ENZYMATIC MODIFICATION OF AMADUMBE FLOUR FOR GLUTEN-FREE APPLICATIONS

By

Vimbainashe Edina Manhivi

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Department of Biotechnology and Food Technology

Faculty of Applied Sciences

Durban University of Technology, Durban, South Africa

Supervisor: Professor Tukayi Kudanga

Co-supervisor: Professor Eric Oscar Amonsou

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DECLARATION

I hereby declare that the work reported in this thesis and submitted at the Department of Biotechnology and Food Technology 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.

[redacted] 17/08/2018

Vimbainashe E Manhivi Date

Student

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

[redacted] 17/08/2018

Prof Tukayi Kudanga Date

Supervisor

[redacted] 17/08/2018

Prof Eric Amonsou Date

Co-Supervisor

DEDICATION

This thesis is dedicated to the Almighty God, for men cannot receive any good thing unless it comes from above. I also like to dedicate this thesis to my husband Menard, my son and my parents!

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I express my sincere gratitude to him who was and is to come, the Lord Jesus, for the strength and life. I also wish to appreciate my supervisor, Professor Tukayi Kudanga for believing in me, training me and guiding me throughout this programme. I really appreciate. To Professor Eric Amonsou, thank you for teaching me to really appreciate science through your constructive criticism and zero tolerance to mediocre work. I wish to thank the Durban University of Technology (DUT), for the scholarship towards remission of fees and the research funding for the programme. The financial support of Agricultural Research Council and the National Research Foundation is also appreciated. I also wish to appreciate the entire staff of the Department of Biotechnology and Food Technology. I want to also thank my husband, Eng Menard Manhivi, the love of my life, for allowing me to be away from our home and supporting me wholeheartedly. I also appreciate my son, Munotidaishe Lloyd, who was deprived of motherly love, care and attention as I got drained by my work. I also wish to appreciate my parents, Fungai and Cornelius Dube, who taught me that I could do all things through Christ who gives me strength. My Pastors, Mr and Mrs Jiriji, for the prayers and spiritual guidance. My colleagues and friends, Faith Seke, Faith Matiza Ruzengwe (Chaminuka), Ruth Mwakanjumba, Betty Ajibade, Omotola Olagunju, Agnes Mukurumbira, Bruce Mawoyo, Zikhona Nyawose, Nyasha Busu, Rudean Van Wyk, Melvin Makolomakwa, Kabange Kasumbwe, Sithembile Shongwe, Bodine Mazibuko, Sandile Ngubane, Blessing Nemadziva and others who one way or the other contributed to the success of this programme. My sisters and brothers in love, Susan and Last Masuka and Eustina and Lloyd Manhivi, thank you for babysitting and the support. I appreciate you.

ABSTRACT

The production of gluten-free bread from gluten-free flours remains a technological challenge. Different strategies have been employed to improve the dough rheological properties. Enzymatic modification of the proteins in dough may result in polymers that mimic gluten. In this study gluten-free amadumbe flour was modified using single and optimised multiple crosslinking enzyme systems for the improvement of rheological properties and bread quality.

Specifically, compositional, rheological and thermal properties of amadumbe and cactus mucilages were investigated as potential hydrocolloids and as substrates for crosslinking enzymes in gluten-free bread production. The effects of laccase, tyrosinase and transglutaminase on amadumbe dough rheology were also investigated. Model reactions were used to demonstrate the different enzymatic reactions occurring in amadumbe dough treated with the crosslinking enzymes. The combination of enzymes was then optimised using response surface methodology (RSM), to produce dough with improved G' and G". Xanthan gum, amadumbe mucilage or cactus mucilage were then added to the dough with an optimum enzyme combination. The effect of these enzymes and hydrocolloids were studied on the bread properties.

The mucilages had a similar composition of monosaccharides and amino acids, except for the absence of rhamnose in amadumbe mucilage. Cactus mucilage showed a pseudoplastic flow behaviour whilst amadumbe mucilage showed a Newtonian flow behaviour at up to 5% (w/v) concentrations. The mucilages contained phenolics and amino acids such as lysine, tyrosine and glutamine, which are potential enzyme substrates. *Trametes versicolor* laccase catalysed the crosslinking of phenolics and thiols producing a wide range of crosslinking products which included homo- and hetero-conjugates, as demonstrated by mass spectroscopy. Thiol and total phenolic contents of dough decreased by up to 28% and 93%, respectively, as laccase activity was increased (0-3 U/g flour), confirming crosslinking reactions. Laccase-catalysed modification of amadumbe dough increased dough viscoelasticity, as shown by the increase in G' and G". Tan δ decreased with increase in laccase activity indicating an increase in the elastic character of the dough. Tyrosinase oxidation resulted in a 7.7 – 39.4% decrease in dough free amine and a 16.8 – 46.3% decrease in the dough thiol content as activity was increased (0-80 U/g flour).

Transglutaminase treatment decreased the dough free amino groups by 10 – 38.1% as activity was increased from 0 to 2 U/g flour. An increase in dough G' and G", showed that both transglutaminase and tyrosinase improved dough viscoelasticity. Evidence of transglutaminase and tyrosinase crosslinking was provided by relevant model reactions monitored by mass spectrometry. Reaction model data showed the formation of the glutamyl-lysine bond due to transglutaminase crosslinking, whilst tyrosinase crosslinking resulted in disulphide and dityrosine bond formation. The viscosity and elasticity of amadumbe dough containing soy protein were optimised using a central composite design and the enzyme combination resulting in maximum G' and G", and minimum Tan δ was selected and verified. The predicted optimal enzyme activities (LAC, 1.78 U/g flour), (TYR, 79 U/g flour) and TG, 1.97 U/g flour) resulted in amadumbe dough that had a higher G' and G", as well as bread with a higher specific volume and lower crumb hardness compared to the dough without enzymes or with a single enzyme system. Addition of cactus and or amadumbe mucilage to the dough containing the optimum enzyme combination further improved dough viscoelasticity, improved bread specific volume, and significantly ($p < 0.05$) reduced bake loss and crumb moisture loss. The better bread was produced from dough with an optimum enzyme combination and 2% cactus mucilage. Sensory evaluation revealed that enzymes and cactus mucilage improved bread texture, appearance and overall acceptability but did not significantly affect bread aroma and taste.

Overall, the combined effect of multiple enzyme-catalysed modification of gluten-free amadumbe flour and amendment with hydrocolloid resulted in more acceptable bread quality than single enzyme systems or unmodified flour. Therefore the optimised combination of enzymes have potential for application in gluten-free bread production.

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 celiac disease, gluten-free bread production, hydrocolloids and a discussion on enzymatic modification of gluten-free flours. Chapter three presents the characterisation of amadumbe and cactus mucilages as potential hydrocolloids in gluten-free bread making. Chapter four reports on the effect of laccase on amadumbe dough rheology. The effect of transglutaminase and tyrosinase on amadumbe dough rheology is covered in Chapter five. Chapter six reports on the optimization of the three enzymes (transglutaminase, laccase and tyrosinase) using response surface methodology and the effect of addition of selected hydrocolloids to the optimum dough. Chapter seven is a general discussion of the entire findings. Chapter eight concludes the thesis and suggests recommendations for future studies.

