206.565 ± 3.175^a	0.0923 ± 0.0053^d	0.99
1 U/g	387.400 ± 2.843^c	0.0591 ± 0.0026^b	0.99
2 U/g	468.035 ± 1.704^d	0.0368 ± 0.0012^a	0.99
3 U/g	353.830 ± 1.909^b	0.0698 ± 0.0017^c	0.99

Values are means \pm SD ($n=2$). Different superscripts within columns indicate significant differences ($p < 0.05$) at varying laccase activities. G^* is the complex modulus, A_n is a measure of the strength of the crosslinking polymer network and n^* is a measure of the crosslinks in the protein network.

4.3.2. Scanning electron microscopy

BPI gel micrographs showed the formation of lath sheet-like structures which are interconnected (Figure 4.3). The image of the BPI gel made in the absence of laccase shows the gel structure could have been disrupted during dehydration hence confirming that a weak gel had been formed (Farahnaky *et al.*, 2010). The porosity and homogeneity of the lath sheet-like structures formed increased with increase in laccase activity used for the BPI modification. BPI gels with a more porous, dense and homogeneous network were formed when laccase activity at 2 U/g protein was used. Previous studies have shown that the use of crosslinking

enzymes results in the formation of a protein gel with a more homogeneous, dense and porous network with a smoother surface (Wen *et al.*, 2018). Improvement in the homogeneity of the network structure could have led to the highest strength recorded upon the use of these enzyme activities. Higher laccase activity (3 U/g protein) reduced the homogeneity of the BPI gel network. This is consistent with the decrease in strength observed at the same enzyme level. Protein gels with a less uniform network and reduced strength have been reported when high levels of the crosslinking enzyme transglutaminase were used (Guo *et al.*, 2013; Chapter 5).

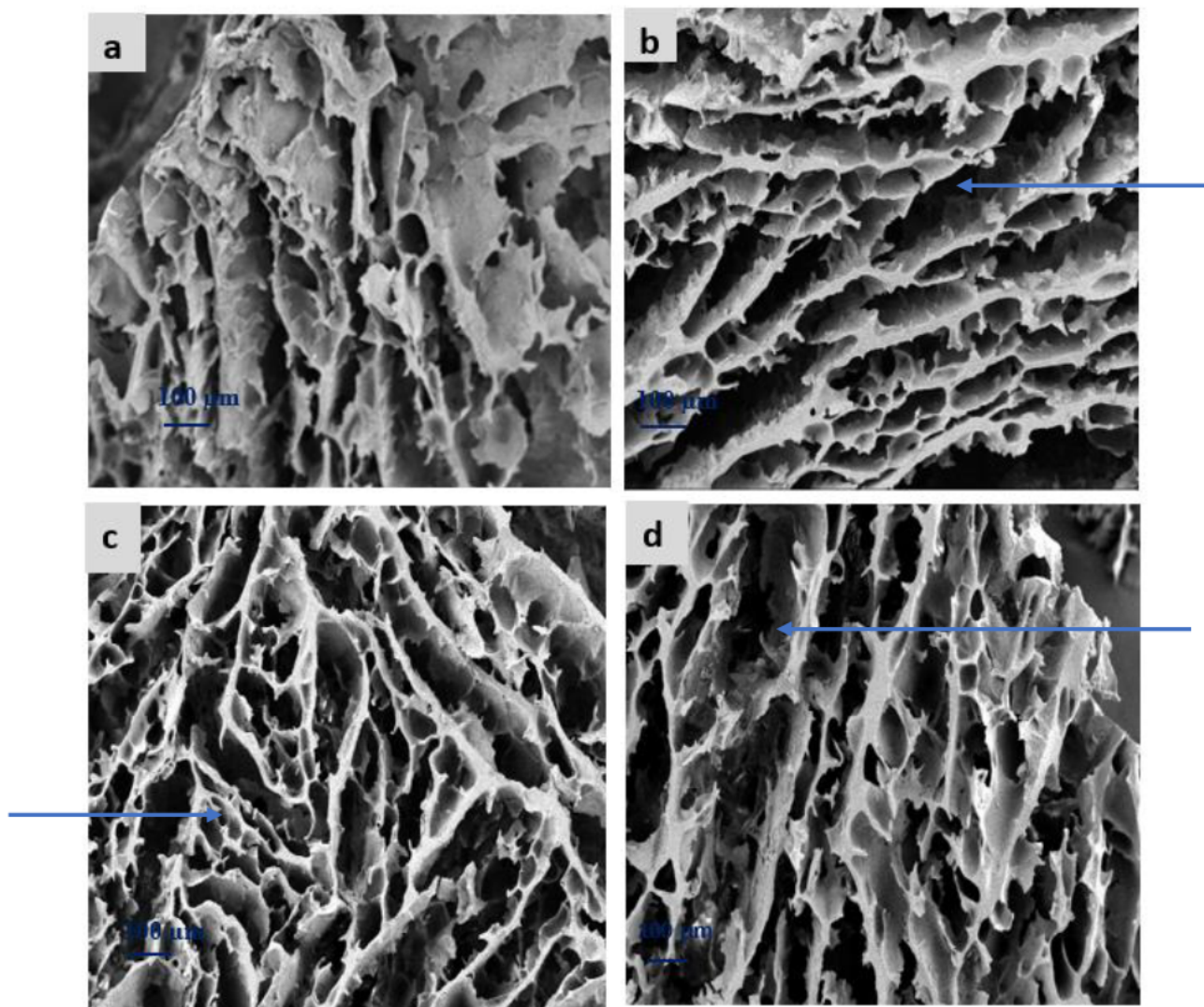


Figure 4.3: SEM micrographs of Bambara groundnut protein isolate gels made at different laccase activities; (a) 0 U/g (b) 1 U/g (c) 2 U/g and (d) 3 U/g protein. Arrows show differences in porosity.

4.3.3. Effect of laccase on free thiol content of the gels

Laccase addition resulted in a significant decrease in the free thiol groups (Figure 4.4). Laccase incubation facilitates the formation of inter-and intra- molecular disulphide bonds. Additionally, the decrease in the free thiol content suggests the formation of thiol-phenolic conjugates (Manhivi *et al.*, 2018). However, a further increase in the laccase activity from 1 U/g to 3 U/g protein had no significant effect ($p > 0.05$) on the thiol content. Disulphide bond formation was also confirmed with the model reactions (section 4.3.7).

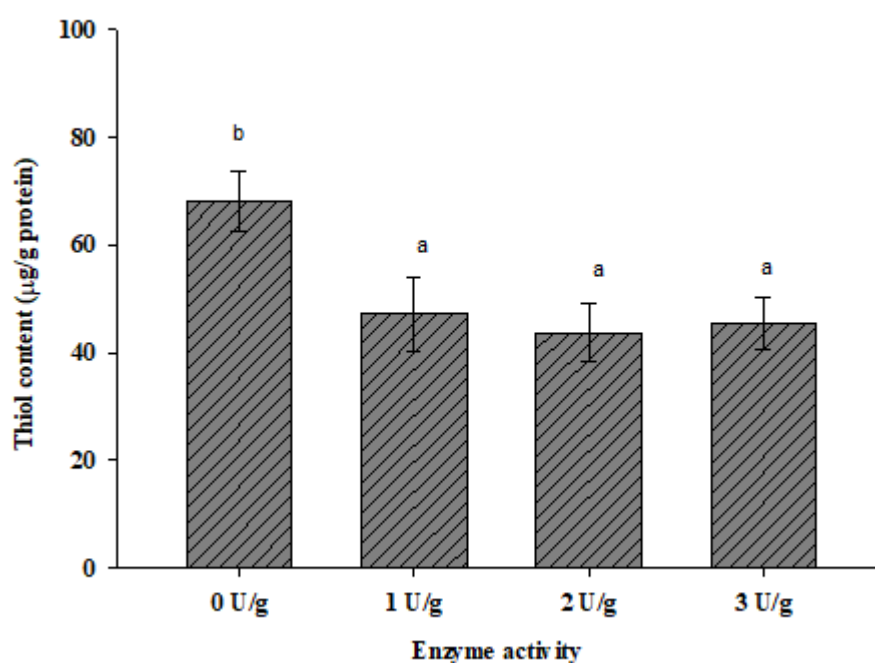


Figure 4.4: Effect of laccase activity on the free thiol content of Bambara groundnut protein isolate gels. Values are means \pm SD ($n=3$). Bars with different letters differ significantly ($p < 0.05$).

4.3.4. Phenolic quantification

The total phenolic content of the BPI gels significantly decreased ($p < 0.05$) upon laccase addition and with increase in the laccase activity (Figure 4.5). This could be attributed to laccase crosslinking of phenolic compounds as observed by the formation of phenolic

conjugates in the model reactions (section 4.3.7). Free tyrosine residues in lactalbumin (Ma *et al.*, 2020) and casein (Selinheimo *et al.*, 2008) were reported to decrease after incubating with laccase.

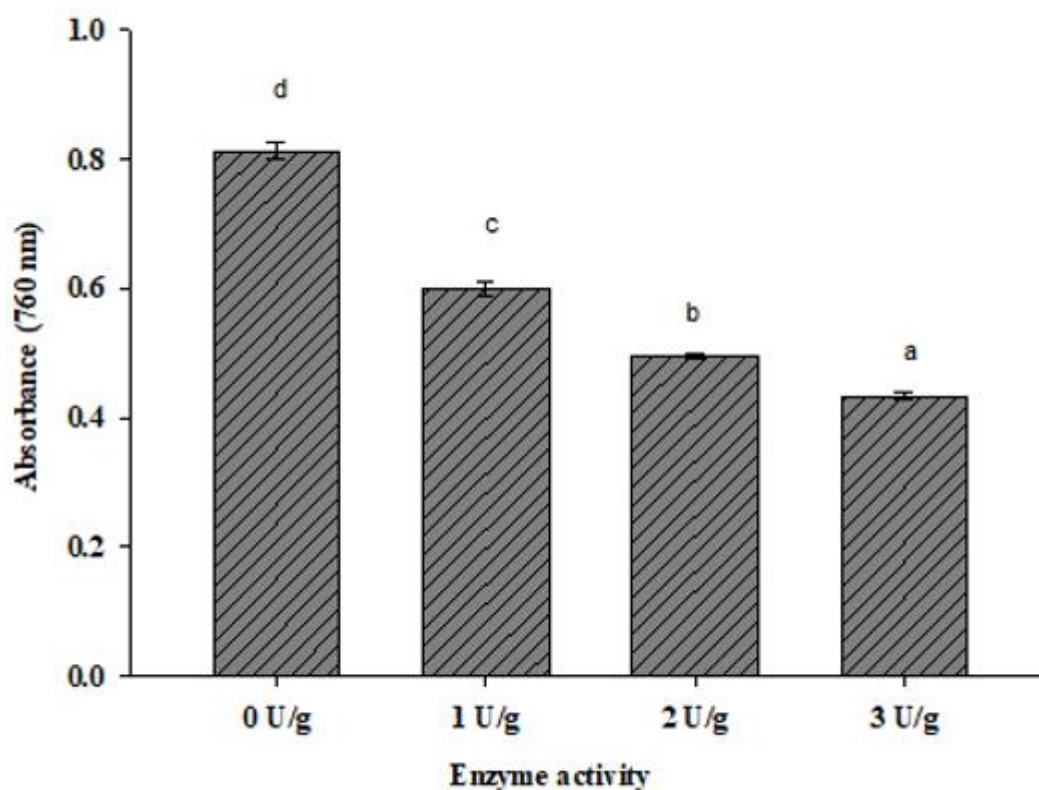


Figure 4.5: Effect of laccase activity on the total phenolic content of Bambara groundnut protein isolate gels. Values are means \pm SD (n=3). Bars with different alphabets differ significantly ($p < 0.05$).

4.3.5. Surface hydrophobicity (H_o)

Generally, laccase (1 – 3 U/g protein) significantly ($p < 0.05$) increased the H_o of BPI gels as compared to those made in the absence of laccase (0 U/g protein) (Figure 4.6). This suggests that laccase induced the unfolding of the BPI structure, thereby exposing more hydrophobic groups to the aqueous environment, previously buried in the internal domain of the molecules. The highest H_o was observed in the BPI gel made with 1 U/g protein of laccase activity. A significant ($p < 0.05$) decrease in the H_o was observed upon using a laccase activity at > 1 U/g.

H_o reflects the extent that proteins unfold and the exposure of hydrophobic groups or regions. Hence the increase in H_o could facilitate hydrophobic interactions leading to protein polymerisation and the formation of gels (He *et al.*, 2014). Furthermore, the protein gels formed can be much denser and compact. Excessive enzyme crosslinking leads to the aggregation of the exposed hydrophobic groups and regions; hence they become buried again in the protein network causing the observed reduction in H_o (Wen *et al.*, 2018).

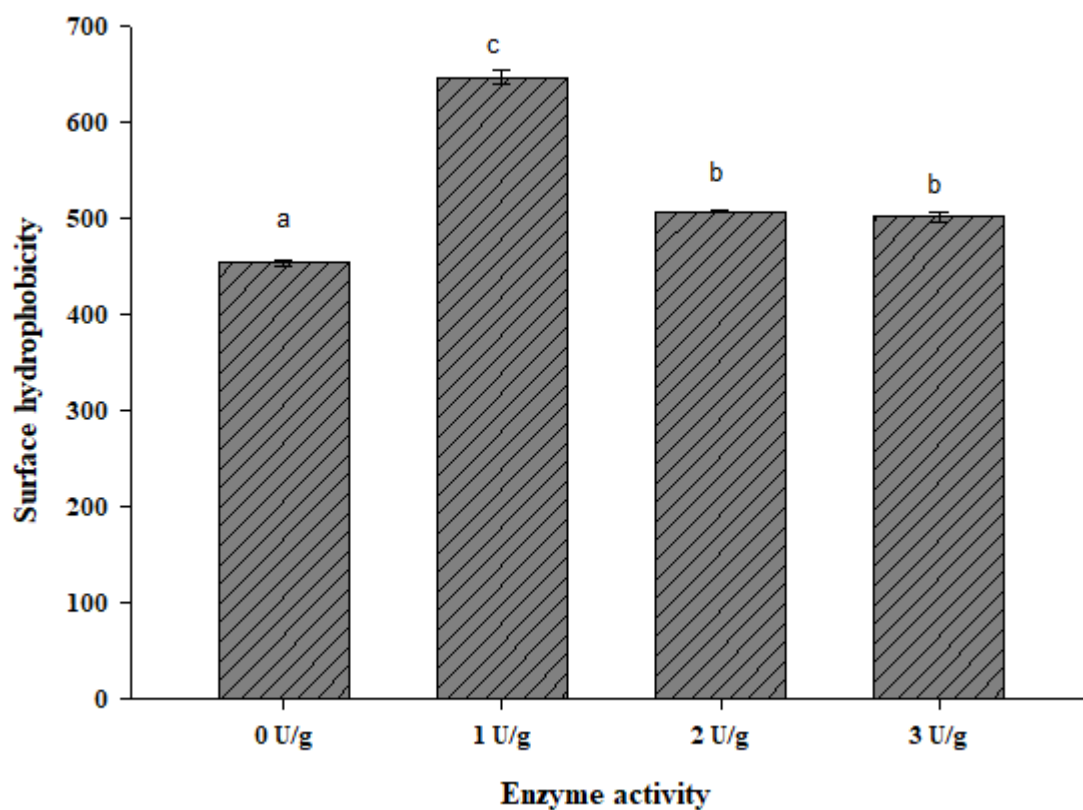


Figure 4.6: Surface hydrophobicity (H_o) of Bambara groundnut protein isolate gels made at different laccase activities. Values are means \pm SD (n=3). Bars with different alphabets differ significantly ($p < 0.05$).

4.3.6. Non- covalent interactions

Laccase addition generally decreased the non-covalent bonds, that is, non-specific associations, ionic bonds and hydrogen bonds (Table 4.2) in the BPI gels. Specifically, the decrease was

observed upon the addition of 1 U/g protein of laccase. However, a further increase in the laccase activity (2 and 3 U/g protein) caused a significant increase ($p < 0.05$) in the non-specific associations and hydrophobic interactions. There were no significant differences ($p > 0.05$) observed in the ionic bonds upon increasing the laccase activity from 1 to 3 U/g protein. The hydrogen bonds significantly decreased with the further increase in the laccase activity (from 1 to 2 U/g protein). Hydrogen bonds changes may lead to protein molecules rearrangement during gelation; such changes, transform the α -helices to β -sheets and β -turns leading to alterations in the gel networks, and increase in gel strength (Chen & Han, 2011; Chen *et al.*, 2016). According to Jia *et al.* (2016), the decrease in the non-covalent interactions could be due to the high binding energy and steric hindrance effects of covalent bond interactions such as disulphide bonds.

Table 4.2: Non- covalent interactions in Bambara groundnut pprotein isolate gels made at different laccase activities

Enzyme activity	Soluble protein (g/L)			
	Non-specific associations	Ionic bonds	Hydrogen bonds	Hydrophobic interactions
0 (U/g)	17.68 ± 0.88^c	5.05 ± 0.28^b	4.03 ± 0.46^c	34.21 ± 0.35^c
1 U/g	9.03 ± 0.08^a	1.75 ± 0.14^a	2.35 ± 0.13^b	29.81 ± 0.24^a
2 U/g	11.93 ± 0.48^b	1.84 ± 0.20^a	1.17 ± 0.08^a	32.62 ± 0.31^c
3 U/g	11.77 ± 0.17^b	1.90 ± 0.14^a	1.43 ± 0.04^a	31.02 ± 0.34^b

Values are means \pm SD (n=3). Different superscripts within columns indicate significant differences ($p < 0.05$) at varying laccase activities.

4.3.7. Model reactions

Laccase crosslinking of potential substrates was studied using model amino acids; cysteine, glutathione and tyrosine. No products were observed from mixing any of the amino acids in the absence of laccase. The resulting products from the laccase-catalysed reactions were analysed by mass spectrometry (MS).

Laccase-catalysed crosslinking of cysteine and glutathione was observed in the presence of tyrosine. Laccase cannot directly oxidise thiols; hence phenolic molecules act as mediators for the oxidation. The oxidation resulted in the formation of disulphide bonds between cysteine molecules (m/z [M-H]⁻ = 239) (Figure 4.7A), glutathione and cysteine (m/z [M-H]⁻ = 425) (Figure 4.7B) and between glutathione molecules (m/z [M-H]⁻ = 611) (Figure 4.7C). These results agreed with those observed by Manhivi *et al.* (2018) at similar conditions. Polymers with different molecular masses have been observed in laccase-treated tyrosine-containing peptides as analysed by MS (Mattinen *et al.*, 2005; Ma *et al.*, 2020). The results suggest that the addition of laccase results in the BPI crosslinking and the formation of disulphide bonds during gelation. Laccase modification of BPI would have been facilitated by the substantial amount of tyrosine (2.9 g/100g) present in the protein (chapter 5). Disulphide bond formation caused the observed increase in the BPI gel strength.

The oxidation of tyrosine by laccase resulted in the formation of dityrosine ([M]⁺ = 360) (Figure 4.7D). Dityrosine formation has been observed in laccase oxidation of lactalbumin (Steffensen *et al.*, 2008; Ma *et al.*, 2020). Tyrosyl radicals also formed conjugates with cysteine m/z [M-H]⁻ = 299 (Figure 4.7E) and glutathione m/z [M-H]⁻ = 485 (Figure 4.7F). Laccase catalyses the oxidation of tyrosine residues to form tyrosyl radicals which polymerise, to produce crosslinked proteins through dityrosine bond formation (Geiger, 1987, Ma *et al.*, 2020).

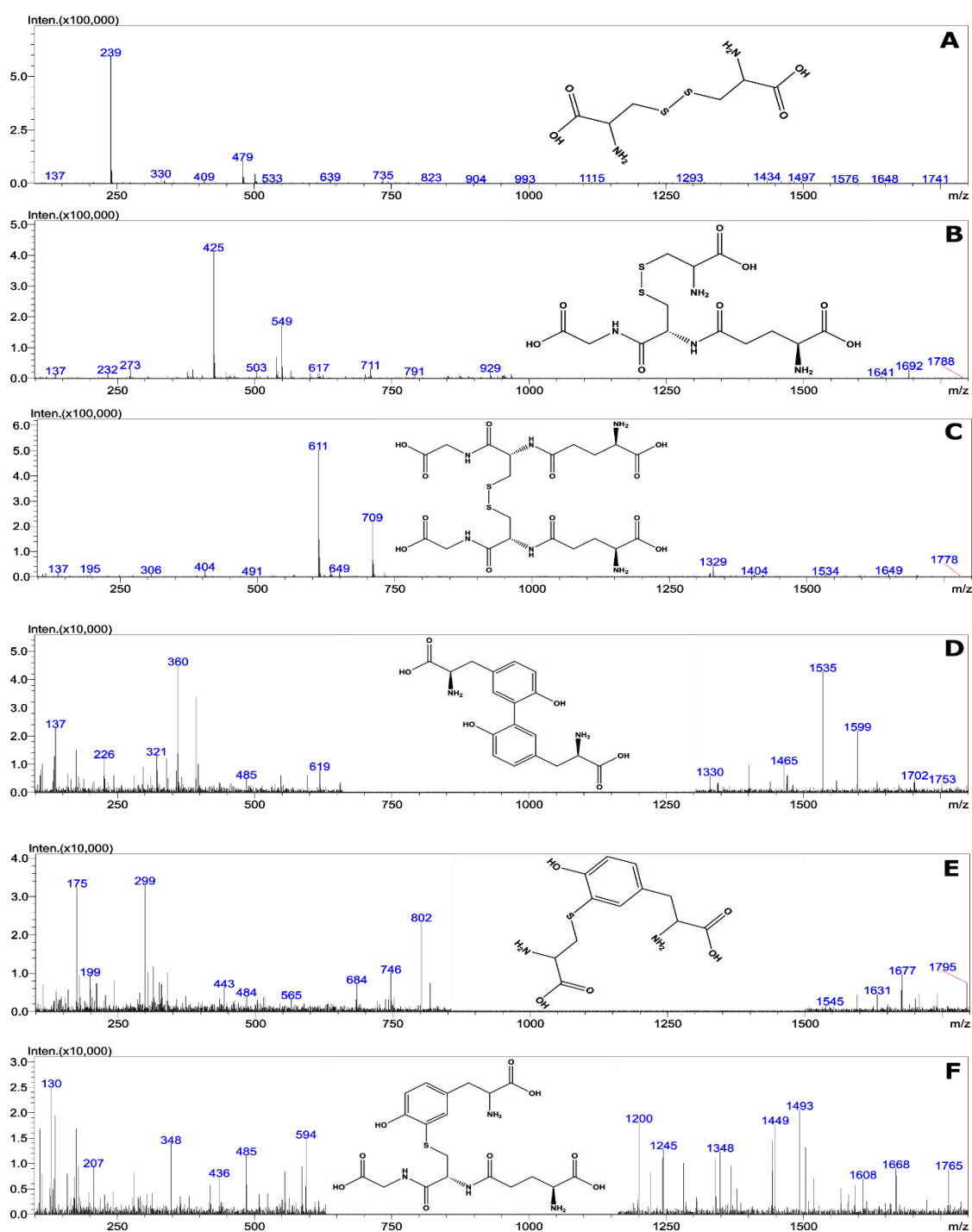


Figure 4.7: Mass spectra and proposed structures of (A) cysteine-cysteine (B) glutathione-cysteine (C) glutathione-glutathione (D) tyrosine-tyrosine (E) tyrosine-cysteine (F) tyrosine-glutathione conjugates formed through laccase treatment.