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ABBREVIATIONS

Anti-tTG: Anti-Tissue Transglutaminase Antibodies

CD: Celiac Disease

CMC: Carboxymethylcellulose

GF: Gluten-free

GuHCI: Guanidine Hydrochloride

HLA: Human Leukocytic Antigens

HPMC: Hydroxypropyl methylcellulose

IBS: Irritable Bowel Syndrome

Ig: Immunoglobulin

LAC: Laccase

SH: Sulfhydryl

SPI: Soy Protein Isolate

SS: Disulphide

TG: transglutaminase

Tris-Gly: Tris-Glycine

TYR: tyrosinase

LIST OF TABLES

PUBLICATIONS AND CONFERENCES

Publications

Manhivi, V. E., Venter, S., Amonsou, E. O. & Kudanga, T. (2018). Composition, thermal and rheological properties of polysaccharides from amadumbe (*Colocasia esculenta*) and cactus (*Opuntia spp*.). *Carbohydrate Polymers,* 195**,** 163-169.

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. (2018). Transglutaminase and tyrosinase as potential crosslinking tools for the improvement of rheological properties of gluten-free amadumbe dough. *Submitted to Food Research International*.

Manhivi, V. E., Amonsou, E. O., & Kudanga, T. (2018). Effect of multiple crosslinking enzyme systems and hydrocolloids on gluten-free amadumbe dough rheology and bread quality. *Submitted to Journal of Food Engineering*.

Conferences

Manhivi, V. E., Amonsou, E. O., & Kudanga, T. Composition, thermal and rheological properties of amadumbe (*Colocasia esculenta*) and cactus (*Opuntia spp.*) as potential hydrocolloids in gluten-free dough systems. South African Association of Food Scientist and Technologist (SAAFOST), Cape Town, September 2017.

CHAPTER ONE

1.0 Introduction

The incidence of celiac disease (CD) has been increasing in recent years mainly because of the high frequency of consumption of gluten-containing foods such as bread and pasta, and the availability of more advanced diagnostic techniques (Rubio-Tapia et al., 2012). The only way of managing celiac disease is a gluten-free diet. Some health-conscious consumers have also eliminated gluten from their diets. It has therefore become necessary to continuously develop gluten-free products to meet the growing demand. Bread is one of the most consumed bakery product. However, the development of a dough from gluten-free flours is technologically challenging. Gluten-free dough is of a more fluid consistency compared to wheat flour dough. The consequent viscosity and rheological properties resemble that of cake batters (Nitcheu Ngemakwe et al., 2014). This is because gluten is responsible for water absorption, dough strength, extensibility, elasticity and viscosity (Renzetti et al., 2008). Gluten is also responsible for a satisfactory texture on consumption and a spongy bread with a honeycomb structure (Wieser, 2007).

In order to simulate gluten viscoelasticity in gluten-free dough, diverse ingredients have been proposed. Since gluten-free cereals and starches are low in proteins, dairy proteins, egg/ egg white proteins and legume proteins such as soybean and pea proteins have been used to increase the protein amount and modify the dough-forming properties of gluten-free flours (Renzetti et al., 2008). However, these proteins still lack a viscoelastic character. Consequentially, bread with a low specific volume and dense crumb texture is obtained. This has prompted the use of gums and hydrocolloids such as guar, xanthan, pectin, carrageenan, alginate and carboxymethyl cellulose (CMC) (Lazaridou et al., 2007) in gluten-free products since they increase the viscosity of gluten-free dough, preventing gas bubbles from coalescing. However, dough hardening and bread with a lower specific volume have been observed as hydrocolloid concentration is increased. Hydroxypropyl methylcellulose (HPMC) addition in dough preparation resulted in bread with relatively high specific volume and better texture (Lazaridou et al., 2007). This was attributed to the ability of the HPMC to modify viscosity and also act as an emulsifier due to the presence of methyl groups. Mucilages, such as those from amadumbe, may have a similar surface active effect

owing to the existence of hydrophilic carbohydrates and hydrophobic proteins in them. Although hydrocolloids have resulted in improvement in bread properties, gluten-free bread remains relatively inferior. Enzymes are therefore being considered for crosslinking and modification of gluten-free dough.

The crosslinking enzymes that are most promising for gluten-free dough modification are laccases, tyrosinases and transglutaminases. Laccase is a multi-copper containing polyphenol oxidase that has a wide substrate specificity. It catalyses the oxidation of various phenolic compounds, phenolic and alkyl amines as well as thiols, through one-electron removal, generating unstable phenolic radicals (Kudanga et al., 2011). The radicals are stabilised through radical-radical coupling. The crosslinking of ferulic acid side chains attached to arabinoxylan via ester bonds strengthens the dough and increases its gas holding capacity (Joye et al., 2009). Disulphide cross-links, which are important as the backbone for gluten elasticity, may also be formed between cysteine and glutathione residues (Flander et al., 2011). Tyrosinase is a copper-containing phenol oxidase which catalyses the oxidation of mono and diphenols to a corresponding *o*-quinone (Flander et al., 2011). Quinones are highly unstable and can further react to form higher molecular weight polymers. Tyrosinase increased the visco-elasticity of wheat dough (Renzetti et al., 2010), and the loaf volume and decreased hardness of wheat bread (Selinheimo et al., 2007) and oat bread (Renzetti et al., 2010). Tyrosinase also increased the loaf volume of gluten-free oat bread (Flander et al., 2011). Transglutaminase (TG) has been widely used in foods because of its ability to modify protein functionality. TG forms covalent cross-links between L-lysine and L-glutamine amino acid residues, resulting in new inter and intra-molecular peptide bonds (Onyango et al., 2010). This increases the molecular weight of proteins, mimicking the higher molecular weight fraction of gluten which is responsible for dough elasticity (Smerdel et al., 2012). In the absence of free amines, TG deamidates glutamine to glutamate, increasing the hydrophilic properties of the dough (Onyango et al., 2010).

The use of a single enzyme may limit the effectiveness of enzymatic processes. Transglutaminase is a highly specific enzyme and lysine is usually a limiting amino acid. Although laccase and tyrosinase have a wider substrate range, availability of reactive substrates may still be limited in gluten-free flours. Laccase effectiveness is limited in the absence of phenolics whilst tyrosinase

reacts exclusively with tyrosine or its hydroxylated form. The use of a combination of enzymes may increase their crosslinking efficiency. Furthermore, it is necessary to use a starch base that contains the necessary substrates for these enzymes. A traditional underutilised crop, such as amadumbe (*Colocasia esculenta*) is a potential source of gluten-free flour. The flour already contains mucilage, a hydrocolloid with potential enzyme substrates, which may make it unnecessary to amend it with additional hydrocolloids.

Therefore, the aim of this research was to determine the effect of laccase, transglutaminase and tyrosinase, separately and combined, on gluten-free amadumbe dough rheology. Evidence of the enzymatic reactions was demonstrated for the first time using model reactions. In addition, the effect of adding a hydrocolloid (amadumbe or cactus mucilage) to the optimum enzyme combination was investigated on dough rheology and bread characteristics. Cactus cladode utilisation has been deemed necessary in order to prevent the invasiveness of the plant (Novoa et al., 2015). Hence, the potential of cactus mucilage as a hydrocolloid in amadumbe gluten-free dough is also investigated.

CHAPTER TWO

2.0. Literature Review

The following literature review gives a brief overview of celiac disease (CD), its prevalence, the biochemistry and physiology of the disease. Also discussed in this chapter are the challenges faced in the food industry whilst trying to simulate gluten functionality in gluten-free baked products and the ways that have been proposed to improve the quality of gluten-free foods.

2.1 Celiac disease

CD is a permanent, immune-mediated, inflammatory enteropathy which is characterised by damage to the jejunal mucosa as a response to ingested gluten-containing cereals in individuals who are already genetically predisposed (Badenhorst, 2014). Gluten is a protein occurring in wheat, rye, and barley. Therefore, it is also found in many commonly consumed food items such as bread and pasta. Gluten proteins have been classified into two major solubility fractions, the gliadins and glutenins, both of which are believed to contain peptides that activate the disease (Wolter et al., 2013). Gliadins are more immunogenic compared to glutenins (Badenhorst, 2014).

Gliadin and glutenin undergo incomplete digestion. Gliadin has been observed to be resistant to degradation by gastric, pancreatic and other digestive enzymes produced by the intestinal brush border membrane due to the high proline content. This causes peptide derivatives to persist in the intestines after gluten ingestion (Badenhorst, 2014). Since glutenins and gliadins contain a high amount of glutamine and are low in lysine, they are preferentially deamidated by transglutaminase. Furthermore, the gliadin epitopes recognized by T cells are the tissue transglutaminase targeted residues for deamidation (Gayathri and Rashmi, 2014).

Transglutaminase, therefore, further deaminates gluten proteins and hence increases the immunogenicity of the peptide derivatives to patients with celiac disease by introducing negative charges (Ludvigsson et al., 2013). The human leukocytic antigens (HLA), HLA-DQ2 (specific to gliadins) or HLA-DQ8 (specific to glutelins) bind to negatively charged residues in peptides with a great affinity (Badenhorst, 2014). Therefore, CD4 T cells in people with celiac disease are

activated by deamidated gluten residues presented by HLA-DQ2 and DQ8 molecules, causing their proliferation. After accepting the gluten residues presented by HLA, the T cells further trigger the humoral mediated immune pathway and activate B cells to produce antibodies against the gluten peptides, tissue transglutaminase and an array of cytokines (Gayathri and Rashmi, 2014). CD is believed to be the outcome of both environmental (gluten) and genetic factors (Cataldo and Montalto, 2007). In order for a gluten immunological response to occur, the presence of (HLA) mainly HLA-DQ2 or HLA DQ-8 molecules, which are genetically coded for, and the production of auto-antibodies to tissue transglutaminase are necessary to hasten the occurrence of the disease (Gayathri and Rashmi, 2014). Cytokines produced by people with celiac disease make the enteric lymphocytes as cytotoxic cells, these damage the enteric cells. This promotes inflammatory response of the upper small intestine. In normal small intestinal mucosa villi and crypts can be distinguished under a light microscope (Fig 2.1). However, villi in celiacs appear as flattened and have an increased number of cytotoxic lymphocytes (Mendoza and McGough, 2005).

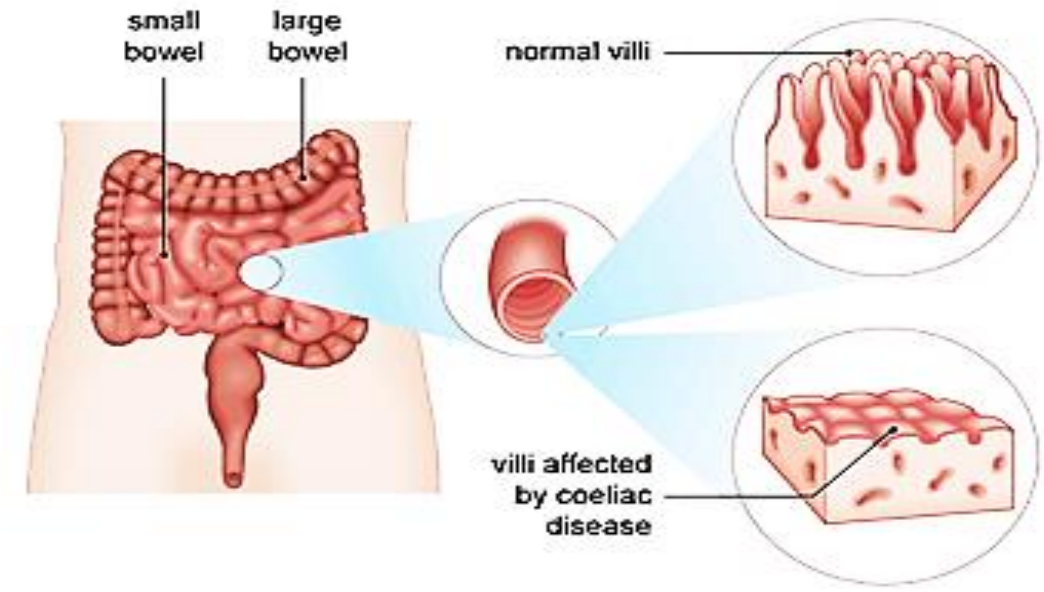


Figure 2.1: Effects of celiac disease on villi (Picture adapted from Arotec Diagnostics, 2016)

2.1.1 Epidemiology of Celiac Disease

Risk analysis for CD is based on the frequency of gluten ingestion and the genetic predisposition in people. This is because the world geographical distribution of CD has seemed to be related to the spread of wheat consumption as well as the migration patterns of human beings. CD occurs in

approximately 1% of the population in western countries and the genetic predisposition is high in people with European ancestry (Badenhorst, 2014). Occurrence in Africa has been estimated at 0.33% and might possibly increase in the near future due to a high frequency of gluten consumption and better diagnostic tools (Seguchi et al., 2012).

In Northern African populations, surprisingly high occurrences of CD have previously been reported both in the general people and in high risk groups (Cataldo and Montalto, 2007; Gujral et al., 2012). The association between CD and insulin dependent diabetes mellitus in Algeria was observed as 16.4%, the highest frequency in the world (Boudraa et al., 1996). Furthermore, the prevalence of anti-endomysium antibodies as observed from a recent serological screening in 2 500 Tunisian blood donors which seemed healthy showed that they are present in 1:355 of the general population, which is close to the occurrence in Europeans (Mankai et al., 2006). The highest known prevalence of CD in the world, of 5.6%, has been observed in the Saharawi people of North Africa. They are of Arab and Berber origin and live as refugees in Algeria (Cataldo and Montalto, 2007). This shows that CD can occur in people of any ethnic group.

There are no reported statistics on the exact prevalence of CD in South Africa. However, a modelled estimate study conducted by Byass et al (2010) suggested that globally 2.2 million children, under the age of five, were living with celiac disease. An estimated mortality of 42 000 was noted and a higher mortality was estimated for the African region than other regions as defined by WHO (Byass et al., 2010). Therefore, CD must be considered a public health problem occurring worldwide, affecting all the racial groups in every part of the world where there is regular consumption of wheat (Cataldo and Montalto, 2007).

The diagnosis of celiac disease is believed to follow an iceberg model (Fig 2.2). This is a familiar model used to depict a clearer picture of the epidemiology of CD. The majority of patients have silent CD, which may continue undiagnosed as the disease has no gastrointestinal symptoms. This may have also caused an underestimation of the prevalence of the disease.