4.4. Conclusion

Laccase modification of BPI led to an increase in crosslinking degree, consequently improving the gel strength. Increase in laccase activity reduced the gel point temperature. Laccase activity at 2 U/g protein could be recommended as further increase significantly disrupted the protein structure leading to reduced final gel strength. Laccase catalysed the crosslinking of BPI, as demonstrated by the decrease in thiol and phenolic content, increased surface hydrophobicity and crosslinking of amino acids (glutathione, cysteine and lysine) in model reactions. Hydrophobic interactions were the major non-covalent interactions in BPI gel formation. BPI modification with laccase promoted the formation of a gel with a homogeneous and dense network. Laccase can be used in texture improvement of Bambara groundnut pprotein-based food products and encapsulation of heat sensitive bioactive compounds.

CHAPTER FIVE

Transglutaminase-mediated crosslinking of Bambara groundnut protein hydrogels: Implications on rheological, textural and microstructural properties

Abstract

Interest in plant protein-based hydrogels with desirable strength has been increasing in recent years. In this study, Bambara groundnut protein isolate (BPI) was crosslinked with transglutaminase (TGase) (0 – 25 U/g protein) during gelation and the rheological, textural and microstructural properties of the resulting hydrogels were investigated. Treatment with TGase up to 15 U/g protein resulted in the formation of hydrogels with small pores and an organised homogeneous network. G' of TGase-treated BPI hydrogels was more than ten-fold higher than G'' throughout the frequency range of 0-100 rad/s, suggesting dominance of the elastic like behaviour. BPI hydrogel with the highest G' (6967 Pa) and hardness (5.60 N) was formed at 15 U/g TGase activity. The hydrogel had a higher distribution of β -sheets (53.52%) and α -helixes (26.17%) as compared to the β -turns and random coils. However, a further increase in TGase activity did not improve the hydrogel properties. Transglutaminase mediated crosslinking of BPI hydrogel was demonstrated by the reduction in amine and thiol groups and the formation of a new protein band (56 kDa) in crosslinked hydrogels. Overall, TGase promoted the formation of a strong gel with an organised network.

5.1. Introduction

Hydrogels are polymeric materials with a three-dimensional network structure stabilised by covalent and non-covalent macromolecular interactions (Kopecek, 2007; Clark & Ross-Murphy, 2009). They have the ability to absorb large amounts of water and biological fluids (Calo and Khutoryanskiy, 2015). Hydrogels made from natural polymers have recently been

gaining attention in biomedical applications such as in drug and bioactive compound delivery due to their biocompatibility and biodegradability (Hu *et al.*, 2015). Hence the increased utilisation of globular proteins such as soy in hydrogel production (Guo *et al.*, 2013; Hu *et al.*, 2015). However, the use of globular proteins in hydrogels present some limitations associated with weak network structures.

Research efforts to improve the strength of the network structures in hydrogels from globular proteins include the use of chemicals such as glyceraldehyde and glutaraldehyde (Caillard *et al.*, 2008). Of these, glutaraldehyde forms hydrogels with enhanced mechanical strength but has limited application due to the toxicity of the chemical (Caillard *et al.*, 2008). Hence studies have successively shifted to investigating enzymatic crosslinking since enzymes are generally regarded as safe (GRAS). Transglutaminase (TGase) is the most commonly used enzyme in plant protein hydrogel production. TGase is a transferase enzyme that catalyses the deamidation and crosslinking reactions between protein molecules. The crosslinking reaction occurs through an acyl transfer reaction between a carboxamide group of a protein or peptide bound glutamine (acyl donor) and an amino group of a lysine residue (acyl acceptor) forming a relatively protease resistant inter- or intra-molecular (glutamyl) lysine isopeptide bond (Heck *et al.*, 2013; Hu *et al.*, 2015). TGase crosslinking significantly improves the strength of hydrogels, for example, from soy protein (Hu *et al.*, 2015) and pea protein (Djoullah *et al.*, 2018).

The rapid rise in population growth coupled with environment concerns around animal protein production and its excessive consumption, is anticipated to put enormous pressure on available food resources (Ranganathan *et al.*, 2016). The demand for plant protein is expected to significantly increase as the global population approaches 9 billion by 2050 (United Nations,

2017). Consequently, sustainable alternatives to conventional protein sources (e.g. soya bean) will be required to meet the needs for human consumption and industrial applications. One of the biggest challenges for the global food system is meeting the protein demand despite constraints imposed by environmental contexts. Multiple plant protein sources must be explored to address the shortage in different regions. Bambara groundnut (*Vigna subterranea*) is a drought-tolerant legume of African origin (Thammarat *et al.*, 2015), which has a competitive advantage over leguminous grains such as soya bean, pea and lupin. For instance, lupin and pea are more suitable for cultivation in temperate environment, whilst soya bean cannot thrive in harsh dry environments (Stagnari *et al.*, 2017). Bambara groundnut, just like soya and pea, is a good source of protein by comparison to WHO/ FAO (2007) standards. In addition to adequate level of lysine (6.0–6.8 g/ 100 g), Bambara groundnut protein also seems to have a fairly high methionine content (1.3–1.8 g/100 g) (Ijarotimi & Esho, 2009; Adebawale *et al.*, 2011; Arise *et al.*, 2017), which is unusual with legumes including soya. The knowledge of protein functionality is important to define food uses of candidate proteins.

The potential use of Bambara groundnut proteins in making gels has been recently investigated. The first study by Diedericks *et al.* (2019) reported the gelation of vicilin, the major storage protein fraction of Bambara groundnut. Fitting rheological data into the percolation and fractal scaling model indicated a weak link gel, translating to an inhomogeneous aggregated structure (Diedericks *et al.*, 2019). Since variations in compositional subunits may impact gel properties, the previous study on Bambara groundnut protein gel formation rather focused on the isolate and the optimisation of process parameters such as pH and NaCl concentrations in an attempt to improve gel strength (chapter 3). Though homogeneous aggregate structures were observed at pH 6.0 and 0.5 M NaCl concentration, the higher dependence of storage and loss moduli on frequency sweep indicated that the formed gels were still weak (chapter 3). Since gel strength

is a prerequisite for various industrial applications, there is a need to further improve Bambara groundnut protein isolate (BPI) gel strength for potential use as a soy protein alternative, which can be achieved through using TGase.

Currently, there are no reports on the use of TGase in the improvement of BPI gel properties. A couple of studies reported that the addition of BPI and TGase to sardine (*Sardinella albella*) surimi gels significantly increased the hardness of the surimi gels as compared to those made with either TGase or BPI only (Kudre & Benjakul, 2013; Kudre & Benjakul, 2014). This suggests that TGase has the potential to crosslink Bambara groundnut proteins. However, there is a need to understand the effect of TGase on BPI hydrogel properties, particularly the implications on microstructure, structure and functionality. Based on previous studies the impact of TGase on the hydrogel properties are dependent on the protein type, protein susceptibility to enzymatic crosslinking (Djoullah *et al.*, 2018) and substrate and enzyme dose (Flanagan & FitzGerald, 2003). Bambara groundnut protein differs from pea, cowpea and even soya bean protein in compositional subunit, and this difference may significantly influence TGase crosslinking and hydrogel properties. Therefore, the aim of the present study was to investigate the effect of TGase crosslinking on the texture, rheology, structure, and microstructural properties of BPI hydrogels.

5.2. Materials and methods

5.2.1. Materials

Transglutaminase (Activa TG-S-NF, 87 U/g) was kindly donated by Maccallum & Associates (Durban, South Africa, representing Ajinomoto). Ortho phthalaldehyde (OPA), Guanidine hydrochloride, Tris, glycine, 4-Dithiothreitol (DTT), *Ellman's reagent* (5,5' dithio-bis-[2-nitronenzoic acid]), BSA standard, Coomassie Brilliant Blue G-250, Bradford reagent and

potassium bromide were purchased from Sigma-Aldrich, South Africa. Bambara groundnuts were purchased from Jozini, KwaZulu Natal (KZN) province, South Africa.

5.2.2. Extraction of Bambara groundnut protein isolate

Bambara groundnut protein was extracted from the flours using the modified isoelectric precipitation procedure by Boye *et al.* (2010) as described in section 3.2.3.

5.2.3. Bambara protein isolate amino acid analysis

Bambara groundnut protein isolate (BPI) amino acid content was determined using the AccQ-Tag derivatisation method as described by Busu & Amonsou (2019). The BPI sample was placed in a 0.5 ml hydrolysis tube and dried down. The hydrolysis tube was then placed in a vessel containing a hydrolysis solution (6 M HCl). To prevent oxidation during hydrolysis, nitrogen was used for flushing the vessel and placed at 110°C for 24 h. A Shimadzu UPLC system (Shimadzu, Kyoto, Japan) was used to analyse the amino acid content in the BPI. Amino acid content was expressed as g/100 g.

5.2.4. Preparation of Bambara groundnut protein isolate hydrogels

Bambara groundnut protein isolate (BPI) hydrogels were prepared using the method by Djoullah *et al.* (2018) with some modifications. BPI solutions of 100 g/L (w/v) were prepared in 0.5 M NaCl solution and pH was adjusted to pH 6.0 using 1 M NaOH. Low NaCl concentration reduces electrostatic repulsion between proteins through charge screening, thus promoting protein-protein interactions during gelation. Centrifugation (Eppendorf 5810R Centrifuge, Germany) was then carried out at $4000 \times g$ for 5 min. Thereafter the supernatant (80 g/L) was placed in falcon tubes and heated at 95°C for 10 min in a water bath for partial denaturation (to expose reactive sites). Protein solutions were then cooled rapidly under

running cold water and TGase was added at different activities (0, 5, 10, 15, 20 and 25 U/g). This was followed by incubation at 35°C for 1 h and then the samples were placed in the cold room at 4°C for 12 h. With the exception of samples for rheological and texture analysis, the BPI hydrogels were frozen at -80°C in a Bio freezer (Evosafe series VF720-86, Snijders labs, Netherlands), freeze-dried using a freeze drier (Christ, Germany) and kept in a cold room at 4°C until further analysis.

5.2.5. Free amino quantification

The degree of TGase crosslinking was estimated using a modified method by Caillard *et al.* (2008) to measure absorbance variations of OPA, which binds to the free amino groups (-NH₂). BPI hydrogels (40 mg) were mixed with 1 ml of sodium dodecyl sulfate (1%, w/v) and stirred for 1 h at 25°C and centrifuged for 10 min at 8000 × g. Hydrogel supernatant (150 µl) was then mixed with 2.5 ml of OPA reagent. The samples were incubated at 35°C for 2 min, and their absorbances were measured at 340 nm using a Genesys 150 UV-Visible spectrophotometer (Thermo Scientific, USA). Absorbance values were used to estimate the changes in free amino groups in the crosslinked BPI hydrogels (Hu *et al.*, 2011).

5.2.6. Thiol quantification

The variations in the thiol groups were measured using a previously described method in section 3.2.11.

5.2.7. SDS-PAGE

This was performed under reducing conditions, as described by Busu & Amonsou (2019), with minor changes. Samples (20 mg/ml) were prepared by mixing the freeze-dried BPI hydrogels and the loading buffer (4 ml of 1.5 M Tris-HCl buffer, pH 6.8, 10 ml glycerol, 1 ml of 1%

bromophenol blue and 2 g SDS) which contained 1,4-dithiothreitol (DTT). The BPI hydrogels were then heated in a water bath for 5 min at 95°C. The samples were then centrifuged at $15000 \times g$ for 5 min. A separating gel of 12% (pH 8.8) and stacking gel of 4% (pH 6.8) was prepared as previously described (Laemmli, 1970). BPI hydrogel samples (20 μ l) and molecular weight marker (3 μ l) were loaded in separate lanes. The molecular weight marker was a mixture of standard proteins ranging from 10 to 250 kDa. Coomassie Brilliant Blue G-250 (0.25%, w/v) was used to stain the gel after electrophoresis.

5.2.8. Fourier transform infrared (FTIR) spectroscopy

The changes in the secondary structures of the BPI hydrogels prepared at varying TGase activities were estimated using the Perkin Elmer 2000 FT-IR spectrometer (Perkin Elmer, USA) following a previously described method in section 3.2.12.

5.2.9. Rheological properties of Bambara groundnut protein hydrogels

Rheological properties of the BPI hydrogels were determined using a rheometer (MCR102 Rheometer, Anton Paar, Austria), as previously described by Wu *et al.* (2017). The measuring system used comprised of a parallel plate (50 mm diameter, 1.15 mm gap). The cylindrical BPI gels were placed between the plates using a spatula, and the test was started after the sample had rested for 3 min. Constant temperature of 25°C and 0.5% strain within the linear viscoelastic region were used for the analysis. Frequency sweep tests were performed between 0.1 and 100 rad/s, and the storage modulus (G') and loss modulus (G'') values were recorded.

5.2.10. Texture analyses

The texture analyses of BPI hydrogels were measured using an EZ-LX/E2-SX series Texture Analyser (Shimadzu, Japan) as described by Li *et al.* (2018) with some modifications. BPI

hydrogel of 10 mm thickness was compressed twice to 50% of its original height using a cylindrical stainless-steel probe (25 mm diameter and trigger force of 500 N) using a crosshead speed of 1 mm/s and return speed of 1 mm/s. A 5 s gap was allowed to pass between the two compression cycles. The cohesiveness and hardness were recorded.

5.2.11. Water holding capacity

BPI hydrogel water holding capacity was measured as previously described in section 3.2.10.

5.2.12. Syneresis

The syneresis of the BPI hydrogels was measured using the method previously described by (Zand-Rajabi & Madadlou, 2016). Fresh BPI hydrogel sample in 50 ml falcon tubes were stored upside down for 120 min at room temperature and the expelled water was weighed. Syneresis was calculated using the following equation:

$$\text{Syneresis} = \frac{B}{A} \times 100$$

where *B* is the weight of expelled water from samples after 120 min and *A* is weight of hydrogel samples.

5.2.13. Scanning electron microscopy (SEM)

BPI hydrogel structures were investigated using SEM (ZEISS ULTRA PLUS FEG-SEM, Zeiss, Germany), as previously described in section 4.2.5.

5.2.14. Amino acid model reactions

Model reactions were performed using relevant/reactive amino acids in the BPI to predict possible reactions that would occur in BPI hydrogels when TGase was added. Glutamine (5

mM) or asparagine was mixed with 5 mM lysine or histidine in 50 mM ammonium acetate buffer (pH 5.5). The reaction was initiated by adding transglutaminase (2 U) to the reaction mixture. The reactions were incubated for 1 h at 25°C. Thereafter, an equal volume of ice-cold methanol was added to each reaction and kept on ice for 20 min to precipitate TGase.

5.2.15. LC-MS analysis of reaction products

LC-MS analyses of reaction products were performed on a Shimadzu UHPLC system, coupled with a Shimadzu ESI mass spectrophotometer (Shimadzu, Kyoto, Japan) as previously described in section 4.2.11.

5.2.16. Statistical analysis

All experiments were conducted in triplicate except where stated. The data were analysed using analysis of variance (ANOVA) and mean differences compared using Fischer's Least Significant Differences Test ($p < 0.05$ and $p < 0.01$).

5.3. Results and discussion

5.3.1. Amino acid composition

BPI is a good source of amino acids such as aspartic acid, lysine and histidine (Table 5.1) with the amounts similar to those in soy protein (Adebowale *et al.*, 2011). Although the glutamic acid content was also high in BPI, it is lower than the amount in soy protein (Adebowale *et al.*, 2011). Glutamine and asparagine are derivatives of glutamic acid and aspartic acid, respectively. The high content of glutamic acid (including glutamine) and lysine in the Bambara groundnut protein suggests that when used in hydrogel formation, its structure can be stabilised by TGase-mediated crosslinking. This is because TGase has been proposed to catalyse the crosslinking reaction between the carboxamide group of glutamine (acyl donor)

and the amino group of lysine and other primary amines (acyl acceptor) (Hu *et al.*, 2015). Thiol group-containing amino acids like cysteine are also present in BPI, and these are involved in disulfide bond formation during TGase crosslinking (Wen *et al.*, 2018). Hence, the presence and abundance of these amino acids could favour TGase crosslinking of BPI, resulting in the formation of a structured network.

Table 5.1: Amino acid composition of Bambara groundnut protein isolate (g/100g)

Amino acid	g/100g	FAO/WHO children (2007)	FAO/WHO adults (2007)
Threonine	5.0	3.4	0.9
Lysine	6.1	5.8	1.6
Histidine	2.5	1.9	1.6
Methionine	1.2	2.7	1.7
Valine	4.4	3.5	1.5
Isoleucine	3.2	2.8	1.3
Leucine	6.9	6.6	1.9
Phenylalanine	6.1	6.3	1.9
Serine	4.3		
Arginine	7.2		
Glycine	2.8		
Tyrosine	2.9		
Aspartic acid	10.5		
Glutamic acid	15.5		
Alanine	3.8		
Proline	3.3		
Cysteine	1.4		

5.3.2. Free amino groups

Free amino groups significantly decreased in the presence of TGase (Figure 5.1), with the highest being observed in the heated BPI solution that did not have the enzyme. The reduction of the free amino group in the presence of TGase suggested the formation of glutamine-lysine isopeptide bonds. The covalent crosslinking (through glutamine-lysine isopeptide bonds) of peptide chains promoted the formation of the protein hydrogel (Wen *et al.*, 2018). The free amino groups in the BPI hydrogels decreased with increase in TGase activity, suggesting increased protein crosslinking. However, no significant decrease was observed after the addition of 20 U/g protein or 25 U/g protein TGase. This may be due to the exhaustion of amino acids such as lysine in protein chains of close proximity to those with the peptide or protein bound glutamine (Manhivi *et al.*, 2020). These results are in agreement with Wen *et al.* (2018), who reported a decrease in the free amino groups of bitter apricot kernel protein with increasing TGase activity up to 20 U/g. The reduction in the free amino groups suggests their involvement in the formation of peptides.

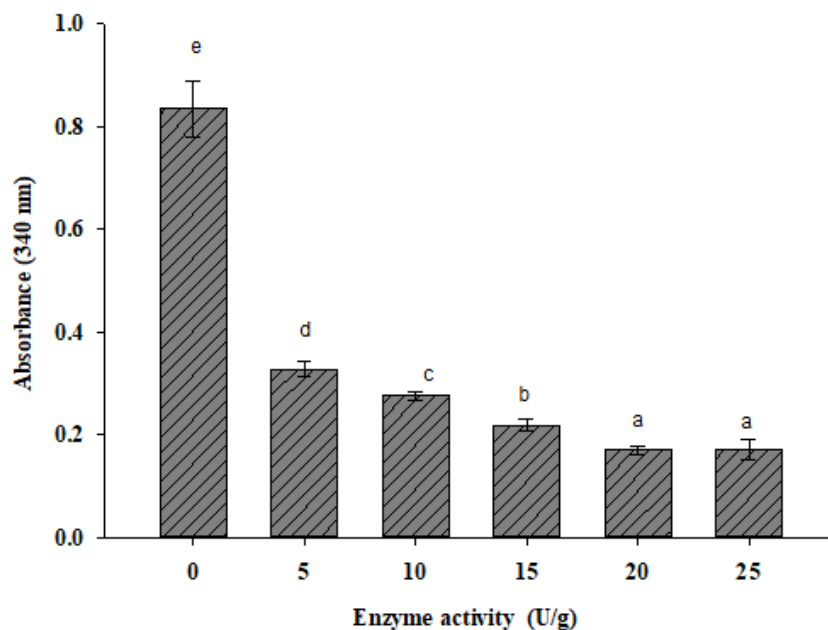


Figure 5.1: Effect of transglutaminase activity on the free amine content of Bambara groundnut protein isolate hydrogels. Values are means \pm SD (n=3). Bars with different letters differ significantly ($p < 0.05$).

5.3.3. Thiol content

The thiol content of the Bambara groundnut protein hydrogels decreased with increase in TGase activity (Figure 5.2). Heated Bambara groundnut protein solution with no TGase had the highest thiol content. This is because no TGase-induced crosslinking could take place in the absence of the enzyme. However, the use of TGase induced protein unfolding and conformational changes hence exposing some of the buried thiol groups resulting in the formation of inter- or intra-molecular disulfide bonds (Wen *et al.*, 2018) and thus, a decrease in the free thiol groups. The observed reduction could be further attributed to some of the free thiol groups being buried within the high molecular weight aggregates formed as a result of TGase-catalysed crosslinking; thus, they were no longer measurable (Zhang *et al.*, 2016).

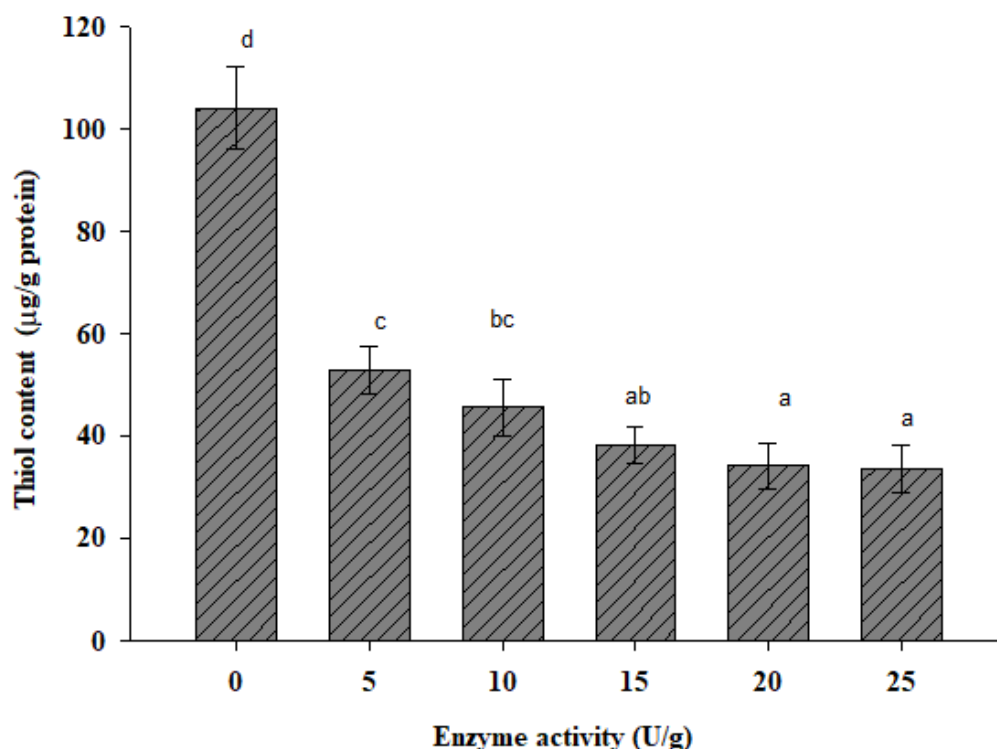


Figure 5.2: Effect of transglutaminase activity on the thiol content of Bambara groundnut protein isolate hydrogels. Values are means \pm SD (n=3). Bars with different letters differ significantly ($p < 0.05$).

5.3.4. SDS-PAGE

The heated BPI solution had prominent bands within the molecular weight range of 15 to 100 kDa (Figure 5.3a). The bands around 49, 70 and 80 KDa corresponded to the vicilin, convicilin and legumin, respectively. TGase was observed at a band with a molecular weight of approximately 38 kDa (Figure 5.3b). The addition of TGase activity > 10 U/g protein led to the disappearance of most of the low molecular weight bands, confirming that BPI is an excellent substrate for TGase crosslinking (Wen *et al.*, 2018). A new band was observed at 56 kDa upon the addition of ≥ 10 U/g protein TGase activity and this could suggest the crosslinking of low molecular weight proteins to form this band. Higher molecular weight bands of the BPI hydrogels were observed at the top of the separating gel, and this could be attributed to the bigger protein aggregates formed following TGase-induced intermolecular bond formation

(Djoullah *et al.*, 2018). The formation of high molecular weight bands confirms crosslinking and is in agreement with the reduction in free amine and thiol groups.