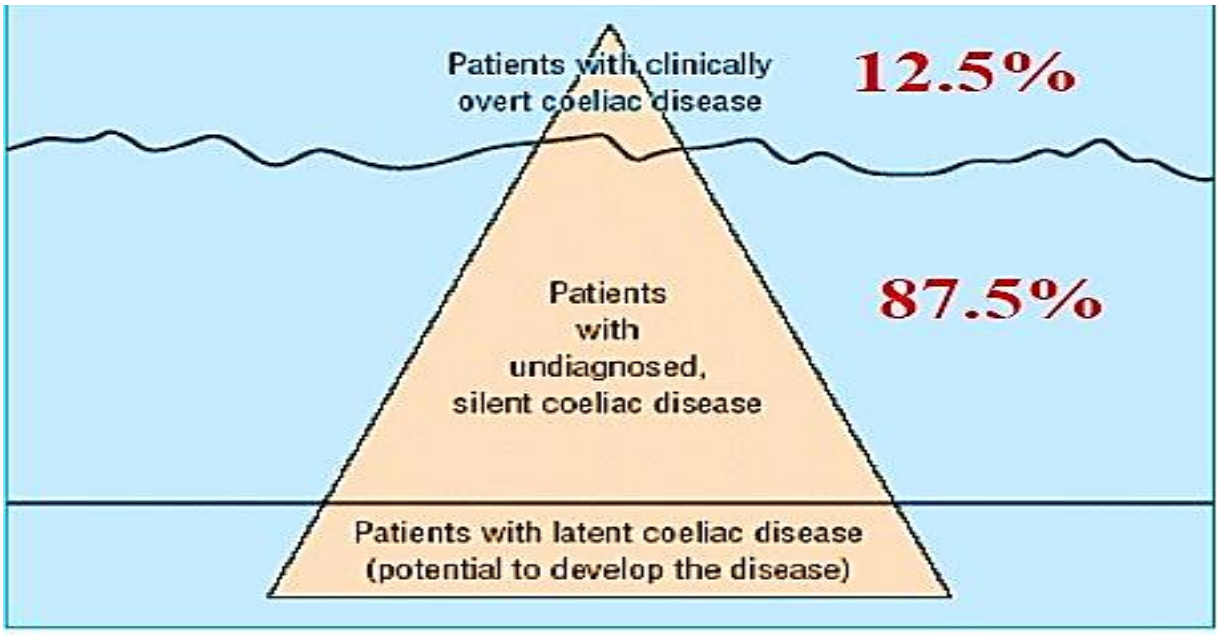


Figure 2.2: Iceberg model which shows the occurrence of celiac disease (Feighery, 1999**)**

2.1.2 Problems associated with celiac disease

CD in children is often observed by retarded growth, abnormal stools, possible vomiting, no weight gain, abdominal distension, muscle wasting, irritability, dental enamel defects and a poor appetite (Mendoza and McGough, 2005). In severe cases, chronic diarrhoea, stunting, delayed puberty, anaemia and mortality are also observed (Seguchi et al., 2012). The onset of the symptoms is usually gradual. Occasionally, constipation may occur and alternate with diarrhoea in some children (Mendoza and McGough, 2005).

The diverseness and vagueness of adult symptoms might be a reason for the underdiagnosis of CD as these indications can often be mistaken for other medical conditions such as Irritable Bowel Syndrome (IBS) (Mendoza and McGough, 2005). Almost 36% of patients with CD are misdiagnosed as suffering from IBS, enduring more than 11 years with symptoms before being diagnosed correctly (Badenhorst, 2014). The majority of patients (45-85%), display gastrointestinal symptoms, while vitamin and protein deficiencies, joint pain, fatigue and elevated liver enzymes may also be present in others (Bao et al., 2012; Smith and Goodfellow, 2011).

However, some have been diagnosed after they had problems with iron deficiency anaemia, osteopenia, osteoporosis and other symptoms not normally associated with the disease, such as pain in the upper abdomen, gastroesophageal and reflux dyspepsia (Badenhorst, 2014). Other non-intestinal complications were also noted to occur in people with CD. In 10 to 20% of the patients, dermatitis herpetiformis, a pathognomonic rash occurs. It is an intensely pruritic condition with papulovesicular eruptions usually proportionally occurring in areas such as the hairline, neck, buttocks, back, scalp, groin or face (Hull et al., 2008).

Although rare, CD may also manifest as a neurological disease characterised by a symmetrical distal sensory neuropathy, cerebellar ataxia or a migraine. It may also cause blood abnormalities such as high amounts of serum amylase and hypoalbuminaemia with a large sedimentation rate such as that above 100 (Green, 2005). Untreated CD may also result in infertility and malignant tumours of the bowel (Araújo and Araújo, 2012; Smith and Goodfellow, 2011). Some other symptoms are reported in Table 2.1.

Table 2.1: Celiac disease signs and symptoms

Common signs and symptoms of celiac disease	
Anaemia	Easy bruising
Deficiencies of vitamins A, D, E, K	Bone or joint pain
Abdominal bloating	Swollen limbs
Indigestion,	Mouth ulcers
Recurring diarrhoea	Infertility in both sexes
Constipation	Depression
Extreme weakness/fatigue	Migraine headaches
Weight problems	Neurological conditions
Nausea	Miscarriages

(Araújo and Araújo, 2012)

2.1.3 Diagnosis and treatment of CD

The diagnosis of CD starts with a blood test, which if positive, characteristic mucosal damage checked by using a biopsy and the confirmation of diagnosis is obtained (Badenhorst, 2014). The occurrences of anti-endomysial antibodies (IgA-EMA) and anti-tissue transglutaminase antibodies (IgA-tTG) are the basis for blood tests. Anti-gliadin antibodies are, however, tested as they are more accurate in children under 2 years (Badenhorst, 2014). The classic presence of the mucosal enterocytes combined with villi and crypts which appear as flattened structures with a mosaic pattern when observed under a light microscope form the basis of the biopsy method used to diagnose CD (Badenhorst, 2014). The only known treatment of CD is by adhering to a gluten-free diet (Mendoza and McGough, 2005). It is therefore necessary to develop alternative food products for people with CD.

2.2 Non-Celiac Gluten Sensitivity (NCGS)

Non-Celiac Gluten Sensitivity (NCGS) is an enteropathy typically occurring as gastrointestinal and/or extra-intestinal symptoms. These symptoms only occur relative to dietary uptake of gluten-containing food, in people that are not known to have either CD or wheat allergy (WA) (Catassi et al., 2015). The occurrence and causes are not yet fully understood. However, the reported symptoms are listed in Table 2.2. The treatment of this sensitivity is also an exclusively gluten-free diet.

Table 2.2: Non-Celiac Gluten sensitivity signs and symptoms

The signs (clinical) of Non-Celiac Gluten Sensitivity (NCGS)		
Abdominal pain	Diarrhoea	Epigastric pain
Nausea	Constipation	Alternating bowel habits
Foggy mind	Aerophagia	GER Joint/muscle pain
Stomatitis	Headache	Numbness
Skin rash/dermatitis	Anxiety	Anaemia
Weight loss	Anal fissures	Loss of balance
Depression	Rhinitis/asthma	Weight increase
Interstitial cystitis	Ingrown hairs	Lack of wellbeing
Sensory symptoms	Disturbed sleep pattern	Hallucinations
Mood swings	Tiredness	Bloating

(Catassi et al., 2015)

2.3 Challenges in the production of gluten-free baked products

Gluten-free flour produces a dough that does not have the ability to become cohesive and elastic as compared to wheat dough. Gluten-free dough has been noted to be sticky, less elastic, less extensible and tough to handle especially in production processes, with it often called batter instead of dough (Matos and Rosell, 2015). The consequences of the absence of gluten in bread are a crumbling texture, poor volume of the loaf, unattractive colour and of a generally poor quality (Gujral and Rosell, 2004). This is because gluten-free bread has a lack of an ability to retain the carbon dioxide that is produced during proofing and the batter formed during mixing is a result of a low water holding capacity. Gluten-free bread therefore has a short shelf life as staling occurs faster due to the high starch content, low water holding capacity of the dough and low moisture retention ability of the bread. Another key constraint in the production of gluten-free products from gluten-free starch bases is the high carbohydrate content which may considerably reduce the amount of dietary protein (Deora et al., 2015).