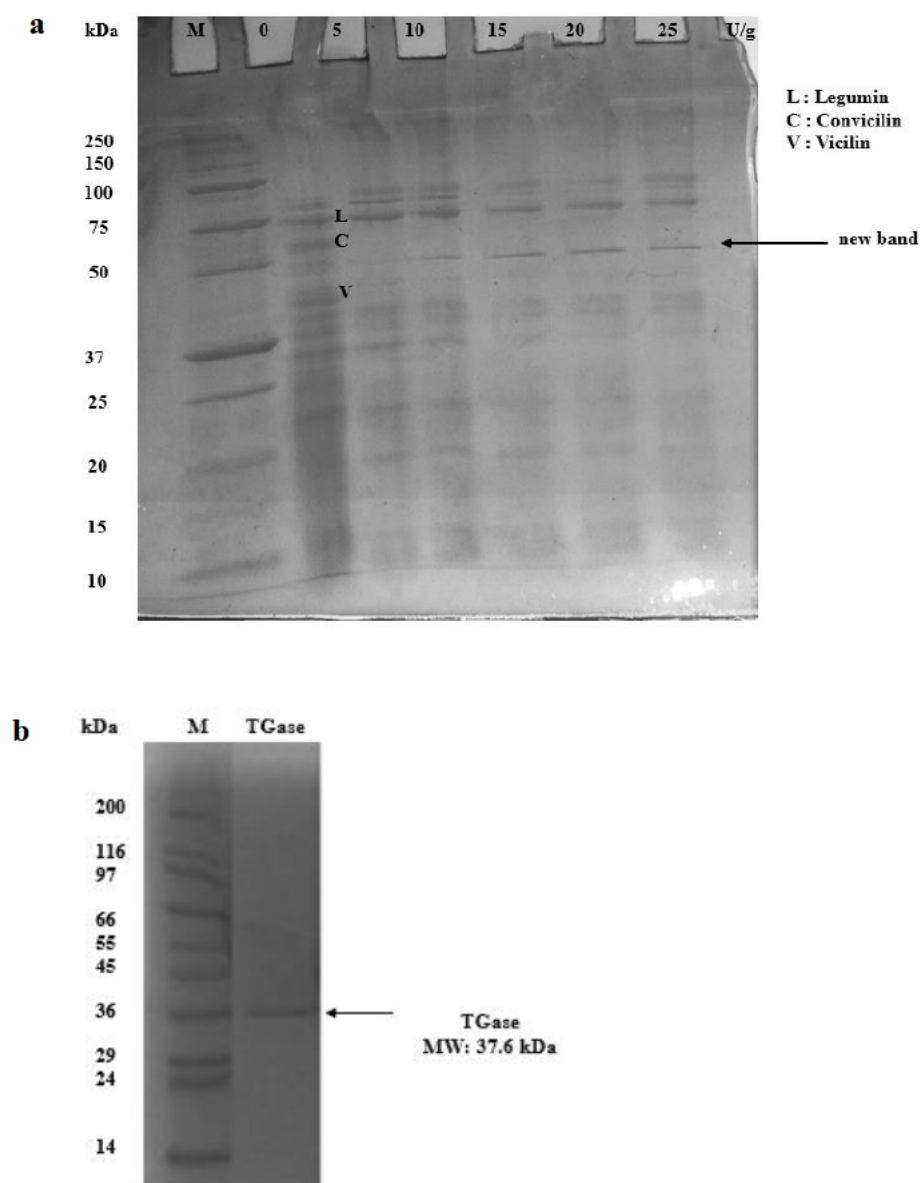


Figure 5.3: SDS-PAGE of (a) Bambara groundnut protein isolate hydrogels made at varying transglutaminase activity and (b) transglutaminase enzyme, Lane M: marker

5.3.5. FTIR

The FTIR spectra of TGase-treated BPI hydrogels showed broader peaks, corresponding to amide I ($1700\text{--}1600\text{ cm}^{-1}$) and amide II ($1600\text{--}1500\text{ cm}^{-1}$) regions, respectively (Figure 5.4). The BPI hydrogels had major peaks at 1633 cm^{-1} and within the $3207\text{ cm}^{-1}\text{--}3280\text{ cm}^{-1}$ range, which can be associated with the strong bending vibration of the -OH and -NH_2 of the peptide chains, respectively (Zaleska *et al.*, 2001).

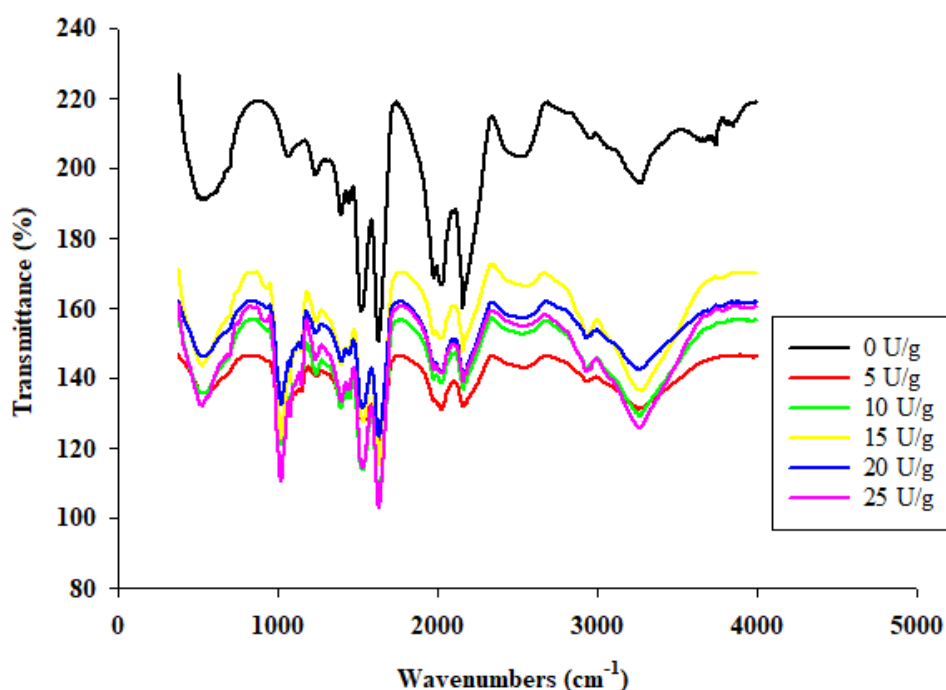


Figure 5.4: FTIR spectra of Bambara groundnut protein isolate hydrogels made at varying transglutaminase activities.

The deconvoluted amide I region (Table 5.2) of the infrared spectra was used to quantify the secondary structural changes of the BPI hydrogels (Carbonaro & Nucara, 2010; Wang *et al.*, 2017). Varying the TGase activity resulted in changes in the percentages of β -sheets, β -turns, α -helix, and random coils. This can be attributed to the rearrangement of the protein structure

after enzymatic crosslinking. Generally, the β -sheets increased with increase in the TGase activity from 5 U/g protein to 20 U/g protein, coupled with a decrease in the content of α -helixes suggesting the conversion of the α -helixes to β -sheets and increased protein crosslinking and subsequent aggregation (Carbonaro & Nucara, 2010). Protein unfolding, dissociation and rearrangement of Bambara groundnut protein molecules during TGase crosslinking could have caused the observed decrease in the α -helixes content (Wang *et al.*, 2017).

Table 5.2: Effect of transglutaminase activity on the percentage secondary structures of Bambara groundnut protein isolate hydrogels

Secondary structures		Enzyme		Activity (U/g)			
Peak range (cm ⁻¹)		0	5	10	15	20	25
β-sheets	1612-1614	52.45	50.56	39.00	53.52	56.46	47.94
	1629-1632						
	1670-1694						
β-turns	1662-1684	19.29	20.58	22.47	20.16	21.81	24.00
α-helix	1630	25.93	28.86	15.17	26.17	21.73	21.11
	1648-1660						
Random coil	1640-1650	12.33	0	23.38	0.14	0	16.95

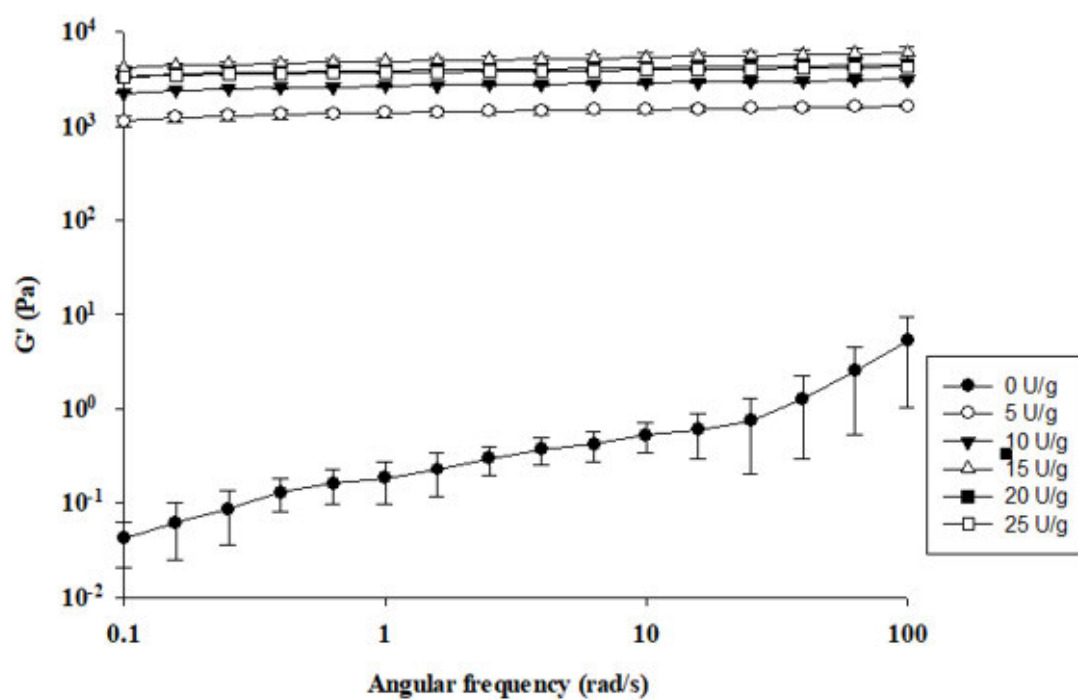
5.3.6. Effect of Transglutaminase activity on rheological properties of BPI hydrogels

The G' of the TGase-treated BPI hydrogels were more than ten-fold higher than the G'' values over the frequency range 0.1 - 100 rad/s (Figure 5.5), suggesting the dominance of elastic like behaviour. The large difference of more than tenfold in the G' and G'' values also indicate that

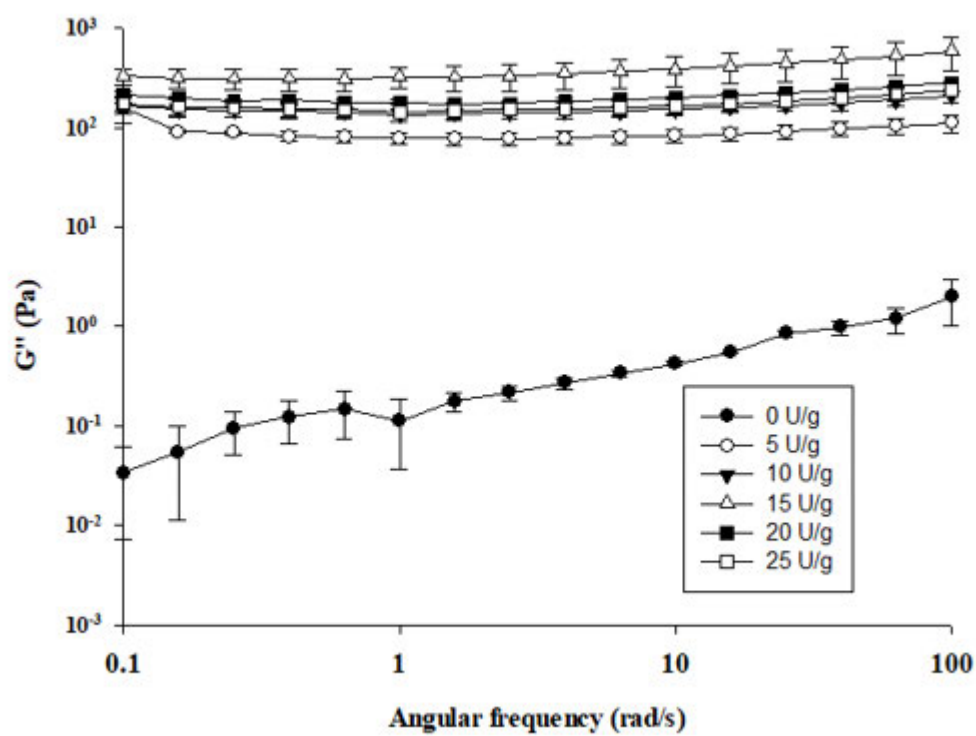
the hydrogel network was stable (Deng *et al.*, 2018). In addition, the two moduli had little dependence on the angular frequency, which suggests that the formed hydrogels were strong. The lowest G' value observed in the BPI heated solution with no enzyme was due to the viscous flow nature of the sample as no gel was formed in the absence of the enzyme. Upon the addition of TGase, crosslinks were formed within the Bambara groundnut proteins resulting in the formation of hydrogels and an increase in the G' . TGase-catalysed crosslinking results in the formation of covalent bonds within the hydrogel network (Hu *et al.*, 2015). The highest G' was observed at 15 U/g protein TGase activity, which is consistent with the observed SEM images (section 5.3.7). However, a further increase in TGase activity (≥ 20 U/g protein) caused a significant reduction in G' which can be attributed to the disorganised hydrogel network. The decrease in G' can also result from an increase in protein-protein interactions coupled with a decrease in water-protein interactions (Chen & Han, 2011) at high TGase activities.

The heated BPI solution had the highest $\tan \delta$ value (Figure 5.5C). TGase crosslinking of BPI hydrogels resulted in a decrease in the $\tan \delta$ values. The low $\tan \delta$ values recorded suggest the dominance of a more elastic than the viscous system in the hydrogels. Djoullah *et al.* (2018), reported the highest $\tan \delta$ value of less than 0.1 (recorded within a frequency range of 0.01 to 10 Hz) for pea protein gels formed using thermal pre-treatment and subsequent TGase crosslinking. These pea protein gels were further categorised as strong based on the $\tan \delta$ of <0.1 . In the present study, at an angular frequency of 10 rad/s, the $\tan \delta$ values were <0.1 hence further supporting that the TGase-crosslinked gels obtained were strong. Clark & Ross-Murphy (1987) defined a strong gel as a gel that has the G' and G'' parameters which are independent of the oscillatory frequency and with the $G' > 10G''$ ($\tan \delta < 0.1$).

A



B



C

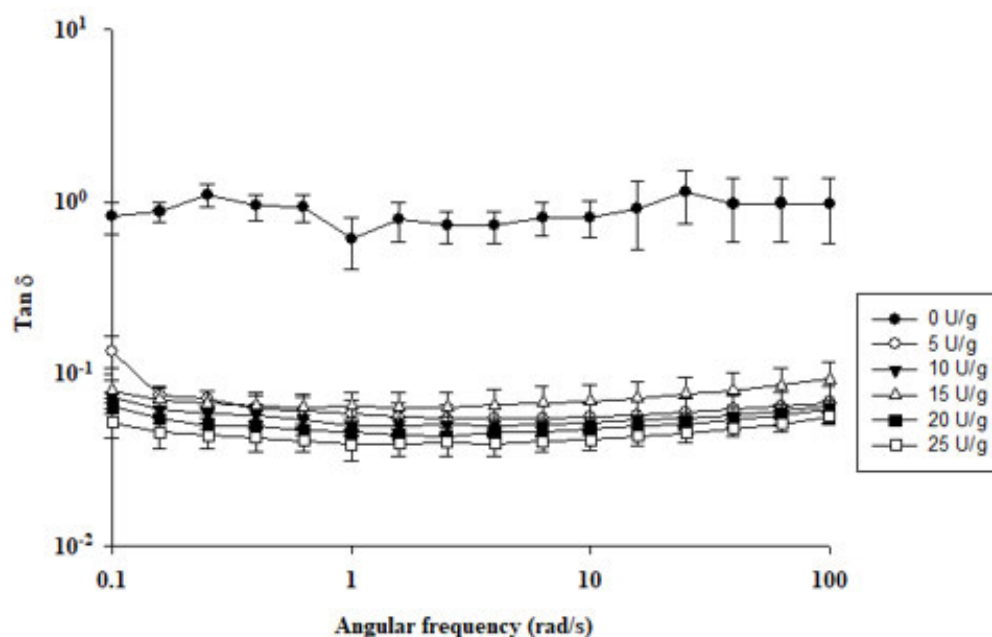


Figure 5.5: Effect of transglutaminase activity on (A) storage modulus (G'), (B) loss modulus (G'') and (C) $\tan \delta$ as a function of angular frequency of Bambara groundnut protein isolate hydrogels.

5.3.7. Texture analysis

The hardness of the BPI hydrogels increased as the TGase activity was increased from 5 to 15 U/g protein (Table 5.3). This highlighted that TGase increased the formation of BPI hydrogels with an organised and stable matrix (Wen *et al.*, 2018). The maximum hydrogel hardness was obtained at 15 U/g protein. The increase in the hardness could be attributed to the increased covalent bonds that are formed as a result of TGase crosslinking. Chen & Han (2011) reported that the glutamine-lysine isopeptide bonds are more than 20 times stronger than non-covalent bonds. There were no significant differences in the cohesiveness of the BPI hydrogels formed at the different enzyme activities (Table 5.3). Although the BPI hydrogels made at ≥ 20 U/g protein had the same cohesiveness as those made at 15 U/g protein, their hardness was significantly lower. High TGase activity (≥ 20 U/g protein) led to an increase in the protein-

protein interactions and decreased protein-water interactions, which results in water loss (Chen & Han 2011) and this could have contributed to the observed hydrogel cohesiveness and reduced hardness. The syneresis results (Table 5.4) obtained in the present study highlighted an increase in the water loss as the enzyme activity was increased. The reduction in the water holding capacity (Table 5.4) with increase in enzyme activity indicate a decrease in protein-water interactions. Furthermore, the reduction in the hardness could be attributed to the non-homogeneous hydrogel network as shown by the SEM images (section 5.3.7). Excessive crosslinking led to a disordered hairtail muscle protein gel network, thereby decreasing the hardness (Hu *et al.*, 2015).

Table 5.3: Effect of transglutaminase activity on hardness and cohesiveness of Bambara groundnut protein isolate hydrogels

Transglutaminase activity	Hardness (N)	Cohesiveness
5 U/g	2.93 ± 0.17^a	0.47 ± 0.0071^a
10 U/g	5.38 ± 0.97^{bc}	0.47 ± 0.0570^a
15 U/g	5.60 ± 1.54^c	0.52 ± 0.0035^a
20 U/g	4.22 ± 0.78^{abc}	0.52 ± 0.0035^a
25 U/g	3.95 ± 0.30^{ab}	0.52 ± 0.0280^a

Values are mean \pm SD (n=3); different superscripts within columns indicate significant differences ($p < 0.05$) among the different transglutaminase activities.

Table 5.4: Effect of varying transglutaminase activity on the water holding capacity and syneresis of Bambara groundnut protein isolate hydrogels.

Transglutaminase activity	Water holding capacity (%)	Syneresis (%)
5 U/g	86.58 ± 1.04^c	1.43 ± 0.33^a
10 U/g	84.83 ± 0.87^b	4.31 ± 0.45^b
15 U/g	84.86 ± 0.75^b	6.91 ± 1.15^c
20 U/g	81.25 ± 1.14^a	13.93 ± 1.28^d
25 U/g	81.45 ± 0.83^a	13.67 ± 1.32^d

Data is shown as mean \pm SD (n=3), different superscripts indicate significant differences ($p < 0.05$)

5.3.8. Scanning electron microscopy

The scanning electron microscope images of BPI hydrogels varied with the TGase activity added during hydrogel formation (Figure 5.6). A heterogeneous irregular matrix was observed for the heated BPI solution. However, the addition of TGase resulted in the improvement of the hydrogel matrix due to crosslinking. A more homogeneous, porous network with a smoother surface was observed upon the addition of 15 U/g protein. Wen *et al.* (2018) observed a denser and more homogeneous network as TGase activity was increased in apricot kernel protein gels. The addition of TGase activity >15 U/g protein in BPI hydrogels resulted in a disorganised BPI hydrogel matrix, which can be attributed to excessive crosslinking. The formation of BPI hydrogel with a disordered matrix could explain the decrease in the G' values and hardness recorded upon the addition of TGase activity >15 U/g. In the study by Guo *et al.* (2013), excess TGase caused the soy protein gel to contract after gelation, as well as a decrease in strength and decline in pore size homogeneity, thus, showing a less uniform network.

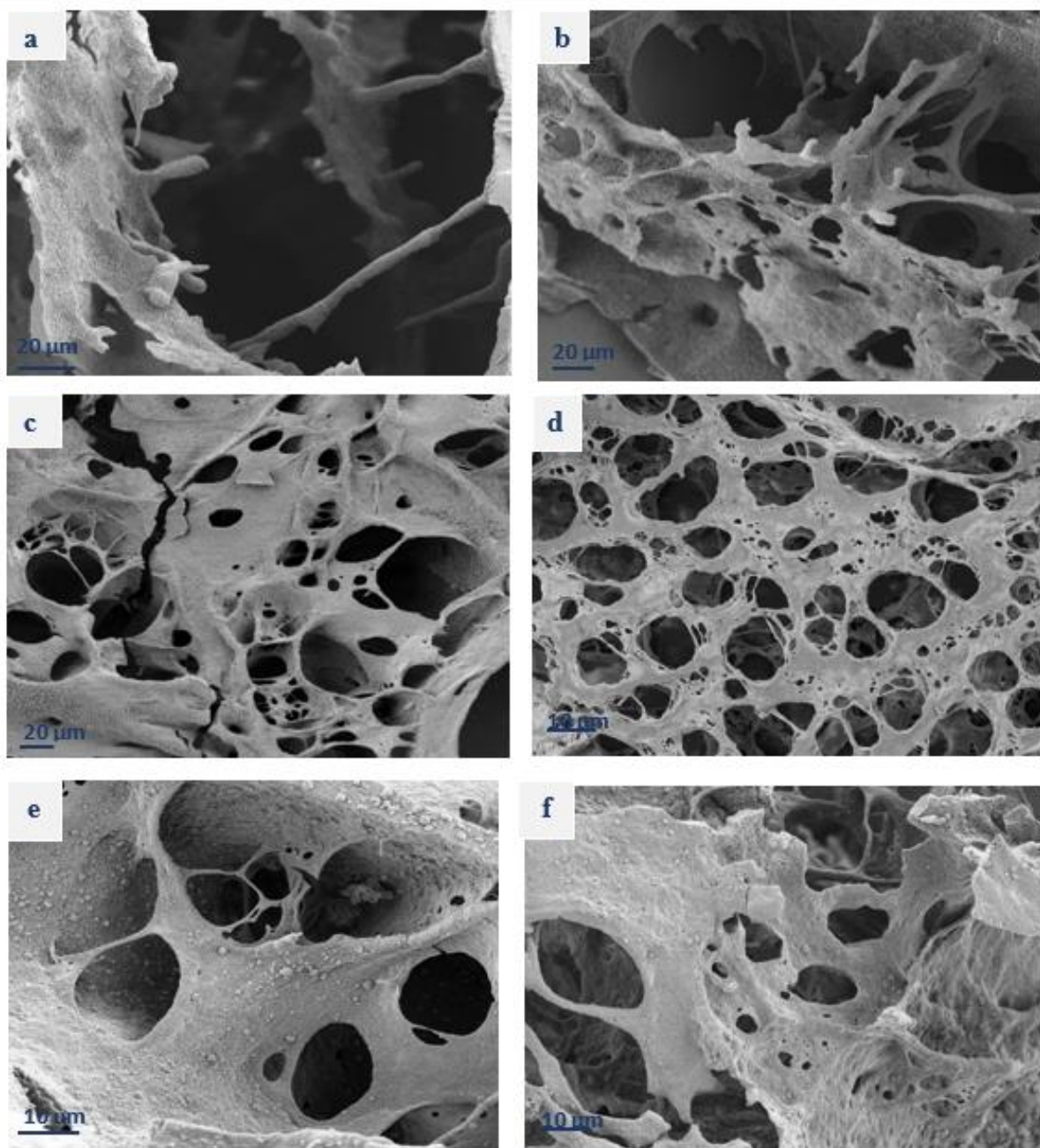


Figure 5.6: SEM micrographs of Bambara groundnut protein isolate hydrogels made at varying transglutaminase activities; (a) 0 U/g (b) 5 U/g (c) 10 U/g (d) 15 U/g (e) 20 U/g and (f) 25 U/g protein.

5.3.9. Model reactions

The amino acid profile informed the model reactions performed in this study. Therefore, the potential of TGase to crosslink BPI was studied using model amino acids; glutamine an acyl

donor and lysine an acyl acceptor. Products of the reactions were analysed by mass spectrometry (MS). TGase-catalysed reaction between glutamine and lysine resulted in the formation of a crosslinked peptide (m/z $[M-H]^- = 273$) linked through a (glutamine) lysine isopeptide bond (Figure 5.7). Lysine ϵ -amino group react with the side chain amide group of glutamine resulting in the formation of ϵ -(γ -glutamyl) lysine (Manhivi *et al.*, 2020). The acyl donor capacity of asparagine as well as the acyl acceptor capability of histidine were not observed. This could be because glutamine and lysine are the most preferred substrates for catalysis by TGase. These results suggested that TGase can catalyse crosslinking reactions of Bambara groundnut protein which could explain changes in hydrogel properties described in this study. In essence, these model reactions indicate protein crosslinking.

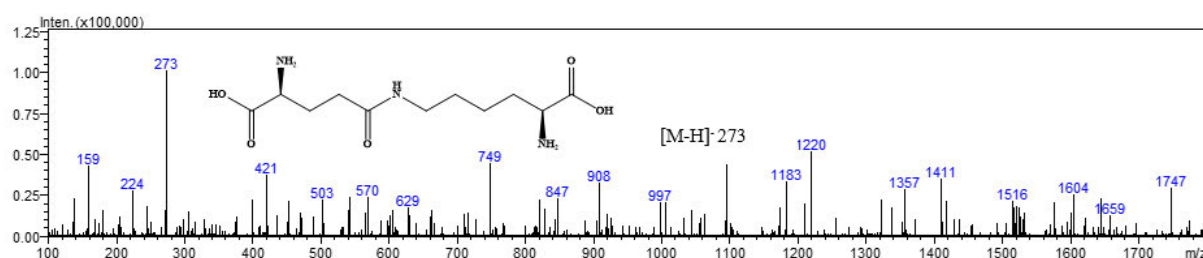


Figure 5.7: Mass spectra and proposed structures of glutamine-lysine crosslinked peptides formed through transglutaminase treatment.

5.4. Conclusion

TGase promoted the formation of an organised network with better hydrogel strength. TGase activity up to 15 U/g protein could be recommended as further increase had no major effect on the hydrogel strength. The structure of the network is TGase activity-dependent up to 15 U/g protein with the treated hydrogel displaying numerous smaller-sized pores and a homogeneous network. Transglutaminase mediated the crosslinking of BPI hydrogel, as demonstrated by a reduction in amine and thiol groups and SDS PAGE. Transglutaminase crosslinking can,

therefore, be used to improve the rheological, textural and microstructural properties of BPI hydrogels. The approach used in the present study enabled gel formation at low temperatures thus the produced hydrogels can potentially be used for the encapsulation of heat-sensitive bioactive compounds.

CHAPTER SIX

Effect of combining laccase and transglutaminase on rheological, textural, encapsulation and delivery properties of Bambara groundnut protein hydrogels

Abstract

Dual crosslinking strategies designed to increase crosslinks between interacting protein molecules have recently been gaining a lot of scientific interest. In this study, the effect of combining laccase (0 -1 U/g protein) and transglutaminase (TGase) (0 – 15 U/g protein) crosslinking on the rheological, textural, encapsulation and delivery properties of Bambara groundnut protein isolate (BPI) cold set induced hydrogels were investigated. BPI hydrogels produced with TGase only or a combination of TGase and laccase showed a $G' > 10G''$ over a frequency range of 0 – 100 rad/s suggesting the dominance of the elastic behaviour. BPI hydrogel with the highest hardness was formed at 15 and 0.5 U/g protein of TGase and laccase activities, respectively. This hydrogel had the highest riboflavin encapsulation efficiency of 98.8%. Furthermore, the hydrogel had the lowest swelling capacity attributed to the lowest release kinetic constants in both simulated gastric fluid and simulated intestinal fluid, in the presence of digestive enzymes, which indicated that riboflavin release was due to diffusion and swelling. TGase and laccase crosslinking can be successfully used for the production of Bambara groundnut protein cold-set hydrogels for encapsulating and preventing the early release of heat sensitive compounds in the stomach while making them available in the small intestines.

6.1. Introduction

Bioactive compounds also known as nutraceuticals are substances that have been reported to have a physiological benefit. These substances possess anti-oxidative, anti-inflammatory, anti-

hyperlipidemic and antihypertensive properties hence they offer protection against noncommunicable diseases and delay the ageing process (Aditya *et al.*, 2017). An example of such bioactive compounds is riboflavin which protects against cardiovascular diseases and cancers (Powers, 2003). Although riboflavin has been shown to be involved in a number of metabolic reactions, humans are not able to synthesise it. Hence, it is supposed to be obtained as a nutrient via intestinal absorption (Yoshimatsu *et al.*, 2014). Delivery systems have therefore been designed to protect such bioactive compounds against conditions in the gastrointestinal tract and control their retention, stability and release within the specific targeted locations (McClements, 2017).

The most widely used delivery systems are hydrogels. Hydrogels are hydrophilic, three-dimensional network polymers with the ability to absorb large amounts of water and biological fluids while maintaining their structure (Bahram *et al.*, 2016). Hydrogels can trap, protect the entrapped compound from the environmental conditions in the gastrointestinal tract and deliver at the targeted site (Yan *et al.*, 2020). Natural polymer-based hydrogels, especially from plant proteins have recently gained attention in delivery applications due to their biocompatibility and biodegradability (Wen *et al.*, 2018). As such this has led to the increased utilisation of plant proteins especially soybean protein (Guo *et al.*, 2013; Hu *et al.*, 2015). There is high demand for soy protein in several applications which has led to the search for alternatives with similar or even better functional properties. Bambara groundnut (*Vigna subterranea*) is an alternative source of protein (19- 25%) (Arise *et al.*, 2017). Studies on the gelation of Bambara groundnut proteins have shown that like with all other plant proteins the hydrogels formed are composed of weak network structures (chapter 3; Diedericks *et al.*, 2020). Hence, limiting their applications as encapsulation materials for delivery purposes. Thus, the need for improving these hydrogel network structures.

Crosslinking enzymes such as transglutaminase (TGase) and laccase form covalent bonds between molecules resulting in the formation of firm network structures within hydrogels. Apart from the improvement in strength, TGase crosslinking has also been reported to significantly improve the release properties of soy protein hydrogels (Song & Zhang, 2008; Hu *et al.*, 2015). In the previous studies, the homogeneity of network structures and the strength of Bambara groundnut protein heat induced gels and cold set induced hydrogels were successfully improved using laccase (chapter 4) and TGase (chapter 5), respectively. However, the hydrogel properties can be enhanced for use in the release of bioactive compounds like riboflavin. Since TGase is a highly specific enzyme, the use of a multiple enzyme system can be a way of increasing the crosslinking density within the protein molecules. The enzyme laccase has a broad range substrate specificity, combining it with TGase has potential to increase the crosslinks within protein hydrogels. Therefore, the current study investigated the effect of combining TGase and laccase crosslinking on rheological, textural, encapsulation and release properties of Bambara groundnut protein hydrogels.

6.2. Materials and methods

6.2.1. Materials

Transglutaminase (Activa TG-S-NF, 87 U/g) was kindly donated by Maccallum & Associates (Durban, South Africa; representing Ajinomoto). *Trametes versicolor* laccase, riboflavin and all other chemicals were purchased from Sigma-Aldrich, South Africa. Bambara groundnuts were purchased from Jozini, KwaZulu-Natal (KZN) province, South Africa.

6.2.2. Extraction of Bambara groundnut protein isolate

Bambara groundnut protein isolate with a protein content of 90 g/100g as determined by Bradford method (Bradford, 1976) was extracted using the modified isoelectric precipitation method (Boye *et al.*, 2010) previously described in section 3.2.3.

6.2.3. Experimental design

The effect of enzymatic crosslinking of BPI in the production of hydrogels was optimised by response surface methodology (RSM) using central composite design (CCD). The independent variables were laccase (0 – 1 U/g) and TGase (0 – 15 U/g). The enzyme activity levels were determined from studies described in the previous chapters 4 and 5. Since laccase is a more robust enzyme an activity slightly lower than the optimal observed in chapter 4 was chosen so as to prevent the cleavage of the covalent bonds and a subsequent reduction in the hydrogel strength. Also, a different method from that used in chapter 4 (heat set method) was used for this study (cold set method) hence a slightly lower laccase activity was chosen. The dependent variable was G' derived from frequency sweep measurement data. Table 1 summarises the experimental design (13 runs) with coded levels. These were generated by statistical software Design Expert version 11 (StatEase Inc., USA). Each experimental run was prepared in a randomised order. A quadratic polynomial model regression (Equation 1) was used to describe system behaviour.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i X_j + \varepsilon \dots\dots\dots \text{Equation (1)}$$

where Y is the response variable, β_0 is a constant; β_i is the linear and β_{ij} the interactive coefficient; X_i is the level of the independent variable and ε is the random error. The

optimisation objective was to maximise G' of the enzyme-induced BPI hydrogels. Design Expert 11 (StatEase Inc., USA) was used for numerical optimisation and this was found at a point that maximises the desirability function.

Table 6.1: Process variables used in the central composite design for Bambara groundnut protein isolate hydrogel preparation.

Factors	Coded (Xi)				
	$-\alpha$	-1	0	+1	$+\alpha$
Transglutaminase	0	0	7.5	15	18.11
Laccase	0	0	0.5	1	1.21

6.2.4. Preparation of Bambara groundnut protein isolate hydrogels

BPI hydrogels were prepared using the method described in section 5.2.4 with minor modifications. BPI solutions of 10% (w/w) were prepared in 0.5 M NaCl solution and pH was adjusted to pH 6 using 1 M NaOH. Centrifugation was then done for 5 min at $4000 \times g$. Thereafter the supernatant was placed in falcon tubes and partially denaturated by heating in a water bath at 95°C for 10 min. Protein solutions were then cooled rapidly under cold running water and enzymes were added at different activities according to the experimental design (Table 6.1). The samples were stored at 4°C in the cold room overnight after 1 h incubation at 35°C . Rheological and textural analysis were carried out on the wet gel. BPI hydrogels samples for the swelling capacity were frozen at -80°C in a Bio freezer (Evosafe series VF720-86, Snijders labs, Netherlands), freeze-dried using a Christ freeze drier (Germany) prior to the analysis.

6.2.5. Rheological properties of enzyme-induced Bambara groundnut protein hydrogels

The frequency sweep tests of the enzyme-induced BPI hydrogels were determined using an MCR102 Rheometer (Anton Paar, Austria), as previously described by Wu *et al.* (2017). The

measuring system used comprised of a parallel plate (25 mm diameter, 1 mm gap). Constant temperature of 25°C and 0.5% strain within the linear viscoelastic region was used for the analysis. Frequency sweep tests were performed between 0.1 and 100 rad/s and the storage modulus (G'), loss modulus (G'') and $\tan \delta$ values were recorded.

6.2.6. Verification and selection for further analysis

The optimum gel, G' actual values were compared to the predicted values for verification purposes at 95% confidence interval. Seven experimental conditions were then chosen from the RSM data for preparation of samples for further analysis.

6.2.7. Textural properties

The texture analyses of BPI hydrogels were measured as described in section 5.2.10 using a 100 N trigger force.

6.2.8. Swelling properties

The swelling properties of the enzyme-induced BPI hydrogels were measured in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) (without digestive enzyme) as the swelling media as described by Caillard *et al.* (2009) with minor modifications. SGF and SIF were prepared in accordance to the United States Pharmacopeial Convention (2004). SGF (pH 1.2) was made up of 2 g NaCl, 7 ml of 37% hydrochloric acid and 1000 ml of double-distilled water. SIF was prepared using 6.8 g of monobasic potassium phosphate dissolved in 250 ml of double-distilled water, 190 ml of 0.2 M NaOH and 400 ml of double distilled water. The final volume of the SIF was brought to 1000 ml with double distilled water after pH was adjusted to 7.5 using 0.2 M NaOH.

Approximately 20 mg freeze-dried BPI hydrogels were immersed in 20 ml of swelling medium. BPI hydrogels were briefly blotted at several time intervals and weighed. Swelling ratio (Sr) was calculated from the difference in mass before and after immersion, and the swelling ratio expressed as a percentage as follows:

$$\text{Sr \%} = \frac{B-A}{A} \times 100$$

where B and A is the hydrogel weight after and before swelling, respectively.

6.2.9. Encapsulation of riboflavin into enzyme-induced Bambara groundnut protein isolate hydrogels

Enzyme-induced BPI hydrogels containing riboflavin were prepared as described above (section 6.2.4) with minor modifications. After heating to partially denature the protein, the BPI solutions were then cooled rapidly under running cold water and riboflavin was added to a final concentration of 0.0048 mg/ml. Different enzyme activities were added according to the experimental design (Table 1). The samples were wrapped with aluminium foil to protect from light and minimise light-induced deterioration of riboflavin. Thereafter, the samples were incubated at 35°C for 1 h and left at 4°C in the cold room overnight. Enzyme-induced BPI hydrogels encapsulated with riboflavin were frozen at -80°C in a Bio freezer (Evosafe series VF720-86, Snijders labs, Netherlands), freeze-dried using a Christ freeze drier (Germany) and kept at 4°C until further analysis.

6.2.10. Determination of riboflavin loading of enzyme-induced Bambara groundnut protein isolate hydrogels

The amount of riboflavin encapsulated in the BPI hydrogels was determined by following the method previously described by Hu *et al.* (2015) with minor modifications. Freeze-dried BPI

hydrogels were ground into powder and approximately 25 mg weighed. Weighed samples were hydrolysed each in 25 ml of SIF containing 10 g/L pancreatin at 37°C and pH 7.5 with vigorous agitation for 6 h. The resulting mixture was centrifuged at $8\,000 \times g$ for 20 min at room temperature. A standard curve prepared from absorbance measurements in a UV-visible spectrophotometer (at 445 nm) was used to determine the riboflavin concentration in the supernatant. The encapsulation efficiency (EE) of the riboflavin was calculated as follows:

$EE = \frac{R_h}{R_t} \times 100$, where R_h is the amount of riboflavin encapsulated in the hydrogels and R_t is the total amount of riboflavin

6.2.11. In-vitro release of riboflavin from enzyme-induced Bambara groundnut protein isolate hydrogels

Encapsulated BPI hydrogels were immersed into SGF and SIF medium (with or without enzymes) in 50 ml tubes. Pepsin (3.2 g/L) and pancreatin (10 g/L) were added to SGF and SIF, respectively (Maltais *et al.*, 2009). Samples were incubated at 37°C for 2 h in SGF with and without pepsin and 6 h in SIF with and without pancreatin in a shaking incubator set at 100 rpm. The amount of riboflavin released was measured every 15 min for 1 h followed by every 30 min when in SGF. In SIF, riboflavin released was measured every 15 min for 1 h followed by 30 min intervals for a period of 1 h and then 1 h intervals until the end of the 6 h (Maltais *et al.*, 2009; Wen *et al.*, 2018).

To simulate the succession of pH conditions and enzymatic activities occurring during the digestive process, enzyme-induced BPI hydrogels were first subjected to gastric conditions with pepsin (pH 1.2) for 30 min. The fluid was immediately changed to the intestinal medium with pancreatin (pH 7.5), in which the dissolution was followed for 5.5 h (Maltais *et al.*, 2009).

The amount of riboflavin released into the media was determined from absorbance measurements at 445 nm.

$$\% \text{ riboflavin release} = \frac{[\text{riboflavin}]_{\text{released}}}{[\text{riboflavin}]_{\text{total}}} \times 100$$

where $[\text{riboflavin}]_{\text{total}}$ is the amount loaded in each BPI hydrogel and $[\text{riboflavin}]_{\text{released}}$ is the amount of riboflavin measured in the dissolution medium.

The kinetics of riboflavin release from the hydrogel matrix was evaluated using the Ritger-Peppas equation (Ritger & Peppas, 1987): $\frac{M_t}{M_\infty} = k t^n$

where M_t/M_∞ is the fraction of riboflavin released at time t relative to the fraction released at infinite time, k is a kinetic constant and n is a diffusional exponent.

6.2.12. Statistical analysis

All experiments were conducted in triplicate except where stated. The data was analysed using analysis of variance (ANOVA) and mean differences compared using Fischer's Least Significant Differences Test ($p < 0.05$ and $p < 0.01$).

6.3. Results and Discussion

6.3.1. Effect of enzymes on the rheological properties of Bambara groundnut protein isolate hydrogels

BPI hydrogels were formed from the treatment of protein solutions with either TGase only or a combination of TGase and laccase. No gels were formed from samples without any enzyme and those treated with laccase only. G'' values of the BPI dispersions without any enzyme and those treated with laccase only were higher than the G' values indicating the dominance of the viscous flow nature of the samples (data not shown). The maximum G' values were 0.18 Pa,

1.53 Pa and 0.5 Pa for BPI dispersions with laccase activity of 0, 0.5 and 1 U/g protein, respectively. These results suggested that laccase causes the crosslinking of protein molecules resulting in the increase in G' however, the crosslinking is not sufficient to form a hydrogel. A further increase in the laccase activity (1 U/g protein) caused a decrease in the G' values thus indicating the weakening of the BPI structure which could be attributed to the fragmentation of the protein molecules at high enzyme dosages (Lannto *et al.*, 2005).

The G' values of the TGase only or a combination of TGase and laccase-treated BPI hydrogels were more than ten-fold higher than the G'' values over the frequency range 0.1 – 100 rad/s (Figure 6.1 a & b). Such a difference indicated both the dominance of an elastic-like behaviour and that the BPI hydrogel formed had a stable network (Deng *et al.*, 2018). G' and G'' moduli values had a slight dependence on the angular frequency indicating the hydrogels formed were strong. A strong gel must have G' and G'' parameters which are independent of the angular frequency and the $G' > 10G''$ (Clark & Ross-Murphy, 1987).

Low G' values were recorded in the BPI hydrogel treated with TGase activity of 7.5 U/g protein in the absence of laccase. However, the combined effect of TGase activity of 7.5 U/g protein and laccase activity of 0.5 U/g protein resulted in an increase in the G' values. Such an increase in the G' could be attributed to the combined effect of laccase and TGase crosslinking. Crosslinking of amino acids such as glutamine and lysine catalysed by TGase and cysteine, glutathione and tyrosine catalysed by laccase was reported in the previous chapters (chapter 4 and 5). Increased crosslinking could have increased the network structure and subsequently the strength of the formed hydrogels. A further increase of the laccase activity to 1.21 U/g protein (at the same TGase activity of 7.5 U/g protein) caused a significant reduction in the G' values of the formed BPI hydrogels. The BPI hydrogel with the highest G' was obtained at TGase

activity of 15 U/g protein in the absence of laccase. Although the combined use of TGase and laccase activities of 15 and 0.5 U/g protein caused a significant reduction in the G' values, a decrease in the $\tan \delta$ values was observed (Figure 6.1 c) suggesting the increase in the elasticity of the hydrogels upon the combined treatment. The $\tan \delta$ values were < 0.10 highlighting that a more elastic than the viscous behaviour was dominant in the BPI hydrogels. $\tan \delta < 0.10$ in hydrogels further suggest that the formed gels are strong (Clark and Ross-Murphy, 1987).

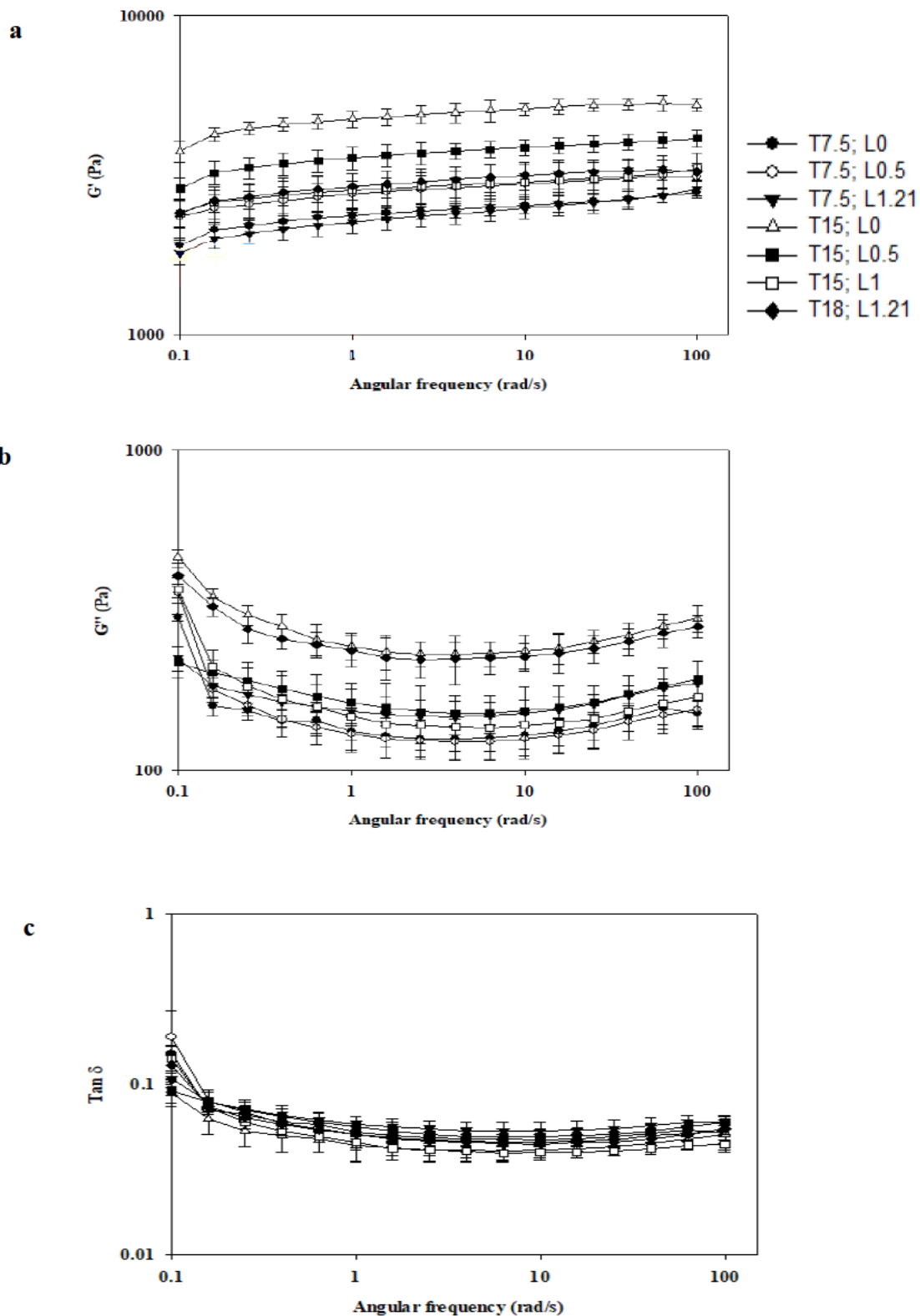


Figure 6.1: Frequency sweep properties of Bambara groundnut protein isolate hydrogels made with varying activities of transglutaminase and laccase (a) Storage modulus (G') (b) Loss modulus (G'') and (c) Tan δ .