2.4 Role of gluten in baked products

In order to replace gluten in gluten-free products, it is necessary to understand gluten and its role in dough. Gluten is an extremely complex seed protein occurring in some cereals such as wheat, barley and rye. When present, it accounts for a high percentage of the cereal protein content. It is made up of two fractions which are the gliadins and glutenins (Fig 2.3). Gliadins contribute fundamentally to the viscosity and extensibility of the dough system whilst glutenins are liable for dough strength and elasticity. The complexity of gluten has raised theories on the actual occurrences in the protein structure which lead to dough formation in wheat dough. These gluten properties are only observed when the flour is hydrated. The extensible and visco-elastic dough has good gas holding properties resulting in bread with an acceptable crumb structure as well as a higher loaf volume. The structural protein for breadmaking is gluten. Gluten-free dough is not able to develop a similar protein network on hydration.

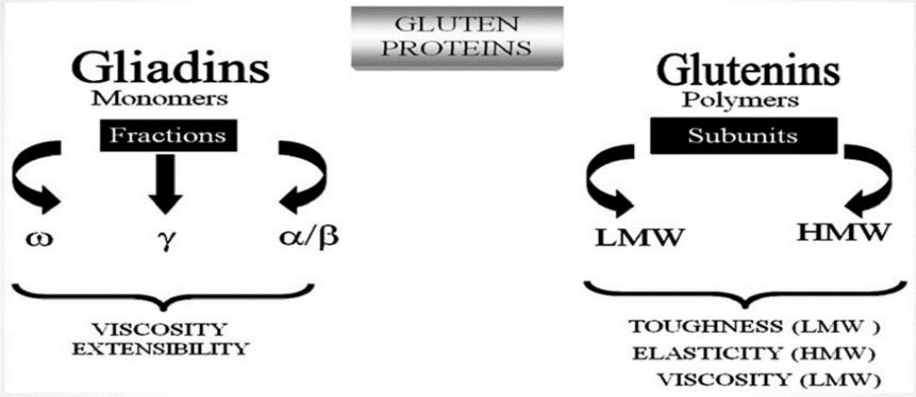


Figure 2.3: Gluten composition (Lamacchia et al., 2014).

2.5 Structure of gluten

2.5.1 Gliadins

These are mainly monomeric protein with molecular weights (MWs) around 28,000-55,000 Daltons. They can have different classes according to their diverse primary structures into the alpha, beta, gamma and omega-type (Fig 2.4). Intrachain disulphide bonds are either absent or present, but interchain disulphide links never occur. These gliadins are soluble in aqueous alcohol.

2.5.2 Glutenins

Glutenins are polymeric, alcohol-insoluble proteins made up of High Molecular Weight (HMW) and Low Molecular Weight (LMW) subunits branching off the HMW backbone (Wieser, 2007). The HMW subunit of glutenin, confers dough elasticity and cohesiveness. The HMW protein polymers are stabilized by interchain disulphide bonds which are believed to be critically essential as the ‘elastic backbone' of gluten. Reduction of disulphide bonds in glutenin result in alcohol-soluble proteins (Wieser, 2007). The HMW subunits have been reported to be about 12% of the total storage protein and they are only found in glutenin polymers. The amounts of these HMW subunits was observed to be positively correlated with dough strength (Dhaka and Khatkar, 2015).

The central part of the HMW is a glutamine-rich repetitive sequence which is therefore hydrophilic (Shewry et al., 2002). FTIR spectroscopy of HMW subunits and of model peptides led to the proposition that hydrogen bonding, which is not as strong as a covalent bond, may be essential in viscoelasticity owing to it holding glutenin subunits and polymers and rendering them an extent

of flexibility (Belton, 1999). This is because the dry glutenin proteins are disorderly with minimal consistent structure. However, on hydration, their ability to move is enhanced and β-sheet structures are formed (Shewry et al., 2002). The protein ability to move further increases if hydration continues and this results in the development of turn-like configurations (Belton, 1999). This led to the loop and train mechanism being proposed. The theory suggests that hydrogen bonding of glutamine residues are the prevailing protein-protein interactions in the β-spiral structures but a plasticised system occurs on continued hydration, where turns in adjacent β-spirals are then organised so that they form structures that are similar to an interchain β-sheet (Wieser, 2007).

If hydration ensues further, fragmentation of some of the interchain hydrogen bonds occurs whilst other hydrogen bonds between glutamine and water are formed. This results in loop regions. Although these loop regions occur, interchain hydrogen bonds are also still present. Therefore, there is a balance between the occurrence of the 'loop' regions and hydrogen-bonded 'chain' regions. The balance depends on the extent of dough hydration (Wieser, 2007). On pulling or extending the dough, stretching of the 'loops' and 'unzipping' of the 'trains' will occur whilst the backbone of this elasticity are the disulphide bonds.

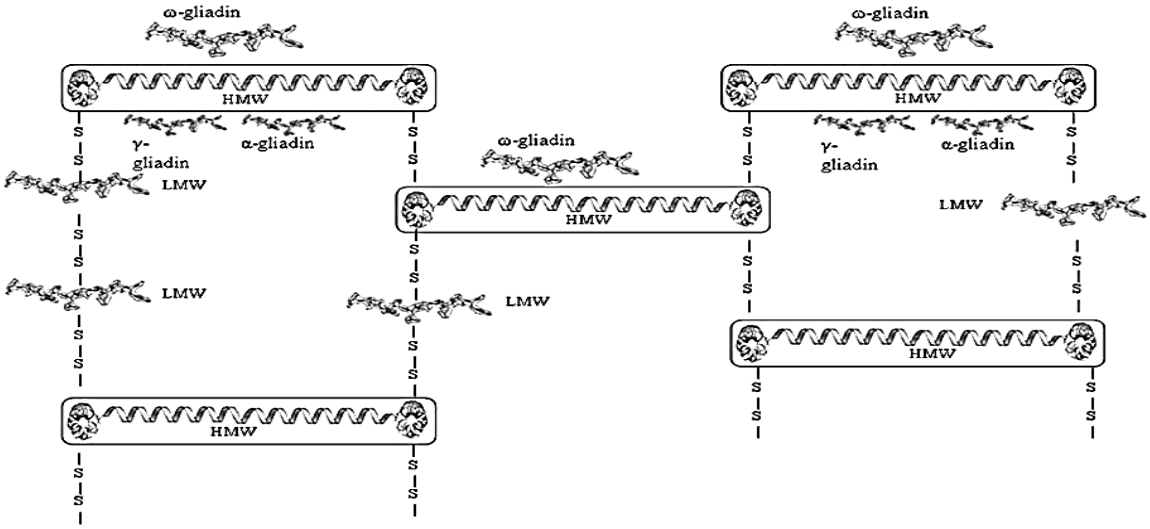


Figure 2.4: Interaction of gliadin and glutenin in gluten through disulphide bonds. A model for wheat gluten in which depicts the HMW subunits with a disulphide-bonded backbone that cooperates with other gluten proteins by –SS bonds, (LMW subunits) and non-covalent interactions (gliadins) (Lamacchia et al., 2014).

2.6 Gluten-free bread making and gluten replacement

2.6.1 Hydrocolloids

The absence of gluten proteins and their lack of a viscoelastic nature in gluten-free bread making probes a need to add hydrocolloids or gums to the formulations so as to mimic gluten. Hydrocolloids are an extended chain of hydrophilic and high molecular weight molecules which form gels in water (Arendt and Moore, 2006). They are normally polysaccharides derived from vegetation such as seeds, fruits, plant extracts, seaweeds or microorganisms (Norton and Foster, 2002). They occur less frequently as proteins.