6.3.2. Model analysis

The quadratic model p-values for G' was 0.0001 suggesting that the model was significant ($p < 0.05$) and adequate to describe the combined effect of crosslinking enzymes (TGase and laccase) on G' parameter measured from the frequency sweep. The lack of fit was not significant ($p > 0.05$) indicating that the model fitted the experimental data well. The independent variables: TGase, laccase and their combination had significant effects on G' ($p < 0.05$) (Table 6.2). The coefficient of determination, R^2 was then used to describe the quality of the polynomial model equation. The R^2 (0.96) was very high, the adjusted R^2 and predicted R^2 were very close and the difference between them less than 0.2. The R^2 values indicated that the model had a high probability of representing the whole sample.

Table 6.2: Quadratic model for G' and linear model regression coefficients for rheological properties of BPI hydrogels.

Coefficients	G'	p-value
Model		0.0001
Intercept	2846.39	
A- TGase	+2535.65	< 0.0001
B- Laccase	-555.53	0.0387
AB	-906.27	0.0139
A^2	-432.16	0.1316
B^2	+181.10	0.4975
Actual R^2	0.96	
Adjusted R^2	0.93	
Predicted R^2	0.76	
Lack of fit		0.2394

The G' responses of the enzymatic crosslinking of BPI are presented in Figure 6.2 as 3-D response surface plots. TGase showed a stronger positive influence in relation to G' increase, with a steady upsurge observed upon increase in the enzyme activity. Laccase on the other hand showed the least significant effect on the G' despite the increase in the enzyme activity as observed from the relatively flat curve. The most pronounced effect of a combination of the enzymes on the G' of the formed BPI hydrogels was observed at high TGase activity approximately 15 U/g protein and low laccase activity close to 0 U/g protein.

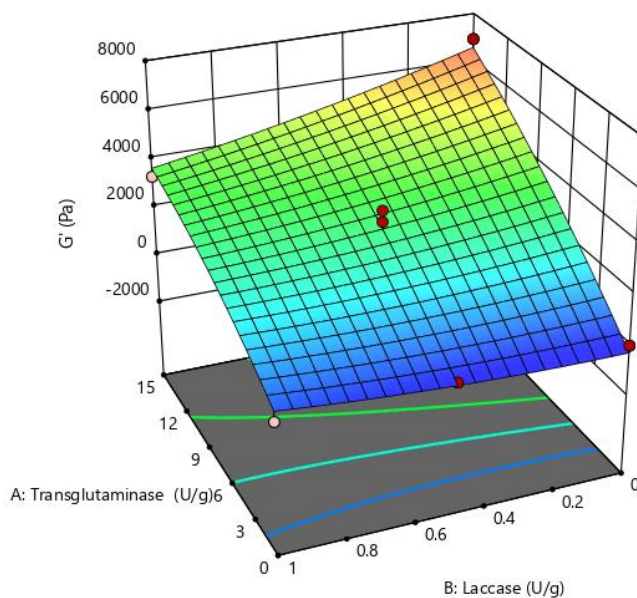


Figure 6.2: 3-D surface plots showing the interaction effects of transglutaminase and laccase on the storage modulus (G') of Bambara groundnut protein isolate hydrogels.

The optimisation goal was to maximise G' of the BPI hydrogels. CCD used for the optimisation predicted that the maximum G' with a desirability of 0.95 will be observed from the hydrogels made with TGase activity of 15 U/g protein and in the absence of laccase. The model predicted the maximum G' of 6593.79 Pa and this value was close to the actual value 6947 Pa measured from the frequency sweep analysis.

6.3.3. Textural properties

The textural properties of the BPI hydrogels were investigated to determine their hardness. Since the frequency sweep results had shown that the highest G' was obtained from the gel made with 15 U/g protein TGase activity in the absence of laccase and the lowest $\tan \delta$ values were recorded in the gels made with combined TGase and laccase, further analysis was done on 7 different combinations including the TGase and laccase activity of 15 and 0.5 U/g protein, respectively which was not part of the RSM optimisation model analysis.

The combined treatment of BPI dispersions with laccase activity of 0.5 U/g protein and TGase activities 7.5 and 15 U/g protein resulted in BPI hydrogels with higher hardness (Table 6.3) as compared to those without laccase. A further increase in the laccase activity to ≥ 1 U/g protein at the same TGase activities resulted in a significant reduction in the hardness of the BPI hydrogels. This could be attributed to gel weakening due to cleavage of covalent bonds at high enzyme dosages (Lancto *et al.*, 2005). The highest hardness was observed in the BPI hydrogel made with TGase and laccase activity of 15 and 0.5 U/g protein, respectively. Increased covalent bonds such as the glutamine-lysine isopeptide bonds and disulphide bonds resulting from the enzymatic crosslinking could have caused the increase in the hardness of the BPI hydrogels.

Table 6.3: Effect of enzyme activities on the hardness of Bambara groundnut protein isolate hydrogels crosslinked with transglutaminase and laccase

Transglutaminase activity (U/g)	Laccase activity (U/g)	Hardness (N)
7.5	0	9.78 ± 0.40 ^a
7.5	0.5	11.85 ± 0.57 ^b
7.5	1	9.19 ± 0.10 ^a
15	0	13.22 ± 0.89 ^b
15	0.5	15.96 ± 0.69 ^c
15	1	9.14 ± 0.07 ^a
18.11	0.5	8.83 ± 0.84 ^a

Values are mean ± SD (n=3); different superscripts indicate significant differences ($p < 0.05$) among the different enzyme activities.

6.3.4. Swelling properties

The behaviour of the swelling properties of the BPI hydrogels at pH 1.2 and pH 7.5 were influenced by the enzyme combination and activity (Figure 6.3). Water uptake was very rapid within the first 15 min in both SGF and SIF for all the samples and thereafter an equilibrium was reached. The swelling of the BPI hydrogels was higher in SIF (pH 7.5) than in SGF (pH 1.2). This could be attributed to the high content of carboxylic acid groups available in BPI contributed by aspartic and glutamic residues reported to be 10.5 g/100g and 15.5 g/100g respectively in chapter 5. During swelling in SGF the carboxylic groups of Bambara groundnut protein were under acidic conditions (pH 1.2) and they remained unionised than when compared to the SIF conditions. The osmotic pressure and electrostatic repulsion of the Bambara groundnut protein molecules were therefore reduced thus resulting in a lower swelling (Maltais *et al.*, 2010). However, in the SIF, the carboxylic group of BPI hydrogels

were in dissociated or ionised state and the osmotic pressure inside the hydrogel increased due to the increased concentration of ions in the form of H^+ and $-COO^-$, hence, promoting the uptake of water. Increase in the swelling is associated with the macromolecular chain repulsion due to the electrostatic repulsion between the negatively charged carboxylic groups of BPI (Hu *et al.*, 2015).

The combination of TGase and laccase resulted in a decrease in the swelling properties of the BPI hydrogels. The swelling properties of BPI hydrogels made with combined enzymes, TGase activity of 7.5 or 15 U/g protein and laccase activity 0.5 U/g protein was lower than those with TGase only. This reduction could be due to increased crosslinking and changes in the 3D structure of BPI hydrogels. According to Caillard *et al.* (2010), the swelling of a crosslinked protein hydrogel strongly depends on the network forces of the crosslinks that counterbalance the swelling forces. Previous studies have also reported decreased hydrogel swelling upon increased crosslinking (Caillard *et al.*, 2010; Hu *et al.*, 2015). At higher laccase activities ≥ 1 U/g protein the swelling properties of the BPI hydrogels increased, and this could be linked to cleavage of covalent bonds that usually predominate at high laccase activity due to the large amounts of radicals formed.

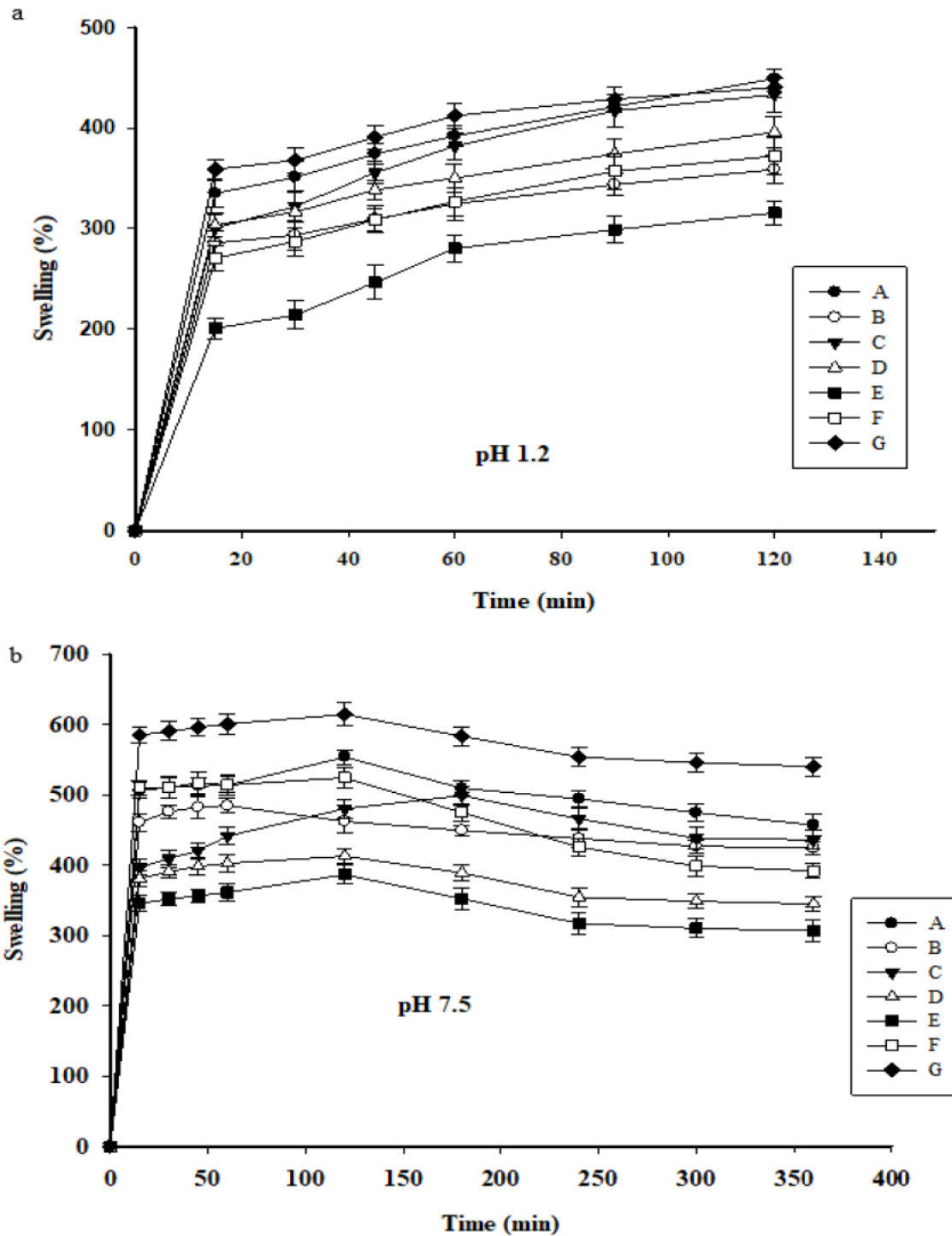


Figure 6.3: Swelling of Bambara groundnut protein isolate hydrogels made with varying activities of transglutaminase and laccase in a) SGF (pH 1.2) and b) SIF (pH 7.5). Values are means \pm SD (n=3). Bars with different letters differ significantly ($p < 0.05$). A. TGase 7.5 U/g & Laccase 0 U/g B. TGase 7.5 U/g & Laccase 0.5 U/g C. TGase 7.5 U/g & Laccase 1.21 U/g D. TGase 15 U/g & Laccase 0 U/g E. TGase 15 U/g & Laccase 0.5 U/g F. TGase 15 U/g & Laccase 1 U/g G. TGase 18.11 U/g & Laccase 0.5 U/g.

6.3.5. Encapsulation efficiency

The use of a combination of enzymes, TGase activities 7.5 or 15 U/g protein and laccase activity of 0.5 U/g protein improved the encapsulation efficiency of the BPI hydrogels (Table 6.4). Hence the BPI hydrogel made with a combination of TGase activity of 15 U/g protein and laccase activity of 0.5 U/g protein had the highest encapsulation efficiency. Further increase in the laccase activity to ≥ 1 U/g protein caused a decrease in the encapsulation efficiency. The increase in the encapsulation efficiency is related to the improved covalent interactions and the dense network structure formed in the presence of both enzymes. Riboflavin has been reported to be a low-molecular weight and partially water-soluble vitamin with no strong interactions with proteins (Chen & Subirade, 2009). Improved covalent interactions and the dense network structure that is formed stabilise the protein-riboflavin solution systems, thus allowing more riboflavin to be encapsulated into the BPI hydrogels (Wen *et al.*, 2018).

Table 6.4: Encapsulation efficiency of riboflavin in Bambara groundnut protein isolate hydrogels crosslinked with transglutaminase and laccase at varying activities

Enzyme activity		Encapsulation Efficiency
TGase	Laccase	
(U/g protein)	(U/g protein)	(%)
7.5	0	97.5 ± 0.1 ^d
7.5	0.5	98.1 ± 0.0 ^e
7.5	1.21	94.5 ± 0.0 ^b
15	0	98.2 ± 0.0 ^e
15	0.5	98.8 ± 0.0 ^f
15	1	95.1 ± 0.0 ^c
18.11	0.5	94.2 ± 0.0 ^a

Values are means ± SD (n=3); different superscripts indicate significant differences ($p < 0.05$) among the different enzyme activities.

6.3.6. In vitro release properties

6.3.6.1. Effect of pH and digestive enzymes on riboflavin release from enzyme-induced Bambara groundnut protein isolate hydrogels

The amount of riboflavin released from the enzyme-induced BPI hydrogels was lower in SGF than SIF in the absence of digestive enzymes (Figure 6.4 a & b). However, in the presence of digestive enzymes the amount of riboflavin released increased in both SGF and SIF (Figure 6.4 c & d). Digestive enzymes have the capability to break down the protein matrix hence accelerating the riboflavin release (Maltais *et al.*, 2010). However, the presence of pepsin in SGF did not quite increase the riboflavin release for all the BPI hydrogels (Fig 6.4 c). Pepsin is known to attack peptide bonds preferentially involving hydrophobic aromatic amino acids (Antonov, 1977), which make up only a small proportion of the BPI, hence the slow digestion.

The high amount of riboflavin released in SIF with pancreatin suggested the extensive action of the digestive enzyme combining the peptide-bond-hydrolysing activities mainly of trypsin, chymotrypsin and elastase (Maltais *et al.*, 2010). As such the release will mainly be due to gel erosion.

Increase in the TGase activity together with the increase in laccase activity caused a reduction in the amount of riboflavin released from the BPI hydrogels. The combined use of TGase activity at 15 U/g protein and laccase activity at 0.5 U/g protein recorded the lowest riboflavin release in the SGF and SIF with or without digestive enzymes. This could be due to reduced swelling which is reported to limit access of digestive enzymes into the BPI hydrogels, resulting in a reduction in matrix erosion and hence decrease in riboflavin release (Hu *et al.*, 2015). In addition, the increased crosslinking density and formation of covalent bonds could reduce the BPI hydrogel erosion.

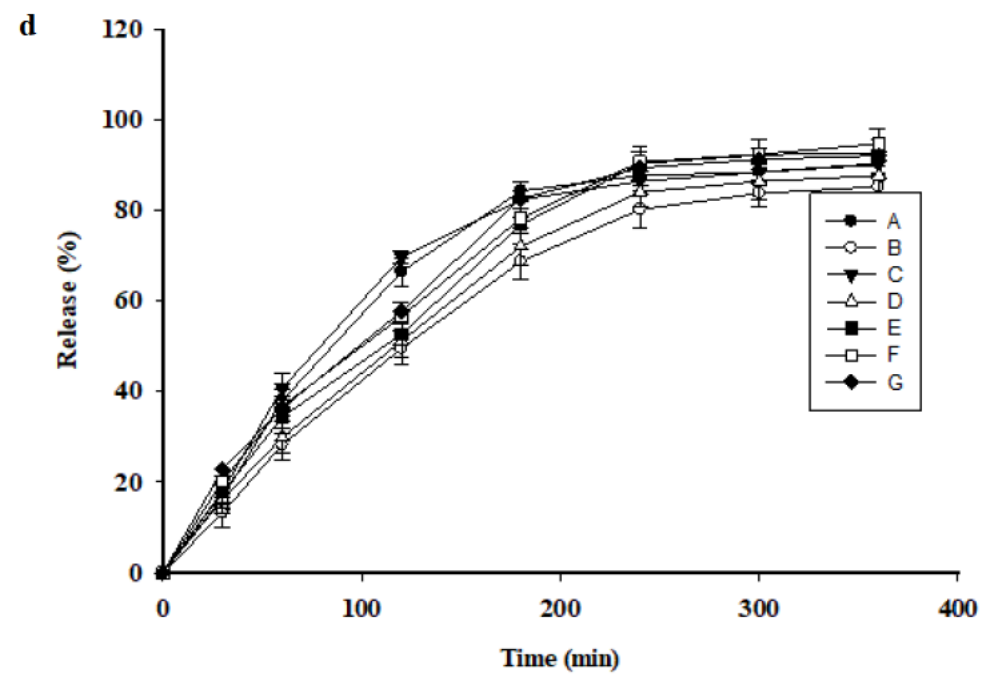
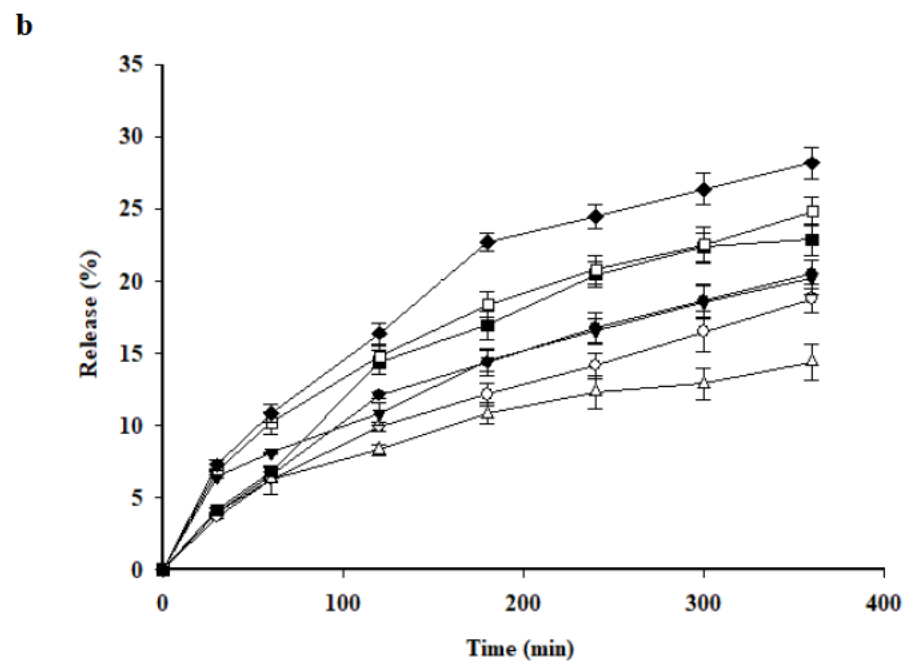
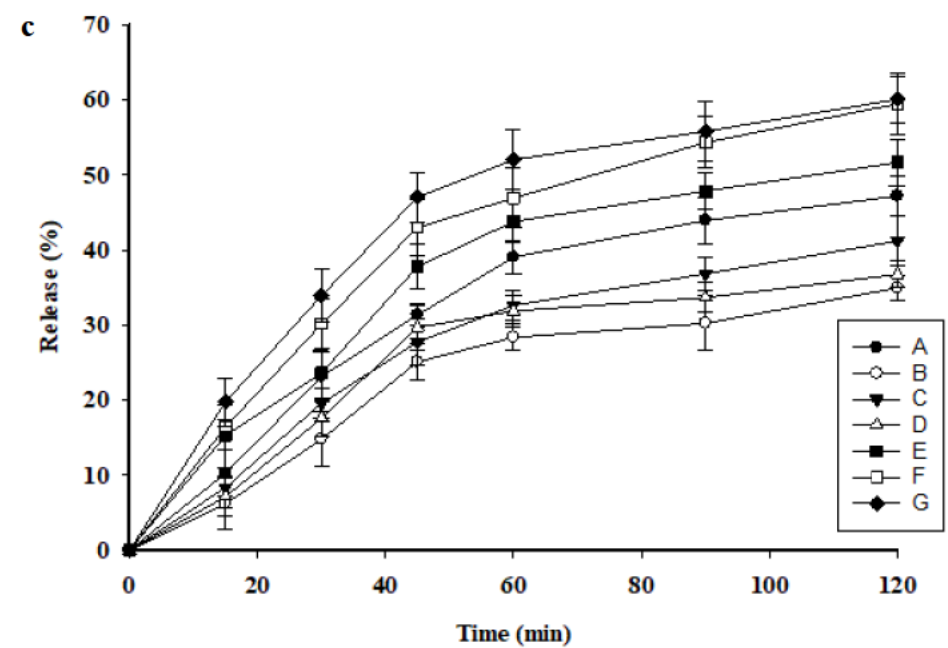
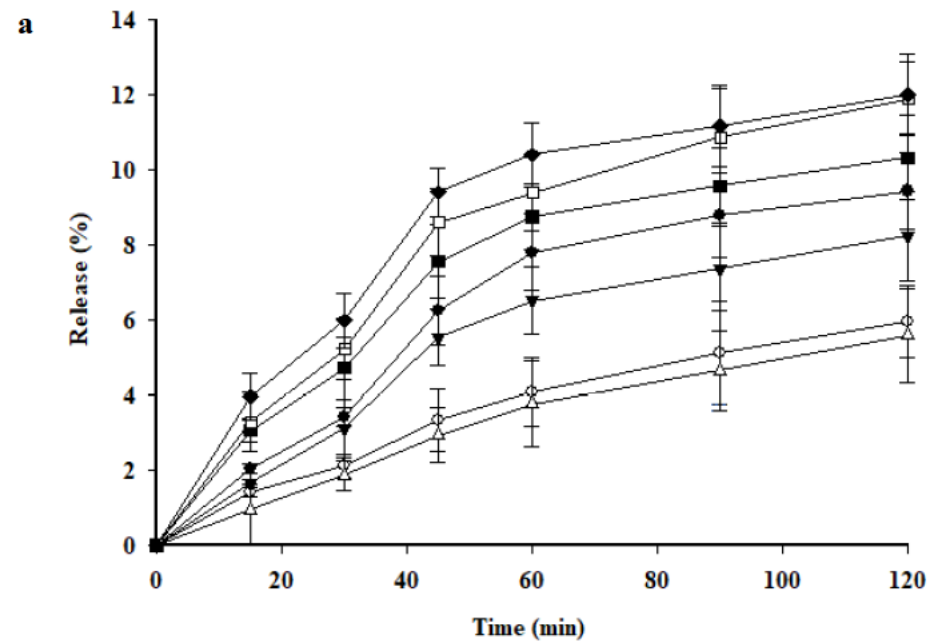


Figure 6.4: Effect of pH and digestive enzymes on the release of riboflavin from enzyme-induced Bambara groundnut protein isolate hydrogels in a) SGF (pH 1.2) b) SIF (pH 7.5) c) SGF (pH 1.2) with pepsin and d) SIF (pH 7.5) with pancreatin. Values are means \pm SD (n=3). A. TGase 7.5 U/g & Laccase 0 U/g B. TGase 15 U/g & Laccase 0 U/g C. TGase 7.5 U/g & Laccase 0.5 U/g D. TGase 15 U/g & Laccase 0.5 U/g E. TGase 7.5 U/g & Laccase 1.21 U/g F. TGase 15 U/g & Laccase 1 U/g G. TGase 18.11 U/g & Laccase 0.5 U/g.

6.3.6.2. Delivery of riboflavin under gastrointestinal conditions

The increase in TGase activity and use of a combination of enzymes influenced the riboflavin release characteristic in SGF with pepsin followed by SIF with pancreatin (Figure 6.5). During the first 30 min in SGF with pepsin, a higher riboflavin release was observed from the BPI hydrogels made with 7.5 U/g protein TGase activity as compared to those with 15 U/g protein. This reduced release in SGF with pepsin could be due to resistance of pepsin digestion as the crosslinking density increased. Previous studies have reported that hydrogels with a compact and dense network structure induced by TGase can inhibit pepsin digestion (Yang *et al.*, 2017; Wen *et al.*, 2018). Monogioudi *et al.* (2011), also reported that TGase crosslinked β -casein enhanced the stability of the gel structure subsequently better resisting enzymatic digestion. As the BPI hydrogels were transferred into the SIF with pancreatin the release of riboflavin accelerated for all the samples. Though an increase was observed for all samples the amount of riboflavin released was low in the BPI hydrogels made with TGase activity of 15 U/g protein and in the presence of laccase. The reduced release could be associated with the compact, dense and low porosity of the hydrogels made from a combination of enzymes (Maltais *et al.*, 2010).

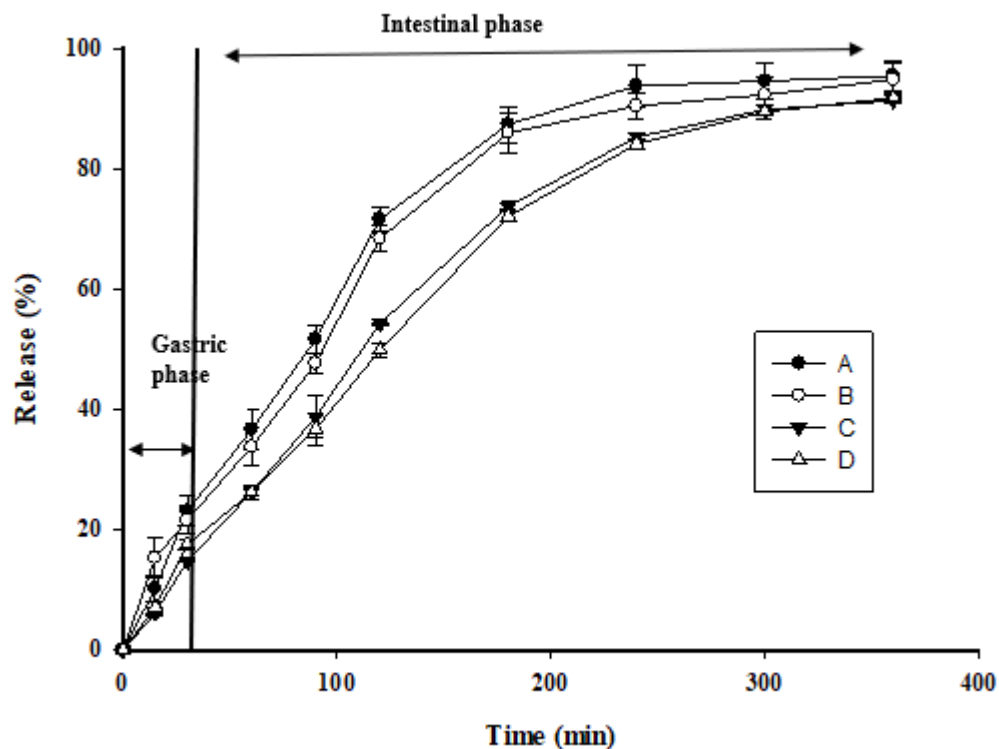


Figure 6.5: In vitro release of riboflavin from enzyme-induced Bambara groundnut protein isolate hydrogels in simulated gastrointestinal conditions. Note 30 mins in SGF with pepsin and 5.5 h in SIF with pancreatin. A. TGase 7.5 U/g & Laccase 0 U/g B. TGase 7.5 U/g & Laccase 0.5 U/g C. TGase 15 U/g & Laccase 0 U/g D. TGase 15 U/g & Laccase 0.5 U/g.

6.3.6.3. Release kinetics

Mechanism of riboflavin release from the enzyme-induced BPI hydrogels was further investigated using the Korsmeyer-Peppas equation. The value of n from the equation indicates the type of release occurring: (1) With Fickian diffusion $n \leq 0.45$ when measured with a non-disintegrated matrix; (2) With non-Fickian diffusion from $0.45 < n < 0.89$ indicating concurrent disintegration and swelling of the matrix (3) With Case-II transport diffusion $n = 0.89$ indicating release from a swelling controlled matrix; (4) $n > 0.89$ indicates super case II transport (Caillard *et al.*, 2009).

Generally, n values for SGF + pepsin were all lower than for SIF + pancreatin (Table 6.5). This could be attributed to the acidic pH in the stomach and the neutral pH in the intestine; the large difference in pH could explain different release profiles (Maltais *et al.*, 2009). Furthermore, pepsin preferentially attack peptide bonds involving hydrophobic aromatic amino acids (Antonov, 1977); these are only a small proportion of Bambara protein molecule structure, hence the slow digestion. In the presence of pancreatin, there is extensive action which combines the peptide-bond-hydrolyzing activities of mainly trypsin, chymotrypsin and elastase (Maltais *et al.*, 2009). In both SGF with pepsin and SIF with pancreatin, the n values for the riboflavin release decreased when high TGase activity (15 U/g protein) was used in combination with laccase at 0.5 U/g protein (Table 6.5). This observation can be linked to the reduced swelling ratios observed at these conditions. The n values for the riboflavin release in SGF with pepsin were between 0.51 and 0.59 showing non-Fickian diffusion, suggesting that diffusion and swelling were responsible for the release of riboflavin (Caillard *et al.*, 2009).

In SIF + pancreatin, the n values for riboflavin release also decreased when high activities of TGase activity were used in combination with laccase. Riboflavin release from BPI hydrogels made with TGase activity at 7.5 U/g of protein in the absence and presence of laccase (0.5 U/g protein) were > 0.89 and 0.89, respectively showing that the release was due to the occurrence of super case II transport and case II transport. In other words, the release from the BPI hydrogel made with 7.5 U/g protein of TGase in the absence of laccase was due to the matrix erosion whilst in the presence of laccase the release was mainly due to the swelling controlled release (Bani-Jaber *et al.*, 2011). At a higher TGase activity of 15 U/g protein the n values for the release were between 0.45 and 0.89, suggesting an anomalous transport involving both disintegration and swelling (Caillard *et al.*, 2009).

Table 6.5: Release kinetic constants of riboflavin from Bambara groundnut protein isolate hydrogels crosslinked with transglutaminase and laccase at varying activities

Enzyme activity		Release kinetics constants			
TGase	Laccase	SGF + pepsin		SIF + pancreatin	
(U/g protein)	(U/g protein)	n ₁	R ₁ ²	n ₂	R ₂ ²
7.5	0	0.59	0.99	0.92	0.99
7.5	0.5	0.57	0.98	0.89	1.00
15	0	0.53	0.98	0.81	1.00
15	0.5	0.51	0.98	0.73	1.00

6.4. Conclusion

Hydrogels were formed by crosslinking Bambara groundnut protein using either TGase only or a combination of TGase and laccase. A combination of TGase and laccase at 15 U/g protein and 0.5 U/g protein, respectively resulted in a hydrogel with the lowest swelling capacity, highest encapsulation efficiency (98.8%), and lowest release kinetics constants. The hydrogels produced from a combination of TGase and laccase prevented the early release of riboflavin in the stomach while making it available in the small intestines. Therefore, a combination of TGase and laccase has great potential in the production of hydrogels for the encapsulation and targeted release of compounds.

CHAPTER SEVEN

General discussion

7.1. General discussion

In this thesis, laccase and transglutaminase were used separately and in combination for the modification of Bambara groundnut protein in the production of hydrogels. The first part of the thesis reported the effect of pH and NaCl concentration on the rheological and microstructural properties of Bambara groundnut protein gels, and the parameters were optimised using response surface methodology (chapter 3). The second part discussed the impact of laccase and transglutaminase on rheological and microstructural properties of Bambara groundnut protein hydrogels using heat set induced and cold set induced methods, respectively (Chapter 4 and 5). The last part focused on the use of a combination of laccase and transglutaminase on the rheological, textural, encapsulation efficiency and release properties of the Bambara groundnut protein hydrogels (chapter 6).

Protein gelation is affected by an interplay of factors such as protein concentration, pH, ionic species, and heating temperature and time (Shand *et al.*, 2007). The influence of these process parameters is dependent on the protein source, composition and conformational structure. Variations in pH and NaCl concentration were investigated to gain knowledge on the relationship between the structural and gelation properties of Bambara groundnut protein and to establish baseline conditions for enzymatic treatment. Frequency sweep measurements showed that the G' values were greater than G'' values irrespective of pH and NaCl concentration used in BPI gel preparation. The difference between G' was less than $10G''$ and the values were highly dependent on angular frequency, indicating the formed gels were weak. Optimisation of the process parameters showed that BPI gels with the highest G' were observed at slightly acidic pH as compared to the neutral and slightly basic pH, within the NaCl

concentration range studied. The model predicted a maximum G' of 3755.73 Pa and G'' of 663.516 Pa which were close to the actual G' of 3800 Pa and G'' of 667 Pa, confirming the adequacy of the model. The high G' values recorded at slightly acidic pH and in the presence of 0.5 M NaCl concentration were attributed to the more compact aggregated network structure that was observed from the SEM images and disulphide bond formation as shown by the low free thiol content. Increased disulphide bond formation strengthens the gels formed (Wang & Damodaran 1990, Wang *et al.*, 2017). In addition, NaCl addition resulted in the screening of electrostatic repulsion between polypeptides which facilitates protein-protein interactions in the gel formation (Renkema *et al.*, 2002).

Modification of Bambara groundnut protein with laccase caused an initial breakdown in structure at an enzyme dependent dose as observed from the change of flow behaviours from $G' > G''$ to $G'' > G'$. This was immediately followed by structure build up during the heating and cooling phase which was more pronounced in the presence of enzyme than in the absence of enzyme. The gelation point of the BPI dispersions reduced with increase in laccase activity. At laccase activity of 3 U/g protein, the gel point temperature of Bambara groundnut protein (29°C), was approximately 3 folds lower than that of the protein without laccase (85°C). The enzyme laccase performs one electron oxidation of its substrates such as tyrosine, forming reactive radicals (Ma *et al.*, 2020). Reactive radicals formed can cause various chemical modification to proteins such as aggregation, polymerisation and fragmentation and the extent of these effects on protein is dependent on enzyme dosage (Lantto *et al.*, 2005). In the present study, at a lower enzyme activity of 1 U/g protein the fragmentation rate was lower than the rate of polymerisation hence G' values were $> G''$ values at the onset of the period of incubation. As the laccase activity was increased to 2 U/g protein the mixture showed $G'' > G'$ at the onset which can be attributed to fragmentation, however the G' increased at faster rate

relative to G'' and this could be attributed to polymerisation exceeding fragmentation hence causing the build-up in the network structure (Lantto *et al.*, 2005). Overall, modification of Bambara groundnut protein resulted in gels with a stable network structure and an increased strength as deduced from the higher G' values, G' and G'' difference of approximately 1 log, reduced dependency on angular frequency and higher G^* values (Sun & Arntfield, 2010; Deng *et al.*, 2018). Laccase activity at 2 U/g protein produced the gel with the highest strength and degree of crosslinking. These high G' values recorded in the laccase-modified BPI gels can be attributed to the polymerisation of protein molecules resulting in increased disulphide bond formation as shown by the low free thiol content observed. Model reactions indicated that laccase-catalysed crosslinking of proteins could be due to various reactions such as dityrosine bond formation, thiol phenolic conjugation and thiol-thiol conjugation. Microscopy images showed laccase treated BPI gels formed were composed of homogeneous lath sheet-like structures which were interconnected.

Transglutaminase-mediated crosslinking of partially denatured Bambara groundnut protein dispersions resulted in hydrogel formation. The presence and abundance of amino acids such as glutamine and lysine favoured TGase crosslinking of BPI and the formation of a structured network (Hu *et al.*, 2015). Free amino group reduction in the presence of TGase indicated glutamine-lysine isopeptide bonds formation. The covalent crosslinking (through glutamine-lysine isopeptide bonds) of peptide chains promoted the formation of the protein hydrogel (Wen *et al.*, 2018). The dominance of an elastic behaviour, G' more than ten times greater than G'' and little dependence of the moduli parameters on angular frequency indicated the formation of strong hydrogels (Deng *et al.*, 2018). The hydrogel with the highest strength as well as a more homogeneous, porous network with a smoother surface was produced upon use of TGase activity at 15 U/g protein. TGase has also been previously reported to crosslink

apricot kernel protein gel resulting in the formation of denser and more homogeneous network as the enzyme activity was increased (Wen *et al.*, 2018).

The use of a combination of enzymes (laccase and TGase) was optimised by response surface methodology using central composite design. The central composite design model predicted that the hydrogel with the highest G' value was made at TGase activity of 15 U/g protein in the absence of laccase. However, the $\tan \delta$ values suggested that more elastic hydrogels were made after treatment with a combination of TGase (15 U/g protein) and laccase (1 U/g protein). A decision was therefore made to incorporate a lower laccase activity of 0.5 U/g protein in combination with TGase activity at 15 U/g protein which had not been part of the model runs. Further analysis showed that the hydrogel made using these activities had the highest hardness. The highest strength could be due to the effects of the combined enzyme system resulting in the formation of multiple covalent linkages such as the disulphide bonds and isopeptide bonds highlighted in chapter 4 and 5, respectively. The hydrogel made at TGase activity of 15 U/g protein and laccase activity of 0.5 U/g protein had the lowest swelling properties which can be attributed to increased crosslinking and changes in the 3D structure. Previous studies have also reported decreased hydrogel swelling upon increased crosslinking (Caillard *et al.*, 2010; Hu *et al.*, 2015). The highest encapsulation efficiency recorded in the BPI hydrogel made at optimal conditions could be due to increased covalent interactions formed within the gel. According to Wen *et al.* (2018), increased covalent interactions stabilise protein-riboflavin solution systems, thus allowing more riboflavin to be encapsulated into the BPI hydrogels (Wen *et al.*, 2018). The mechanism of riboflavin release as deduced from the Korsmeyer-Peppas equation was shown to be non-Fickian diffusion indicating concurrent disintegration and swelling of the matrix. The values for the release kinetics were 0.51 and 0.73 in SGF with pepsin and SIF with pancreatin, respectively. The lower value in SGF with pepsin suggested that the Bambara

groundnut protein cold-set hydrogels produced from a combination of TGase (15 U/g protein) and laccase (0.5 U/g protein) could effectively encapsulate and prevent the early release of heat sensitive compounds in the stomach while making them available in the small intestines. Yang *et al.* (2017) reported that hydrogels with a compact and dense network structure induced by TGase can inhibit pepsin digestion.

Overall, it was clear that the variations in pH and NaCl concentration influenced the frequency sweep (G' and G'') parameters of the BPI gels. However, the high dependence of G' and G'' on angular frequency indicated weak gels. Optimal conditions of pH 6 and 0.5 M NaCl were then chosen as the ideal starting conditions in the enzymatic treatment studies. Laccase modification of BPI led to an increase in crosslinking degree and consequently improved the strength of the heat induced gels. However, laccase was not able to form BPI hydrogels when using the cold set method. The use of TGase when using the cold set method promoted the formation of an organised network with better hydrogel strength. The use of a combination of laccase and TGase at optimal activities better improved the textural, encapsulation and release properties of the Bambara groundnut protein hydrogels for potential application in the encapsulation of heat sensitive compounds.

CHAPTER EIGHT

Conclusions and recommendations

8.1. General conclusion

Enzymatic modification of plant proteins in the production of natural polymer-based hydrogels has been gaining attention due to their biodegradability and biocompatibility. Transglutaminase has been the most widely used enzyme in plant protein hydrogels and soybean the most frequently used protein source. This study investigated improvement of the microstructural, structural, rheological and mechanical properties of Bambara groundnut protein hydrogels using multiple enzymes for potential application in encapsulation and release of bioactive compounds using riboflavin as a model compound. In addition, the effect of pH and NaCl concentration on the rheological and microstructural properties of Bambara groundnut protein gels were optimised before enzymatic crosslinking.

The specific objectives were as follows:

1. To determine the rheological and microstructural properties of Bambara groundnut protein gels as influenced by reaction conditions (pH and ionic strength) using response surface methodology.
2. To determine the effect of using crosslinking enzymes (transglutaminase and laccase) on the textural, rheological, structural and microstructural properties of Bambara groundnut protein hydrogels.
3. To determine the encapsulation efficiency and release properties of the enzyme (transglutaminase and laccase) crosslinked Bambara groundnut protein hydrogel using riboflavin as a model bioactive compound.

Below is a brief summary of the key findings.

The first part of this research study demonstrated that NaCl concentration and pH variations influence the rheological and microstructural properties of heat-induced Bambara groundnut protein gels. The highest G' and G'' values observed at optimum conditions of pH 6 and 0.5 M NaCl implied the formation of a more rigid gel. Increasing the NaCl concentration at slightly acidic conditions had a stabilising effect on the Bambara groundnut protein structure, as demonstrated by the increase in denaturation temperature and enthalpy of denaturation. The lowest water holding capacity and thiol content of the BPI gel were recorded at the optimum conditions suggesting the participation of more disulphide linkages in the network formation. The α helix conformation appeared to be the most dominant conformation, followed by β sheets and β turns. Microscopy images of the BPI gel showed a large aggregated compact network structure.

Laccase modification of BPI caused an initial structure breakdown followed by a structure build-up during the heating and cooling phases. Increase in laccase activity reduced the gel point temperature. Laccase modified BPI formed gels with improved strength as shown by the increase in the difference between G' and G'' to approximately 1 log and reduced dependency on angular frequency. BPI modification with laccase promoted the formation of a gel with a homogeneous lath sheet like structure. Hydrophobic interactions were the major non-covalent interactions in BPI gel formation. Gels made from laccase-catalysed protein had a reduced thiol and phenolic content as well as higher surface hydrophobicity. The crosslinking of phenolics and thiols was confirmed by model reactions using reactive amino acids glutathione, cysteine and lysine.

TGase addition promoted the formation of cold set hydrogels. The G' of TGase-treated BPI hydrogels was more than ten-fold higher than G'' throughout the frequency range of 0-100 rad/s, suggesting dominance of the elastic like behaviour. An organised network with better hydrogel strength was formed with the increase in the TGase activity. The structure of the network was TGase activity-dependent up to 15 U/g protein with the treated hydrogel showing numerous smaller-sized pores and a homogeneous network. Reduction in free amine and thiol groups as well as formation of a new protein band (56 kDa) in crosslinked hydrogels confirmed TGase crosslinking of Bambara groundnut proteins.

When using the cold set method, laccase on its own could not form a hydrogel although the increase in the enzyme activity had a positive effect on the G' and G'' . BPI hydrogels with TGase only or a combination of TGase and laccase showed a $G' > 10G''$ over a frequency range of 0 – 100 rad/s suggesting the dominance of the elastic behaviour. The combined use of TGase (15 U/g protein) and laccase (0.5 U/g protein) produced BPI hydrogels with a higher elasticity. A combination of TGase and laccase at 15 U/g protein and 0.5 U/g protein, respectively resulted in a hydrogel with the lowest swelling capacity, highest encapsulation efficiency (98.8%), and lowest release kinetic constants. The hydrogels produced from the optimised combination of TGase activity at 15 U/ protein and laccase activity at 0.5 U/g protein prevented the early release of riboflavin in the stomach while making it available in the small intestines.

Overall, the study demonstrated that TGase and laccase can be used in improving the network structures of BPI hydrogels as well as improve gel strength. Furthermore, the use of a combination of the enzymes is a potential strategy for increasing the crosslinking density of protein and improving the rheological, textural, microstructural, encapsulation and release properties of the formed hydrogels.

8.2. Recommendations

In the first part of the present research study, only the effect of two process parameters (pH and ionic species) were investigated. However, the gelation of proteins is reported to be affected by other factors such as protein concentration and heating temperature and time (Shand *et al.*, 2007). As such future studies may determine the combined influence of these factors on Bambara groundnut protein gelation.

Furthermore, high performance size exclusion chromatography (SEC-HPLC) can be used to determine the molecular weights of Bambara groundnut crosslinked hydrogels. SDS PAGE has to be conducted to confirm disulphide bond formation as well as fluorescence spectroscopy to analyse dityrosine content after laccase modification of Bambara groundnut protein.

To facilitate the use of the investigated combination of enzymes and the produced hydrogels in both food and pharmaceutical applications, confirmation of the bioavailability of the encapsulated compound *in vivo* in the gastrointestinal tract is required. In addition, the combination of enzymes can also be used in other hydrogel production systems including those combining Bambara groundnut protein and other polysaccharides as they might have improved properties.