The addition of hydrocolloids has been noted to alter the dough rheology, increase bread volume and improve the texture. Bread with enhanced sensory characters such as a soft crumb texture, improved crumb cell size and delayed starch retrogradation have been obtained as a result of the increase in the moisture retention. Hydrocolloids also affect the swelling, pasting, gelatinization and retrogradation of starch (Rojas et al., 1999). They also mimic gas retention (Fig 2.5) and water absorbing characteristics of gluten (McCarthy et al., 2005).

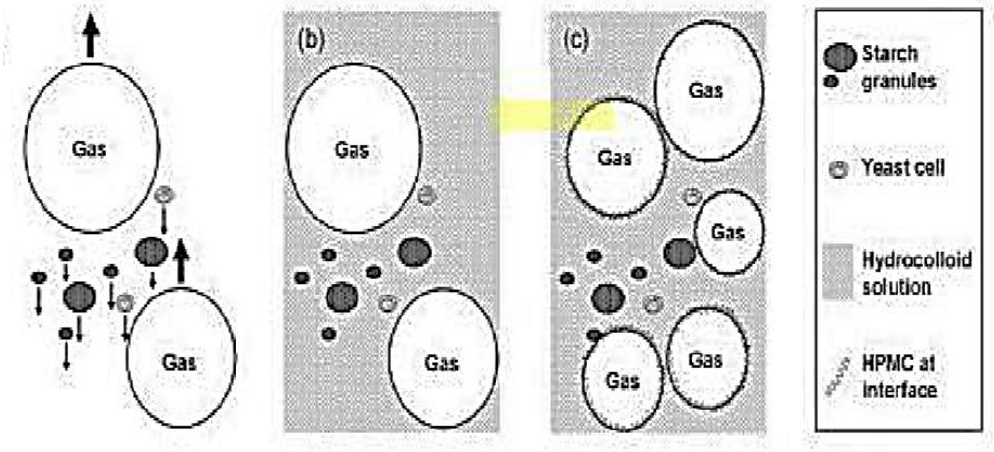


Figure 2.5: The effect of hydrocolloid addition on gas retention in gluten-free dough. The gluten-free dough containing no hydrocolloids is unable to retain gas bubbles produced during fermentation (b) The addition of a hydrocolloid increases the viscosity of the dough (c) Stability conferred to the gas-liquid interface, preventing the coalescence of the gas bubbles (Sly et al., 2014).

Hydrocolloids improve the dough water holding capacity due to their hydrophilic nature. Hydrocolloids such as xanthan, alginate, j-carrageenan, and HPMC were added to wheat flour and they increased the dough water absorption (Rosell et al., 2007, Rosell et al., 2001). Mucilage has also been used to improve bread volume and reduce bread staling (Dickinson, 2003). In gluten-free dough formulated on the basis of using on rice flour, starch and sodium caseinate, it was observed that the addition of xanthan gum caused the highest increase in the water holding capacity when compared to CMC, pectin and agarose. Furthermore, the addition of xanthan gum resulted in a farinograph curve resembling that of a wheat standard farinograph curve when added at 1 or 2% w/w of flour (Lazaridou et al., 2007a). The hydrocolloids reduced dough development time whilst increasing dough consistency and elasticity. The dough consistency and elasticity also reduced with the mixing time, similar to wheat dough. Hydrocolloids vary in composition and are usually used as a combination for enhanced bread characteristics.

There is a current shift to using natural biopolymers in food products. Cactus pear mucilage has been proposed to have the structure shown in Fig 2.6. The yield of cactus pear from the pad was reported to be about 19.4% based on dry weight. The dried mucilage was also observed to have an average of 5.6 % moisture; 7.3% protein; 37.3% ash; 1.14% nitrogen; 9.86% calcium and 1.55% of potassium (Sepulveda et al, 2007). Cactus pear mucilage has a potential to be used as a viscosity modifier in food products due to its high water holding capacity. The high calcium content may also add to the dietary calcium.

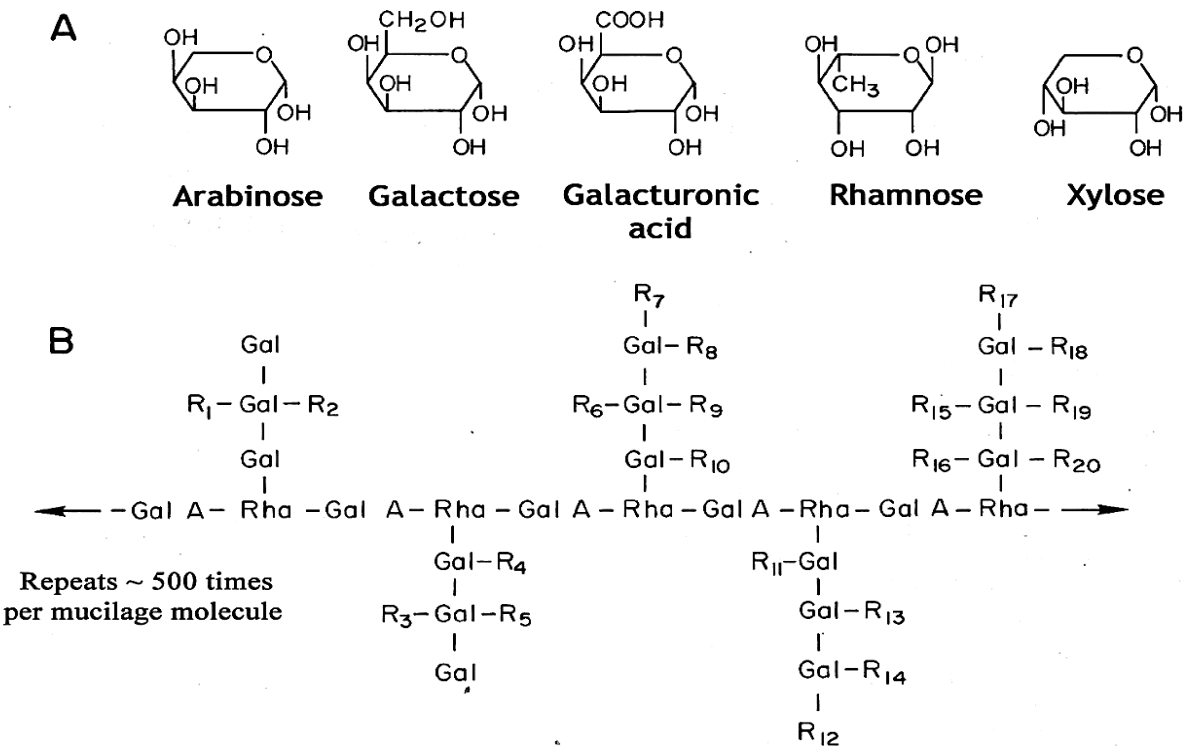


Figure 2.6: A: Mucilage subunit, B: Proposed Opuntia ficus-indica (cactus pear) mucilage structure (McGarvie and Parolis, 1981)

2.6.2 Protein supplementation

2.6.2.1 Dairy proteins

Supplementation of gluten-free dough with dairy proteins has been noted to improve the functionality. The ability of dairy proteins to form linkages and also their desirable ability to swell makes them a protein with functional properties comparable to gluten, (Gallagher, 2009). This has been demonstrated when the addition of yoghurt to gluten-free bread formulas increased the water absorption capacity of dough, thereby enhancing the viscoelastic behaviour of the dough (Gallagher et al., 2004a).

However, a contradictory result was reported by Houben et al. (2012) showed that the main whey proteins in yoghurt, α-lactalbumin (four S-S bonds) and the ß-lactoglobulin, are hydrophobic and compactly folded polypeptide chains. The addition of whey decreased water absorption of dough.

Furthermore, it may not be suitable for supplementation of gluten-free bread for people with manifestations of CD with the high lactose content dairy powders. The significant damage to their intestinal villi may result in secondary lactose intolerance of lactose as the damaged villi stop producing the lactase enzyme (Ortolani and Pastorello, 1997).

2.6.2.2 Legumes

Soybeans and peas are legumes. The soy proteins are made up of 90% globulins and 10% albumins. Soy proteins significantly increased the viscoelastic (G" and G') moduli of rice dough by 69% (Marco and Rosell, 2008). The higher water binding ability of soy proteins may have increased dough viscosity. Including 0.5% soy protein isolate in gluten-free bread improved texture, crumb grain and overall bread acceptability (Sanchez et al., 2002). It was also observed that addition of 5% of pea protein isolate to rice dough substantially increased viscoelasticity (Marco and Rosell, 2008a). The inclusion of pea protein in rice dough also modified dough microstructure as analysis demonstrated that pea proteins where existing as aggregates of unorganized round structures (Marco and Rosell, 2008).

Protein supplementation of gluten-free preparations with soy proteins is nutritionally beneficial and the enhancement of the amounts of free amine groups of the subsequent products are increased. Protein supplementation with legume flour also increases the protein content (Marco and Rosell, 2008a). Legume proteins are supplemented in gluten-free foodstuffs to increase its nutritive benefits because gluten-free foods are of a very low protein content, with lysine being limiting (Marco and Rosell, 2008a). However, not much soy protein should be added as adding 13% soy protein isolate to gluten-free bread lowered the specific volume from 2.1 l/kg to 1.59 l/kg (Smerdel et al., 2012).

2.6.2.3 Egg

The foam stabilising properties of egg albumin have probed its use as a functional ingredient in gluten-free bread. Gas retention and structural stabilisation of the crumb observed when eggs are used are highly dependent on the high foam stabilisation occurring due to the high emulsification capacity of egg proteins (Deora et al. 2015). Addition of egg albumin to rice dough decreased the breakdown by 34% compared to the control and highly augmented the viscosity of gluten-free

dough (Marco and Rosell, 2008a). There was also a minor improvement in G' and G" with frequency (Marco and Rosell, 2008a). Egg white proteins have a high swelling capability in gluten-free dough and a viscous dough with protein structure and function similar to gluten is produced (Houben et al., 2012). The increase in the protein content of gluten-free product resulting from the addition of egg albumin proteins also increases the free amine groups (Marco and Rosell, 2008a). Eggs also considerably modify the rheological properties of gluten-free bread. The bread volume and the number of gas cells per square centimetre of rice bread were noted to increase (Moore et al., 2006). However, eggs may be a costly protein supplement.

2.7 Potential gluten-free flours: Amadumbe

Amadumbe *(Colocasia esculenta),* are gluten-free tubers commonly known by several names, such as amadumbe in South Africa, Taro in the Pacific islands or Cocoyam in West Africa (McEwan et al., 2010). Amadumbe are aroids, grown primarily as edible corms, belonging to the family *Araceae*. The family which has 110 species, is a genus which includes also *Xanthosoma*, *Amorphallus*, *Alocasia* and *Cytosperma* (Owusu-Darko et al., 2014). The tubers are grown in many tropical countries as well as sub-tropical areas.

Amadumbe is extensively cultivated in West Africa although they are an important food crop for more than 400 million people worldwide (Owusu-Darko et al., 2014). It is estimated that the global production of Amadumbe is accounted for by about 60% being from Africa and 40% in Asia, with an average yield of 5.314 mt/ha (Owusu-Darko et al., 2014). In India, where the leaves and corm are used for food, the tuber has different vernacular names used to recognize it locally. These include Alooka or Aloopam (Sanskrit), Kachalu or Khuyya (Hindi), Alu (Marathi), Seppan-Kizhangu (Tamil), Chamadumpa (Telgu) (Kasote et al., 2011).

2.7.1 Utilisation of amadumbe

In India, tubers are primarily consumed as purees mixed with other ingredients. They are easily digestible due to their small starch granules and hence are mashed for baby food in Hawaii and the Pacific (Ammar et al., 2009). In Nigeria, fufu is made from the flour obtained by processing mature edible corms. Fufu is commonly consumed with stew (Coertze and Allenmann, 1996). In Ghana, amadumbe corms are boiled, dried and pounded into a flour. The flour is also used in soup

thickeners and baking flours, in beverages, as porridge and in producing foods for people with gastrointestinal disorders (Owusu-Darko et al., 2014).

In South Africa, amadumbe are important nutritionally and economically to the people of KwaZulu Natal, Mpumalanga and Western Cape (Coertze and Allenmann, 1996). They sell their excess produce for income generation. The corms are also consumed roasted, baked, or fried. After roasting or boiling the corms, they can be consumed with stew or on their own. The boiled corms are also mashed and are given to babies as part of the weaning diet. Locally grown cultivars in KwaZulu Natal are a staple food to the people (Kaushal et al., 2015). These have adapted to the climate and easily grow. However, not much research on the commercial production of amadumbe products has been done.

The use of amadumbe flour as a gluten-free base is currently of interest (Kaushal et al., 2015). The research and development of alternative raw material sources for bread production, other than wheat, is also currently of interest. Cassava (Pasqualone et al., 2010) and yam (Seguchi et al., 2012), have been found to be potential alternative sources of starch for breadmaking and the research on how to utilize them and produce acceptable bread is necessary. Amadumbe have a potential as a substitute starch source in bread making but have been neglected (Mawoyo et al., 2017). It is thought that the neglect can be partially attributed to the assumption by people, that these crops are inferior and for the poor people, due to their lower prices (Ammar et al., 2009). The presence of oxalates, which impart an acrid taste or cause irritation when foods prepared from amadumbe corms are consumed, limits the use of amadumbe (Sefa-Dedeh and Agyir-Sackey, 2004).

2.7.2 Composition of amadumbe flour

Amadumbe flour is composed of many different nutrients which affect its functionality and end use. Some of the major nutritional components are discussed.

2.7.2.1 Carbohydrates

Amadumbe are very rich in carbohydrates. The carbohydrate content of the flour ranges from 73 to 80%, with about 1.4% being crude fibre on Dry Matter (DM) basis (Alcantara et al., 2013). A

starch content of between 70 to 80% of the carbohydrates has also been reported (Ammar et al., 2009, Quach et al., 2001). This is similar to that of yam and potato (Naidoo et al., 2015). Amadumbe starch granules are round and polygonal in shape, and their sizes range from 15 - 40 μm (Owusu-Darko et al., 2014). Starch granules from other root crops such as cassava, true yam and potato are bigger when compared to those from amadumbe (Naidoo et al., 2015, Owusu-Darko et al., 2014). The smaller the starch granule is, the higher its digestibility. Edwards et al. (2002) also observed that increased proportions of small starch granules in dough resulted in the increase of elastic character of the dough. Amadumbe starch was also reported to be hard and adhesive. This was observed when the visco-elastic properties were characterised (Njintang et al., 2007).

Amadumbe starch has a ratio of 1:7 amylose to amylopectin. This amylopectin content is higher than most cereal starches. Taro starch results in a translucent and soft paste comparable to potato starch (Temesgen et al., 2016). Starch gelatinization occurs at temperatures ranging from 63- 73°C and the gelatinization temperature is lower in older corm starches. The starch also contains 0.23-0.52% lipid and 0.017-0.025% phosphorus in the form of phosphate monoester derivatives (Prajapati et al., 2011).