References

- Abd-Rabo, F. H. R., El-Dieb, S. M., Abd-El-Fattah, A. M. & Sakr, S.S. (2010). Natural state changes of cows' and buffaloes' milk proteins induced by microbial transglutaminase. *Journal of American Science*, 6, 612–620.
- Adebowale, Y. A., Schwarzenbolz, U. & Henle, T. (2011). Protein isolates from Bambara groundnut (*Voandzeia Subterranean L.*): Chemical characterization and functional properties. *International Journal of Food Properties*, 14, 758-775.
- Adegbola, O. B. J. & Bamishaiye, E. (2011). Bambara groundnut: an underutilized nut in Africa. *Advances in Agricultural Biotechnology*, 60 - 72.
- Aditya, N. P., Espinosa, Y. G., & Norton, I. T. (2017). Encapsulation systems for the delivery of hydrophilic nutraceuticals: Food application. *Biotechnology Advances*, 35, 450-457.
- Ahmed, E. M. (2015). Hydrogel: Preparation, characterization, and applications: A review. *Journal of Advanced Research*, 6, 105-121.
- Akintayo, E. T., Oshodi, A. A. & Esuso, K.O. (1999). Effects of NaCl, ionic strength and pH on the foaming and gelation of pigeon pea (*Cajanus cajan*) protein concentrates. *Food Chemistry*, 66, 51-56.
- Alhaique, F., Casadei, A. M., Cencetti, C., Coviello, T., Meo, G. D., Matricardi, P., Montanari, E., Pacelli, S. & Paolicelli, P. (2016). From macro to nano polysaccharide hydrogels: An opportunity for the delivery of drugs. *Journal of Drug Delivery Science and Technology*, 32, 88-99
- Alozie, Y., Akpanabiatu, M. I., Eyong, E. U., Umoh, I. B. & Alozie, G. (2009). Amino acid composition of *Dioscorea dumetorum* varieties. *Pakistan Journal of Nutrition*; 8, 103-105.
- Alting, A.C. (2003). Cold gelation of globular proteins. Werkegroepeder, NZIO food research. Wageningen University.

- Anderson, J. M. & Langone, J. J. (1999). Issues and perspectives on the biocompatibility and immunotoxicity evaluation of implanted controlled release systems. *Journal of Controlled Release*, 57, 107-113.
- Antonov, V. K. (1977). New data on pepsin mechanism and specificity. In *Acid Proteases: Structure, Function, and Biology*. Springer, New York, 179-198.
- Aremu, M. O., Olaofe, O. & Akintayo, E. T. (2007). Functional properties of some Nigerian varieties of legume seed flours and flour concentration effect on foaming and gelation properties. *Journal of Food Technology*, 5, 109-115.
- Arise, A. K., Nwachukwu, I. D., Aluko, R. E. & Amonsou, E. O. (2017). Structure, composition and functional properties of storage proteins extracted from bambara groundnut (*Vigna subterranea*) landraces. *International Journal of Food Science and Technology*, 52, 1211-1220.
- Arntfield, S. D. & Murray, E. (1981). The influence of processing parameters on food protein functionality I. Differential scanning calorimetry as an indicator of protein denaturation. *Canadian Institute of Food Science and Technology Journal*, 14, 289-294.
- Bahram, M., Mohseni, N., & Moghtader, M. (2016). An introduction to hydrogels and some recent applications. In *Emerging concepts in analysis and applications of hydrogels*, 6-10.
- Bani-Jaber, A., Al-Aani, L., Alkhatib, H., & Al-Khalidi, B. (2011). Prolonged intragastric drug delivery mediated by Eudragit® E-Carrageenan polyelectrolyte matrix tablets. *Aaps Pharmscitech*, 12, 354-361.
- Barać, M. & Stanojević, S. (2005). The effect of microwave roasting on soybean protein composition and components with trypsin inhibitor activity. *Acta Alimentaria*, 34, 23-31.

- Barimalaa, I. S. & Anoghalu, S. C. (1997). Effect of processing on certain antinutrients in bambara groundnut (*Vigna subterranea*) cotyledons. *Journal of the Science of Food and Agriculture*, 73, 186–188.
- Batista, A. P., Portugal, C. A., Sousa, I., Crespo, J. G. & Raymundo, A. (2005). Accessing gelling ability of vegetable proteins using rheological and fluorescence techniques. *International Journal of Biological Macromolecules*, 36, 135-143.
- Bell, A. J., Deisseroth, W. & Jordan, J. M. (2001). *Taste modified hard confectionery compositions containing functional ingredients*: Google Patents.
- Ben-Harb, S., Panouillé, M., Huc-Mathis, D., Moulin, G., Saint-eve, A., Irlinger, F., Bonnarme, P., Michon, C. & Souchon, I. (2018). The rheological and microstructural properties of pea, milk, mixed pea/milk gels and gelled emulsions designed by thermal, acid, and enzyme treatments. *Food Hydrocolloids*, 77, 75-84.
- Berger, J., Reist, M., Mayer, J. M., Felt, O., Peppas, N. A., & Gurny, R. (2004). Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics*, 57, 19-34.
- Bertrand, T., Jolival, C., Caminade, E., Joly, N., Mougin, C. & Briozzo, P. (2002). Purification and preliminary crystallographic study of *Trametes versicolor* laccase in its native form. *Acta Crystallographica Section D-Biological Crystallography*, 58, 319-321.
- Beveridge, T., Toma, S.J. & Nakai, S. (1974). Determination of SH and SS groups in some food proteins using Ellman's reagent. *Journal of Food Science*, 39, 49-51
- Bikbov, T. M., Grinberg, V. Y., Danilenko, A. N., Chaika, T. S., Vaintraub, I. A., & Tolstoguzov, V. B. (1983). Studies on gelation of soybean globulin solutions. *Colloid and Polymer Science*, 261, 346-358.

- Boye, J., Zare, F. & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*, 43, 414-431.
- Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., Monogioudi, E., Faccio, G., Mattinen, M., Boer, H., Partanen, R., Selinheimo, E., Lantto, R. & Kruus, K. (2010). Crosslinking food proteins for improved functionality. *Annual Review Food Science and Technology*, 1, 113–138.
- Buettner, K., Hertel, T. C. & Pietzsch, M. (2012). Increased thermostability of microbial transglutaminase by combination of several hot spots evolved by random and saturation mutagenesis. *Amino Acids*, 42, 987–996.
- Busu, N. M. & Amonsou, E. O. (2019). Fractionation pH of bambara groundnut (*Vigna subterranea*) protein impacts the degree of complexation with gum arabic. *Food Hydrocolloids*, 87, 653-660.
- Buwalda S. J., Boere, W. M. K., Dijkstra, J. P., Feijen, J., Vermonden, T. & Hennink, W. E. (2014). Hydrogels in a historical perspective: From simple networks to smart materials. *Journal of Controlled Release*, 190, 254–273
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.
- Bryant, C. M. & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science and Technology*, 9, 143-151.
- Buchert, J., Lantto, R., Autio, K., Mattinen, M. & Kruus, K. (2004). Novel protein materials by enzymatic crosslinking. *Abstracts of papers of the American Chemical Society*, 228, U247.

- Caillard, R., Remondetto, G., Mateescu, M. & Subirade, M. (2008). Characterization of amino cross-linked soy protein hydrogels. *Journal of Food Science*, 73, C283-C291.
- Caillard, R., Remondetto, G. E., & Subirade, M. (2009). Physicochemical properties and microstructure of soy protein hydrogels co-induced by Maillard type cross-linking and salts. *Food Research International*, 42, 98-106.
- Caillard, R., Remondetto, G. & Subirade, M. (2010). Rheological investigation of soy protein hydrogels induced by Maillard-type reaction. *Food Hydrocolloids*, 24, 81-87.
- Caló, E. & Khutoryanskiy, V. V. (2015). Biomedical applications of hydrogels: A review of patents and commercial products. *European Polymer Journal*, 65, 252-267.
- Cao, Y., & Mezzenga, R. (2020). Design principles of food gels. *Nature Food*, 1, 106-118.
- Campbell, L. J., Gu, X., Dewar, S. J., & Euston, S. R. (2009). Effects of heat treatment and glucono- δ -lactone-induced acidification on characteristics of soy protein isolate. *Food Hydrocolloids*, 23, 344-351.
- Campo-Deaño, L., Tovar, C. A., Pombo, M. J., Solas, M. T., & Borderías, A. J. (2009). Rheological study of giant squid surimi (*Dosidicus gigas*) made by two methods with different cryoprotectants added. *Journal of Food Engineering*, 94, 26-33.
- Carbonaro, M. & Nucara, A. (2010). Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region. *Amino Acids*, 38, 679-690.
- Chen, H., & Han, M. (2011). Raman spectroscopic study of the effects of microbial transglutaminase on heat-induced gelation of pork myofibrillar proteins and its relationship with textural characteristics. *Food Research International*, 44, 1514-1520.
- Chen, L. Y., Remondetto, G. E., & Subirade, M. 2006. Food protein-based materials as nutraceutical delivery systems. *Trends in Food Science and Technology*, 17, 272- 283.
- Chen, Z., Shi, X., Xu, J., Du, Y., Yao, M., & Guo, S. (2016). Gel properties of SPI modified by enzymatic cross-linking during frozen storage. *Food Hydrocolloids*, 56, 445-452.

- Chen, N., Zhao, M., Chassenieux, C., & Nicolai, T. (2017). The effect of adding NaCl on thermal aggregation and gelation of soy protein isolate. *Food Hydrocolloids*, 70, 88-95.
- Clark, A. H., & Ross-Murphy, S. B. (1987). Structural and mechanical properties of biopolymer gels. *Biopolymers*, 57-192.
- Clark, A. H. & Ross-Murphy, S. B. (2009). *Biopolymer network assembly: measurement and theory*. Elsevier: London, UK.
- Collision, C. T., Sibuga, L. P., Rarimo, A. J. P. & Azam-Ali, S. N. (2000). Influence of sowing date on the growth and yield of Bambara groundnuts landraces in Tanzania. *Experimental. Agriculture*, 36, 1-13.
- Cura, D. E., Lantto, R., Lille, M., Andberg, M., Kruus, K., & Buchert, J. (2009). Laccase-aided protein modification: effects on the structural properties of acidified sodium caseinate gels. *International Dairy Journal*, 19, 737-745.
- Cushnie, T. P. T. & Lamb, A. J. 2005. Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, 26, 343 – 356.
- Dannenberg, F. & Kessler, H.G. (1988). Reaction kinetics of the denaturation of whey proteins in milk. *Journal of Food Science*, 53, 258- 263.
- Davis, H. E. & Leach, J. K. (2011). Designing bioactive delivery systems for tissue regeneration. *Annals of Biomedical Engineering*, 39, 1-13.
- Deng, C., Liu, Y., Li, J., Yadav, M. P. & Yin, L. (2018). Diverse rheological properties, mechanical characteristics and microstructures of corn fiber gum/soy protein isolate hydrogels prepared by laccase and heat treatment. *Food Hydrocolloids*, 76, 113-122.
- Diedericks, C. F., De Koning, L., Jideani, V. A., Venema, P. & Van Der Linden, E., (2019). Extraction, gelation and microstructure of Bambara groundnut vicilins. *Food Hydrocolloids*, 97, 105226.

- Diedericks, C. F., Shek, C., Jideani, V. A., Venema, P., & van der Linden, E. (2020). Physicochemical properties and gelling behaviour of Bambara groundnut protein isolates and protein-enriched fractions. *Food Research International*, 138, 109773.
- Dileep, A. O., Shamasundar, B. A., Binsi, P. K., Badii, F., & Howell, N. K. (2005). Effect of ice storage on the physicochemical and dynamic viscoelastic properties of ribbonfish (*Trichiurus* spp) meat. *Journal of Food Science*, 70, E537-E545.
- Djoullah, A., Husson, F. & Saurel, R. (2018). Gelation behaviors of denaturated pea albumin and globulin fractions during transglutaminase treatment. *Food Hydrocolloids*, 77, 636-645.
- Ebara, M., Kotsuchibashi, Y., Narain, R., Idota, N., Kim, Y. J., Hoffman, M. J., Uto, K. & Aoyagi, T. (2014). Smart biomaterials. *Springer*.
- Eltayeb, A. R. S., Ali, A. O., Abou-Arab, A. A. & Abu-Salem, F. M. (2011). Chemical composition and functional properties of flour and protein isolate extracted from Bambara groundnut (*Vigna subterranean*). *African Journal of Food Science*, 5, 82-90.
- Enguita, F. J., Martins, L. O., Henriques, A. O., & Carrondo, M. A. (2003). Crystal structure of a bacterial endospore coat component a laccase with enhanced thermostability properties. *Journal of Biological Chemistry*, 278, 19416-19425.
- Fairhead' M. & Thöny-Meyer, L. (2010). Cross-linking and immobilisation of different proteins with recombinant *Verrucomicrobium spinosum* tyrosinase. *Journal of Biotechnology*, 150, 546–551.
- Farahnaky, A., Askari, H., Majzoobi, M., & Mesbahi, G. (2010). The impact of concentration, temperature and pH on dynamic rheology of psyllium gels. *Journal of Food Engineering*, 100, 294-301.

- Farjami, T., Madadlou, A. & Labbafi, M. (2015). Characteristics of the bulk hydrogels made of the citric acid cross-linked whey protein microgels. *Food Hydrocolloids*, 50, 159-165.
- Ferreira, P., Coelho, J. F. J., Almeida, J. F. & Gil, M. H. (2011). Photocrosslinkable polymers for biomedical applications, *Biomedical Engineering-Frontiers and Challenges*, 55-74.
- Feyzi, S., Varidi, M., Zare, F. & Varidi, M. J. (2015). Fenugreek (*Trigonella foenum graecum*) seed protein isolate: extraction optimization, amino acid composition, thermo and functional properties. *Journal of the Science of Food and Agriculture*, 95, 3165 - 3176.
- Gan, C. Y., Cheng, L. H. & Easa, A. (2009). Assessment of cross-linking in combined cross-linked soy protein isolate gels by microbial transglutaminase and maillard reaction. *Journal of Food Science*, 74, C141- C146.
- Ganji, F., Vasheghani-Farahani, S. & Vasheghani-Farahani, E. (2010). Theoretical description of hydrogel swelling: a review, *Iran. Polymer Journal*, 19, 375–398.
- Geiger, J. P., Rio, B., Nandris, D., & Nicole, M. (1987). Laccases of *Rigidoporus lignosus* and *Phellinus noxius*. *Applied Biochemistry and Biotechnology*, 12, 121-133.
- Gerrard, J. A. (2002). Protein-protein crosslinking in food: methods, consequences, applications. *Trends in Food Science and Technology*, 13: 391 – 399.
- Gleeson, J. P., Ryan, S. M. & Brayden, D. J. (2016). Oral delivery strategies for nutraceuticals: Delivery vehicles and absorption enhancers. *Trends in Food Science and Technology*, 53: 90-101.
- Gui, Y., Li, J., Zhu, Y., & Guo, L. (2020). Roles of four enzyme crosslinks on structural, thermal and gel properties of potato proteins. *LWT- Food Science and Technology*, 123, 109116.

- Gulrez, S. K. H., Al-Assaf, S. & Phillips, G. O. (2011). Hydrogels: methods of preparation, characterization and applications. *Progress in Molecular and Environmental Bioengineering – From Analysis and Modeling to Technology Applications*. 117–150.
- Guo, J., Jin, Y. C., Yang, X. Q., Yu, S. J., Yin, S. W. & Qi, J. R. (2013). Computed microtomography and mechanical property analysis of soy protein porous hydrogel prepared by homogenizing and microbial transglutaminase cross-linking. *Food Hydrocolloids*, 31, 220-226.
- Gupta, S.C., Kim, J.H., Prasad, S. & Aggarwal, B.B. (2010). Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer Metastasis Rev*, 29, 405–34.
- He, X. H., Liu, H. Z., Liu, L., Zhao, G. L., Wang, Q., & Chen, Q. L. (2014). Effects of high pressure on the physicochemical and functional properties of peanut protein isolates. *Food Hydrocolloids*, 36, 123-129.
- Heck, T., Faccio, G., Richter, M. & Thöny-Meyer, L. (2013). Enzyme-catalyzed protein crosslinking. *Applied Microbiology and Biotechnology*, 97, 461-475.
- Hennink, W. E. & Nostrum, C. F. V. (2002). Novel crosslinking methods to design hydrogels. *Advanced Drug Delivery Reviews*, 54, 13–36.
- Hillocks, R., Bennett, C. & Mponda, O. (2012). Bambara nut: A review of utilisation, market potential and crop improvement. *African Crop Science Journal*, 20, 1-7.
- Ho, M. L., Leu, S. Z., Hsieh, J. F. & Jiang, S. T. (2000). Technical approach to simplify the purification method and characterization of microbial transglutaminase produced from *Streptomyces* *ladakanum*. *Journal of Food Science*, 65, 76–80.
- Hoare, T. R. & Kohane, D. S. (2008). Hydrogels in drug delivery: Progress and challenges. *Polymer*, 49, 1993-2007.

- Hoffman, A. S. (2002). Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews*, 43, 3-12.
- Hongsprabhas, P., Barbut, S. & Marangoni, A. G. (1999). The structure of cold-set whey protein isolate gels prepared with Ca^{++} . *LWT- Food Science and Technology*, 32, 196-202.
- Hongsprabhas, P. & Barbut, S. (1997a). Protein and salt effects on Ca^{2+} - induced cold gelation of whey protein isolate. *Journal of Food Science*, 62, 382–385.
- Hongsprabhas, P., & Barbut, S. (1997b). Structure-forming processes in Ca^{2+} -induced whey protein isolate cold gelation. *International Dairy Journal*, 7, 827–834
- Hu, X., Zhao, M., Sun, W., Zhao, G. & Ren, J. (2011). Effects of microfluidization treatment and transglutaminase cross-linking on physicochemical, functional, and conformational properties of peanut protein isolate. *Journal of Agricultural and Food Chemistry*, 59, 8886-8894.
- Hu, H., Fan, X., Zhou, Z., Xu, X., Fan, G., Wang, L., Huang, X., Pan, S. & Zhu, L. (2013). Acid-induced gelation behavior of soybean protein isolate with high intensity ultrasonic pre-treatments. *Ultrasonics Sonochemistry*, 20, 187-195.
- Hu, H., Zhu, X., Hu, T., Cheung, I. W., Pan, S. & Li-Chan, E. C. (2015). Effect of ultrasound pre-treatment on formation of transglutaminase-catalysed soy protein hydrogel as a riboflavin vehicle for functional foods. *Journal of Functional Foods*, 19, 182-193.
- Hua, Y., Cui, S. W., Wang, Q., Mine, Y., & Poysa, V. (2005). Heat induced gelling properties of soy protein isolates prepared from different defatted soybean flours. *Food Research International*, 38, 377-385.
- Huang, Q., Yu, H. & Ru, Q. 2010. Bioavailability and delivery of nutraceuticals using nanotechnology. *Journal of Food Science*, 75, R60 - R57.

- Janasway, S. & Youngren, S.R. (2012). Hydrocolloid-based nutraceutical delivery systems. *Food and Function*, 3, 503-507.
- Jia, D., Huang, Q., & Xiong, S. (2016). Chemical interactions and gel properties of black carp actomyosin affected by MTGase and their relationships. *Food Chemistry*, 196, 1180-1187.
- Jiang, J., Xiong, Y. L., & Chen, J. (2010). pH shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions. *Journal of Agricultural and Food Chemistry*, 58, 8035-8042.
- Jiang, Z., Yuan, X., Yao, K., Li, X., Zhang, X., Mu, Z., Jiang, L. & Hou, J. (2017). Laccase-aided modification: Effects on structure, gel properties and antioxidant activities of α -lactalbumin. *LWT- Food Science and Technology*, 80, 355-363.
- Jin, M. & Zhong, Q. (2013). Transglutaminase cross-linking to enhance elastic properties of soy protein hydrogels with intercalated montmorillonite nanoclay. *Journal of Food Engineering*, 115, 33-40.
- Ju, Z. Y. & Kilara, A. (1998). Textural properties of cold-set gels induced from heat denatured whey protein isolates. *Journal of Food Science*, 63, 288–292.
- Jus, S., Kokol, V. & Guebitz, G. M. (2008). Tyrosinase-catalysed coupling of functional molecules onto protein fibres. *Enzyme and Microbial Technology*, 42, 535 – 542.
- Kashiwagi, T., Yokoyama, K., Ishikawa, K., Ono, K., Ejima, D., Matsui, H. & Suzuki, E. (2002). Crystal structure of microbial transglutaminase from *Streptoverticillium mobaraense*. *Journal of Biological Chemistry*, 277, 44252– 44260.
- Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et biophysica acta (BBA)-Protein structure*, 624, 13-20.

- Kieliszek, M. & Misiewicz, A. (2014). Microbial transglutaminase and its application in the food industry. A review. *Folia Microbiology*, 59, 241–250.
- Kimpel, F., & Schmitt, J. J. 2015. Review: milk proteins as nanocarrier systems for hydrophobic nutraceuticals. *Journal of Food Science*, 80, 2361- 2366.
- Kopeček, J. (2007). Hydrogel biomaterials: a smart future? *Biomaterials*, 28, 5185-5192.
- Kopecek, J. & Yang, J. (2009). Peptide-directed self-assembly of hydrogels. *Acta Biomaterial*, 5, 805-16.
- Kudanga, T., Nyanhongo, G. S., Guebitz, G. M., & Burton, S. (2011). Potential applications of laccase-mediated coupling and grafting reactions: a review. *Enzyme and Microbial Technology*, 48, 195-208.
- Kudre, T. G., & Benjakul, S. (2013). Combining effect of microbial transglutaminase and bambara groundnut protein isolate on gel properties of surimi from sardine (*Sardinella albella*). *Food Biophysics*, 8, 240-249.
- Kudre, T. G., Benjakul, S. & Kishimura, H. (2013). Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. *Journal of the Science of Food and Agriculture*, 93, 2429-2436.
- Kudre, T. G., & Benjakul, S. (2014). Effects of bambara groundnut protein isolates and microbial transglutaminase on textural and sensorial properties of surmi gel from sardine (*Sardinella albella*). *Food and Bioprocess technology*, 7, 1570-1580.
- Kuraishi, C., Yamazaki, K. & Susa, Y. (2001). Transglutaminase: its utilization in the food industry. *Food Reviews International*, 17, 221–246
- Kwon, D. Y., & Kim, P. S. (1994). The stabilizing effects of hydrophobic cores on peptide folding of bovine-pancreatic-trypsin-inhibitor folding-intermediate model. *European Journal of Biochemistry*, 223, 631-636.

- Labat, E., Morel, M. H., Rouau, X. (2000). Effect of laccase and ferulic acid on wheat flour dough during mixing. *Cereal Chemistry*, 77, 823- 828.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680.
- Lantto, R., Puolanne, E., Kalkkinen, N., Buchert, J., & Autio, K. (2005). Enzyme-aided modification of chicken-breast myofibril proteins: effect of laccase and transglutaminase on gelation and thermal stability. *Journal of Agricultural and Food Chemistry*, 53, 9231-9237.
- Lantto, R., Puolanne, E., Katina, K., Niemistö, M., Buchert, J., & Autio, K. (2007). Effect of laccase and transglutaminase on the textural and water-binding properties of cooked chicken breast meat gels. *European Food Research and Technology*, 225, 75-83.
- Laparra, J.M. & Sanz, Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research*, 61, 219–25.
- Levy-Moonshine, A., Amir, E.A.D. & Keasar, C. (2009). Enhancement of beta-sheet assembly by cooperative hydrogen bonds potential. *Bioinformatics*, 25, 2639-2645.
- Li, J., Li, X., Wang, C., Zhang, M., Xu, Y., Zhou, B. & Yang, Y. (2018). Characteristics of gelling and water holding properties of hen egg white/yolk gel with NaCl addition. *Food Hydrocolloids*, 77, 887-893.
- Li, J., Zhang, Y., Fan, Q., Teng, C., Xie, W., Shi, Y., Su, Y. & Yang, Y. (2018). Combination effects of NaOH and NaCl on the rheology and gel characteristics of hen egg white proteins. *Food Chemistry*, 250, 1-6.
- Liu, J., Pang, Y., Zhang, S., Cleveland, C., Yin, X., Booth, L., Lin, J., Lee, Y. A. L., Mazdiasni, H., Saxton, S., Kirtane, A.R., von Erlach, T., Rogner, J., Langer, R. & Traverso, G. (2017). Triggerable tough hydrogels for gastric resident dosage forms. *Nature communications*, 8, 1-10.

- Liu, M., Zeng, X., Ma, C., Yi, H., Ali, Z., Mou, X., Li, S., Deng, Y. & He, N. (2017). Injectable hydrogels for cartilage and bone tissue engineering. *Bone Research*, 5, 1-20.
- Lu, X., Lu, Z., Yin, L., Cheng, Y., & Li, L. (2010). Effect of preheating temperature and calcium ions on the properties of cold-set soybean protein gel. *Food Research International*, 43, 1673-1683.
- Ma, L., Li, A., Li, T., Li, M., Wang, X., Hussain, M. A., Qayum, A., Jiang, Z., & Hou, J. (2020). Structure and characterization of laccase-crosslinked α -lactalbumin: Impacts of high pressure homogenization pretreatment. *LWT- Food Science and Technology*, 118, 108843.
- Maltais, A., Remondetto, G. E., Gonzalez, R. & Subirade, M. (2005). Formation of soy protein isolate cold-set gels: protein and salt effects. *Journal of Food Science*, 70, C67- C73.
- Maltais, A., Remondetto, G. E. & Subirade, M. (2008). Mechanisms involved in the formation and structure of soya protein cold-set gels: A molecular and supramolecular investigation. *Food Hydrocolloids*, 22, 550-559.
- Maltais, A., Remondetto, G. E. & Subirade, M. (2009). Soy protein cold-set hydrogels as controlled delivery devices for nutraceutical compounds. *Food Hydrocolloids*, 23, 1647-1653.
- Manhivi, V. E., Amonsou, E. O., & Kudanga, T. (2018). Laccase-mediated crosslinking of gluten-free amadumbe flour improves rheological properties. *Food Chemistry*, 264, 157-163.
- Manhivi, V. E., Amonsou, E. O., & Kudanga, T. (2020) Transglutaminase and tyrosinase as potential crosslinking tools for the improvement of rheological properties of gluten-free amadumbe dough. *International Journal of Food Science and Technology*, 1-9.

- Marangoni, A. G., Barbut, S., McGauley, S. E., Marcone, M. & Narine, S. S. (2000). On the structure of particulate gels—The case of salt-induced cold gelation of heat denatured whey protein isolate. *Food Hydrocolloids*, 14, 61–74.
- Marx, C. K., Hertel, T. C. & Pietzsch, M. (2008). Random mutagenesis of a recombinant microbial transglutaminase for the generation of thermostable and heat sensitive variants. *Journal of Biotechnology*, 136, 156–162.
- Matheis, G. & Whitaker, J. R. (1984). Modification of proteins by polyphenol oxidase and peroxidase and their products. *Journal of Food Biochemistry*, 8, 137-162.
- Mattinen, M. L., Kruus, K., Buchert, J., Nielsen, J. H., Andersen, H. J., & Steffensen, C. L. (2005). Laccase-catalyzed polymerization of tyrosine-containing peptides. *The FEBS Journal*, 272, 3640-3650.
- Mattinen, M. L., Hellman, M., Permi, P., Autio, K., Kalkkinen, N., & Buchert, J. (2006). Effect of protein structure on laccase-catalyzed protein oligomerization. *Journal of Agricultural and Food Chemistry*, 54, 883-8890.
- Mayer, A. M. & Staples, R. C. (2002). Laccase: new functions for an old enzyme. *Phytochemistry*, 60, 551–565.
- McClements, D. J. (2017). Recent progress in hydrogel delivery systems for improving nutraceutical bioavailability. *Food hydrocolloids*, 68, 238-245.
- McClements, D. J. (2015). Nanoscale nutrient delivery systems for food applications: improving bioactive dispersibility, stability, and bioavailability. *Journal of Food Science*, 80, 1602-1611.
- McClements, D. J. (2014). *Nanoparticle-and microparticle-based delivery systems: Encapsulation, protection and release of active compounds*. CRC press.
- McClements, D. J. (2012). Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft matter*, 8, 1719-1729.

- McClements, D. J., Decker, E. A., Park, Y. & Weiss, J. 2009. Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition*, 49, 577- 606.
- Mokoonlall, A., Pfannstiel, J., Struch, M., Berger, R. G., & Hinrichs, J. (2016). Structure modification of stirred fermented milk gel due to laccase-catalysed protein crosslinking in a post-processing step. *Innovative Food Science and Emerging Technologies*, 33, 563-570.
- Monogioudi, E., Faccio, G., Lille, M., Poutanen, K., Buchert, J., & Mattinen, M. L. (2011). Effect of enzymatic cross-linking of β -casein on proteolysis by pepsin. *Food Hydrocolloids*, 25, 71-81.
- Moresi, M., Bruno, M., & Parente, E. (2004). Viscoelastic properties of microbial alginate gels by oscillatory dynamic tests. *Journal of Food Engineering*, 64, 179-186.
- Murevanhema, Y. Y. & Jideani, V. A. (2013). Potential of Bambara groundnut (*Vigna subterranea* (L.) Verdc) milk as a probiotic beverage—A review. *Critical Reviews in Food Science and Nutrition*, 53, 954-967.
- Nagano, T., Akasaka, T. & Nishinari, K. (1994a). Dynamic viscoelastic properties of glycinin and β -conglycinin gels from soybeans. *Biopolymers*, 34, 1303–1309.
- Nagano, T., Mori, H. & Nishinari, K. (1994b). Rheological properties and conformational states of β -conglycinin gels at acidic pH. *Biopolymers*, 34, 293–298.
- Ngarize, S., Herman, H., Adams, A. & Howell, N. (2004). Comparison of changes in the secondary structure of unheated, heated, and high-pressure-treated β -lactoglobulin and ovalbumin proteins using Fourier transform Raman spectroscopy and self-deconvolution. *Journal of Agricultural & Food Chemistry*, 52, 6470 - 6477.

- Nguimbou, R. M., Boudjeko, T., Njintang, N. Y., Himeda, M., Scher, J., & Mbofung, C. M. (2014). Mucilage chemical profile and antioxidant properties of giant swamp taro tubers. *Journal of Food Science and Technology*, 51, 3559-3567.
- Nöbel, S., Seifert, B., Daffner, K., Schäfer, J., & Hinrichs, J. (2020). Instantaneous gelation of acid milk gels via customized temperature-time profiles: Screening of concentration and pH suitable for temperature triggered gelation towards 3D-printing. *Food Hydrocolloids*, 106450.
- Okay, O. (2009). General properties of hydrogels. In *Hydrogel Sensors and Actuators*. Springer, Berlin, Heidelberg, 1 - 14.
- Osma, J.F., Toca-Herrera, J. L. & Rodríguez-Couto, S. (2010). Uses of laccases in the food industry. *Enzyme Research*, 1–8.
- Ozer, B., Kirmaci, H. A., Oztekin, S., Hayaloglu, A. & Atamer, M. (2007). Incorporation of microbial transglutaminase into non-fat yogurt production. *International Dairy Journal*. 17, 199 –207.
- Park, M.J., Hur, S.M. & Rhee, H. K. (2002). Online estimation and control of polymer quality in a copolymerization reactor. *AIChE Journal*, 48, 1013 - 1021.
- Peppas, N. A., Bures, P., Leobandung, W. & Ichikawa, H. (2000). Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, 50, 27–46.
- Petrucelli, S. & Añón, M. C. (1995). Thermal aggregation of soy protein isolates. *Journal of Agriculture and Food Chemistry*, 43, 3035– 3041.
- Peyrano, F., de Lamballerie, M., Avanza, M. V., & Speroni, F. (2021). Gelation of cowpea proteins induced by high hydrostatic pressure. *Food Hydrocolloids*, 111, 106191.
- Powers, H. J. (2003). Riboflavin (vitamin B-2) and health. *The American journal of clinical nutrition*, 77, 1352-1360.

- Quan, W., Zhang, C., Zheng, M., Lu, Z., Zhao, H., & Lu, F. (2019). Effects of small laccase from *Streptomyces coelicolor* on the solution and gel properties of whey protein isolate. *LWT- Food Science and Technology*, 101, 17-24.
- Ratnam, D. V., Ankola, D. D., Bhardwaj, V., Sahana, D. K., & Kumar, M. R. (2006). Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. *Journal of Controlled Release*, 113, 189-207.
- Raza, F., Zafar, H., Zhu, Y., Ren, Y., Ullah, A., Khan, A. U., He X., Han, H., Aquib, M. Boakye-Yiadom, A. O. & Ge, L. (2018). A review on recent advances in stabilizing peptides/proteins upon fabrication in hydrogels from biodegradable polymers. *Pharmaceutics*, 10, 16.
- Remondetto, G., Paquin, P. & Subirade, M. (2002). Cold gelation of β -lactoglobulin in the presence of iron. *Journal of Food Science*, 67, 586-595.
- Remondetto, G. & Subirade, M. (2003). Molecular mechanisms of Fe^{2+} - induced β -lactoglobulin cold gelation. *Biopolymers*, 69, 461–469.
- Renkema, J., Knabben, J. & Van Vliet, T. (2001). Gel formation by β -conglycinin and glycinin and their mixtures. *Food Hydrocolloids*, 15, 407-414.
- Renkema, J. M., Gruppen, H. & Van Vliet, T. (2002). Influence of pH and ionic strength on heat-induced formation and rheological properties of soy protein gels in relation to denaturation and their protein compositions. *Journal of Agricultural and Food Chemistry*, 50, 6064-6071.
- Riva, S. (2006). Laccases: blue enzymes for green chemistry. *Trends Biotechnology*, 24, 219-226.
- Rosiak, J. M. & Yoshii, F. (1999). Hydrogels and their medical applications. *Nuclear Instruments and Methods in Physics Research*, 151, 56-64.

- Rubio, L., Motilva, M.J. & Romero, M.P. (2013). Recent advances in biologically active compounds in herbs and spices: a review of the most effective antioxidant and anti-inflammatory active principles. *Critical Reviews in Food Science and Nutrition*, 53, 943–953.
- Rubinstein, M. & Colby, R. H. (2003). *Polymer Physics*, Oxford University Press, Oxford.
- Rui, X., Boye, J. I., Ribereau, S., Simpson, B. K. & Prasher, S. O. (2011). Comparative study of the composition and thermal properties of protein isolates prepared from nine *Phaseolus vulgaris* legume varieties. *Food Research International*, 44, 2497-2504.
- Salvador, P., Toldrà, M., Saguer, E., Carretero, C. & Parés, D. (2009). Microstructure–function relationships of heat-induced gels of porcine haemoglobin. *Food Hydrocolloids*, 23, 1654-1659.
- Selinheimo, E., Lampila, P., Mattinen, M. L., & Buchert, J. (2008). Formation of protein–oligosaccharide conjugates by laccase and tyrosinase. *Journal of Agricultural and Food Chemistry*, 56, 3118-3128.
- Shand, P. J., Ya, H., Pietrasik, Z., & Wanasundara, P. K. J. P. D. (2007). Physicochemical and textural properties of heat-induced pea protein isolate gels. *Food Chemistry*, 102, 1119-1130.
- Shand, P. J., Ya, H., Pietrasik, Z., & Wanasundara, P. K. J. P. D. (2008). Transglutaminase treatment of pea proteins: Effect on physicochemical and rheological properties of heat-induced protein gels. *Food Chemistry*, 107, 692-699.
- Shevkani, K., Kaur, A., Kumar, S. & Singh, N. (2015). Cowpea protein isolates: Functional properties and application in gluten-free rice muffins. *LWT-Food Science and Technology*, 63, 927-933.
- Shin, G. H., Kim, J. T., & Park, H. J. (2015). Recent developments in nanoformulations of lipophilic functional foods. *Trends in Food Science and Technology*, 46, 144-157

- Smetana, K. (1993). Cell biology of hydrogels. *Biomaterials*, 14, 1046-1050.
- Song, F. & Zhang, L.-M. (2008). Enzyme-catalyzed formation and structure characteristics of a protein-based hydrogel. *The Journal of Physical Chemistry B*, 112, 13749-13755.
- Steffensen, C. L., Andersen, M. L., Degn, P. E., & Nielsen, J. H. (2008). Cross-linking proteins by laccase-catalyzed oxidation: importance relative to other modifications. *Journal of Agricultural and Food Chemistry*, 56, 12002-12010.
- Strauss, G. & Gibson, S. A. (2004). Plant phenolics as cross-linkers of gelatine gels and gelatine-based coacervates for use as food ingredients. *Food Hydrocolloids*, 18, 81 – 89.
- Struch, M., Linke, D., Mokoonlall, A., Hinrichs, J., & Berger, R. G. (2015). Laccase-catalysed cross-linking of a yoghurt-like model system made from skimmed milk with added food-grade mediators. *International Dairy Journal*, 49, 89-94.
- Sun, X. D., & Arntfield, S. D. (2010). Gelation properties of salt-extracted pea protein induced by heat treatment. *Food Research International*, 43, 509-515.
- Sun, X. D., & Arntfield, S. D. (2011). Gelation properties of salt-extracted pea protein isolate induced by heat treatment: Effect of heating and cooling rate. *Food Chemistry*, 124, 1011-1016.
- Tang, C. H., Wu, H., Yu, H. P., Li, L., Chen, Z. & Yang, X. Q. (2006). Coagulation and gelation of soy protein isolates induced by microbial transglutaminase. *Journal of Food Biochemistry*, 30, 35-55.
- Tao, Y., Zhang, R., Xu, W., Bai, Z., Zhou, Y., Zhao, S., Xu, Y. & Yu, D. (2016). Rheological behavior and microstructure of release-controlled hydrogels based on xanthan gum crosslinked with sodium trimetaphosphate. *Food Hydrocolloids*, 52, 923-933.

- Teixeira, M. S. L., Feijen, J., Blitterswijk, van A.C. Dijkstra, J.P. & Karperien, M. (2012). Enzyme-catalyzed crosslinkable hydrogels: Emerging strategies for tissue engineering *Biomaterials*, 33, 1281-1290.
- Thalmann, C. & Lötzbeyer, T. (2002). Enzymatic cross-linking of proteins with tyrosinase. *European Food Research and Technology*, 214, 276-281.
- The Lancet. (2016). Global burden of diseases study 2015 assesses the study of the World's health.
- Trespalacios, P. & Pla, R. (2007). Simultaneous application of transglutaminase and high pressure to improve functional properties of chicken meat gels. *Food Chemistry*, 100, 264–272.
- Ullah, F., Othman, M. B. H., Javed, F., Ahmad, Z. & Akil, H. M. (2015). Classification, processing and application of hydrogels: A review. *Materials Science and Engineering: C*, 57, 414-433.
- United Nations. (2017). World population prospects: *The 2017 revision, key findings and advance tables*. Working paper No. ESA/P/WP/248. Department of economic and social affairs, population division. New york.
- Varaprasad, K., Raghavendra, M.G., Jayaramudu, T., Yallapu, M.M. & Sadiku, R. (2017). A mini review on hydrogels classification and recent developments in miscellaneous applications. *Materials Science and Engineering*, 79, 958–971
- Velikov, K. P. & Pelan, E. (2008). Colloidal delivery systems for micronutrients and nutraceuticals. *Soft Matter*, 4, 1964-1980.
- Villalonga, R., Fernandez, M., Fragoso, A., Cao, R., Mariniello, L. & Porta, R. (2003a). Thermal stabilization of trypsin by enzymic modification with b-cyclodextrin derivatives. *Biotechnology and Applied Biochemistry*, 38, 53 –59.

- Wang, C. H. & Damodaran, S. (1990). Thermal gelation of globular proteins: weight-average molecular weight dependence of gel strength. *Journal of Agricultural and Food Chemistry*, 38, 1157-1164.
- Wang, K. Q., Luo, S. Z., Zhong, X. Y., Cai, J., Jiang, S. T. & Zheng, Z. (2017). Changes in chemical interactions and protein conformation during heat-induced wheat gluten gel formation. *Food Chemistry*, 214, 393-399.
- Wang, K., Luo, S., Cai, J., Sun, Q., Zhao, Y., Zhong, X., Jiang, S. & Zheng, Z. (2016). Effects of partial hydrolysis and subsequent cross-linking on wheat gluten physicochemical properties and structure. *Food chemistry*, 197, 168-174.
- Wang, P., Xu, X., Huang, M., Huang, M. & Zhou, G. (2014). Effect of pH on heat-induced gelation of duck blood plasma protein. *Food Hydrocolloids*, 35, 324-331.
- Wen, X., Jin, F., Regenstein, J. M. & Wang, F. (2018). Transglutaminase induced gels using bitter apricot kernel protein: Chemical, textural and release properties. *Food Bioscience*, 26, 15-22.
- Westphalen, A. D., Briggs, J. L. & Lonergan, S. M. (2005). Influence of pH on rheological properties of porcine myofibrillar protein during heat induced gelation. *Meat Science*, 70, 293-299.
- World Health Organisation (WHO). (2018). *Non communicable diseases: fact sheet*. Geneva: World Health Organisation 2018. <http://www.who.int/mediacentre/factsheets/fs355/en/>
- WHO. (2015). *Noncommunicable Diseases Progress Monitor*. Geneva 27, Switzerland.
- World Health Organization. (2014). *Global status report on noncommunicable diseases 2014* (No. WHO/NMH/NVI/15.1). World Health Organization.
- Wu, C., Hua, Y., Chen, Y., Kong, X. & Zhang, C. (2017). Effect of temperature, ionic strength & 11S ratio on the rheological properties of heat-induced soy protein gels in relation to network proteins content and aggregates size. *Food Hydrocolloids*, 66, 389-395.

- Xiao, Y., Kang, S., Liu, Y., Guo, X., Li, M., & Xu, H. Effect and mechanism of calcium ions on the gelation properties of cellulose nanocrystals-whey protein isolate composite gels. *Food Hydrocolloids*, 111, 106401.
- Yan, Y. Z., Zheng, W., Huang, D. Z., Xiao, Z. Y., Park, S. S., Ha, C. S., & Zhai, S. R. (2020). Hierarchical multi-porous carbonaceous beads prepared with nano-CaCO₃ in-situ encapsulated hydrogels for efficient batch and column removal of antibiotics from water. *Microporous and Mesoporous Materials*, 293, 109830.
- Yang, X., Liu, G., Peng, L., Guo, J., Tao, L., Yuan, J., Chang, C., Wei, Y. & Zhang, L. (2017). Highly efficient self-healable and dual responsive cellulose-based hydrogels for controlled release and 3D cell culture. *Advanced Functional Materials*, 27, 1703174.
- Yokoyama, K., Nio, N. & Kikuchi, Y. (2004). Properties and applications of microbial transglutaminase. *Applied Microbiology and Biotechnology*, 64, 447–454.
- Yoshimatsu, H., Yonezawa, A., Yao, Y., Sugano, K., Nakagawa, S., Omura, T., & Matsubara, K. (2014). Functional involvement of RFVT3/SLC52A3 in intestinal riboflavin absorption. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 306, G102-G110.
- Zaleska, H., Ring, S., & Tomasik, P. (2001). Electrosynthesis of potato starch–whey protein isolate complexes. *Carbohydrate Polymers*, 45, 89-94.
- Zhang, P., Hu, T., Feng, S., Xu, Q., Zheng, T., Zhou, M., Chu, X., Huang, X., Lu, X., Pan, S. & Li-Chan, E.C., (2016). Effect of high intensity ultrasound on transglutaminase-catalyzed soy protein isolate cold set gel. *Ultrasonics Sonochemistry*, 29, 380-387.
- Zhang, H., Wang, G. & Yang, H. (2011). Drug delivery systems for differential release in combination therapy. *Expert Opinion on Drug Delivery*, 8, 171-190.

- Zhang, Z., Zhang, R., Chen, L., Tong, Q. & McClements, D. J. (2015). Designing hydrogel particles for controlled or targeted release of lipophilic bioactive agents in the gastrointestinal tract. *European Polymer Journal*, 72, 698-716.
- Zhao, H., Chen, J., Hemar, Y., & Cui, B. (2020). Improvement of the rheological and textural properties of calcium sulfate-induced soy protein isolate gels by the incorporation of different polysaccharides. *Food Chemistry*, 310, 125983.
- Zhao, X., Huebsch, N., Mooney, D. J. & Suo, Z. (2010). Stress-relaxation behavior in gels with ionic and covalent crosslinks. *Journal of Applied Physics*, 1, 107- 114.
- Zhou, J. Z., Zhang, H., Gao, L., Wang, L. & Qian, H. F. (2015). Influence of pH and ionic strength on heat-induced formation and rheological properties of cottonseed protein gels. *Food and Bioproducts Processing*, 96, 27-34.

Appendices

LWT - Food Science and Technology 123 (2020) 109070



Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt



Rheological and microstructural properties of Bambara groundnut protein gels



Faith Matiza Ruzengwe, Eric O. Amonsou, Tukayi Kudanga*

Department of Biotechnology and Food Technology, Durban University of Technology, P.O. BOX 1334, Durban, 4000, South Africa

ARTICLE INFO

Keywords:

Bambara protein isolate
Gel
Rheological properties

ABSTRACT

The increasing demand for plant proteins has stimulated the search for alternative and novel protein sources for various food applications. Rheological and microstructural properties of Bambara protein isolate (BPI) gels, prepared under varying pH and salt conditions, were investigated and optimised using response surface methodology. BPI gels showed $G' > G''$ over a frequency range of 0–100 rad s^{-1} . Although BPI gels displayed the characteristics of weak gels, slightly acidic conditions (pH 5.59–6) coupled with low NaCl concentration (0.5 mol/L) promoted the formation of more rigid gels. At slightly acidic conditions, NaCl had a stabilising effect on the protein structure as demonstrated by the increase in denaturation temperature and enthalpy of denaturation with increasing NaCl concentration. In addition, an increase in α helix and β turns contents and disappearance of random coils were observed when 0.5 mol/L NaCl was added at pH 6. Gels with the highest strength had the lowest water holding capacity and thiol content, suggesting participation of more disulphide linkages during network formation. Microscopy images of gels prepared at the slightly acidic pH in the presence of NaCl showed porous homogeneous aggregates. The BPI gels have potential application for the improvement of food texture.

Food Research International 137 (2020) 109734



Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres



Transglutaminase-mediated crosslinking of Bambara groundnut protein hydrogels: Implications on rheological, textural and microstructural properties



Faith Matiza Ruzengwe, Eric O. Amonsou, Tukayi Kudanga*

Department of Biotechnology and Food Technology, Durban University of Technology, P.O. BOX 1334, Durban 4000, South Africa

ARTICLE INFO

Keywords:

Bambara protein isolate
Hydrogel
Frequency sweep
Hardness
Network structure

ABSTRACT

Interest in plant protein-based hydrogels with desirable strength has been increasing in recent years. In this study, Bambara groundnut protein isolate (BPI) was crosslinked with transglutaminase (TGase) (0 – 25 U/g protein) during gelation and rheological, textural and microstructural properties of the resulting hydrogels were investigated. Treatment with TGase up to 15 U/g protein resulted in the formation of hydrogels with small pores and an organised homogeneous network. G' of TGase-treated BPI hydrogels was more than ten-fold higher than G'' throughout the frequency range of 0–100 rad/s , suggesting dominance of the elastic like behaviour. BPI hydrogel with the highest G' (6967 Pa) and hardness (5.60 N) was formed at 15 U/g protein of TGase activity. The hydrogel had a high distribution β -sheets (53.52%) and α -helices (26.17%) as compared to the β -turns and random coils. However, a further increase in TGase activity did not improve the hydrogel properties. Transglutaminase mediated crosslinking of BPI hydrogel was demonstrated by the reduction in amine and thiol groups and the formation of a new protein band (56 kDa) in crosslinked hydrogels. Overall, TGase promoted the formation of a strong gel with an organised network.