Lipids and proteins at the surface of starch granules have been implicated in the behaviour of starch with respect to pasting properties, wetting and dispersion, and starch suspension stability (Seguchi et al., 2012). Monosaccharide analyses of the whole showed xylose and mannose contents that are indicative of the presence of xyloglucan and glucomannans (Owusu-Darko et al., 2014). Sucrose, fructose, maltose, glucose and raffinose are also present (Temesgen et al., 2016).

2.7.2.2 Non-starch polysaccharides

A gum or mucilage is present in amadumbe. Mucilages are basically exopolysaccharides and glycoproteins which are highly concentrated with hydroxyl groups due to the polysaccharide chain. Past research has shown that up to 10.7% crude taro mucilages can be extracted from taro corms and tubers with boiling water (Gaind et al., 1968). Giant swamp Taro was reported to contain 40.0 g/kg and 51.5 g/kg dry weight after cold water extraction (Nguimbou et al., 2014). The yield of mucilage is also reported to vary from 30 to 190 g/kg when cold water extraction is used (Njintang et al., 2014). The mucilage is also made up of D-galactose (8.70–25.35%), D-glucose (44.95–

78.85%), D-mannose (3.20–10.45%), D-arabinose (2.45–5.20%) and minor contents of uronic acids (Nguimbou et al., 2014). Of the mucilage portion, arabinogalactan-proteins were extracted at yields varying from 5.30 to 8.83 g/kg and they were found to contain mainly arabinose and galactose, with also substantial amounts of glucuronic acid, rhamnose, xylose, and mannose (Nguimbou et al., 2014). The molecular structure of amadumbe mucilage has not been studied.

2.7.2.3 Amadumbe protein

A very low protein content of about 1.12% has been reported for amadumbe (Owusu-Darko et al., 2014). Wills (2006) also reports a range between 0.5 to 2.1% proteins. However, Amon et al (2011) reported a content of 5.88% which is much higher than the content reported by other researchers. Lysine is the first limiting amino acid although the flour contains essential amino acids (Owusu-Darko et al., 2014). Aspartic acid/asparagine (14.4-17.2%) and glutamic acid/glutamine (10.3-13.6%) were dominant in the mucilage and this was similar to the flour composition (Njintang et al., 2014). Taro contains four major protein families, two of which are albumins A1 and A2 (Bezerra et al., 1995). Globulins account for about 80% of amadumbe total soluble tuber proteins and two non-related globulin families, denoted G1 and G2 are present (Monte-Neshich et al., 1995). The G1 family is composed of a large number of isoforms of 12.5 kDa with isoelectric points (pIs) ranging from 5.5 to 9.5 whilst the G2 family is composed of two sets of proteins of 24 kDa (G2a) and 22 kDa (G2b), with pIs near 7.5 (Monte-Neshich et al., 1995). These proteins are specifically found in amadumbe tubers.

2.7.3 Other nutritional benefits of amadumbe

Amadumbe are also a decent source of thiamin, riboflavin, iron, phosphorus and zinc and an excellent source of vitamin B6, vitamin C, niacin, potassium, copper and manganese compared to other root crops. Compared to whole milk, amadumbe contain greater amounts of the vitamin B-complex (Alcantara et al., 2013). Amadumbe consumption can help in the combating of zinc deficiency which is associated with poor growth and stunting. The Trolox equivalent antioxidant capacity (TEAC) of the amadumbe corm was determined as 452 ± 72 mM TEAC/100 g and 244 ± 73 mM TEAC/100 g, by the scavenging activity against ABTS and DPPH radicals, respectively (Simsek and El, 2015). However, the exact phenolics present and their quantities have not been studied.

2.8 Enzymatic modification of dough

After using alternative gluten-free flours, the addition of hydrocolloids and a protein source, gluten-free dough still lacks viscoelasticity. It is therefore necessary to modify its dough forming properties. An interesting way is to introduce new covalent bonds in proteins and polysaccharides. Enzymes have been used recently in gluten-free bread making to achieve this.

2.8.1 Transglutaminase

Enzymes employed in gluten-free baking include transglutaminase (Protein-glutamine γ-glutamyltransferase, EC 2.3.2.13). Transglutaminase is an enzyme that catalyses acyl transfer reaction between a γ-carboxy amide of the peptide or protein-bound glutamine and a primary amine. It catalyses three reactions resulting in deamidation, crosslinking and acyl transfer (Fig 2.7).

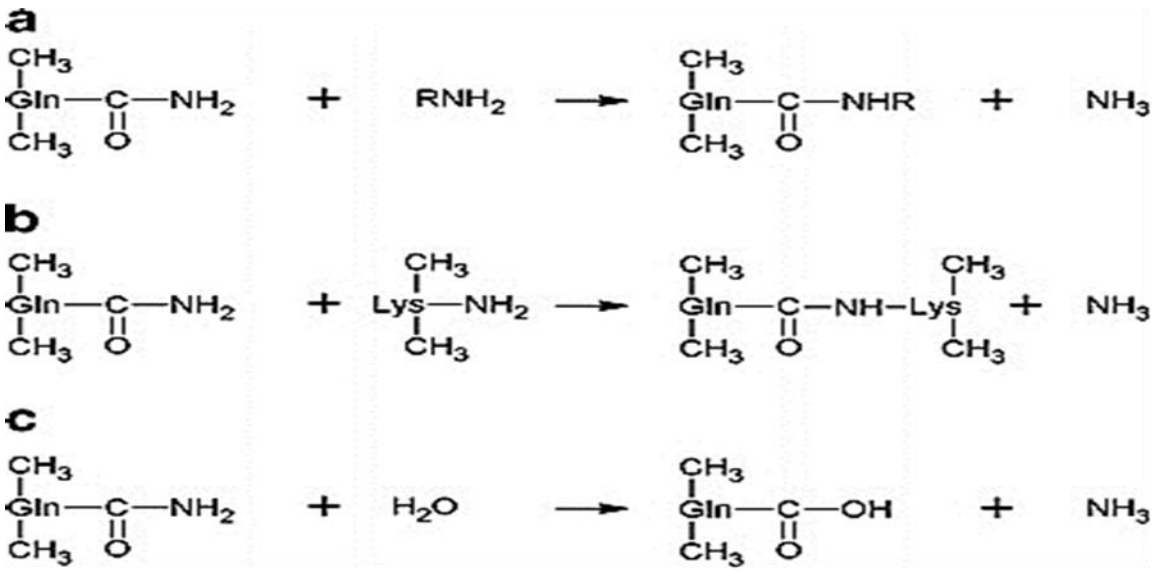


Figure 2.7: a) Acyl transfer reactions by transglutaminase b) When acyl transfer occurs with glutamine as the acyl donor and lysine as the acyl acceptor, crosslinking occurs. c) In the absence of an acyl acceptor, that is a free amino acid, deamidation occurs.

Illustration from (Kieliszek and Misiewicz, 2014)

It is important to note that in lysine-poor proteins such as cereal prolamins, deamidation of glutamine is a dominant reaction (Taylor et al., 2016). Proteins from gluten-free flours generally lack the ability to form a protein network upon baking and therefore there is a need to supplement with functional proteins (Renzetti and Rosell, 2016). Transglutaminase catalyses the creation of polymers among many protein sources which may include whey protein, soybean protein, rice protein, casein, and avenalin resulting in the modification of the elasticity, water holding capacity and additional functional desirable characteristics (Ahn et al., 2005). The effects of transglutaminase on the functional properties of wheat, barley, soy flours and their combinations were investigated. Protein polymerisation was confirmed by an observed decrease in free amino groups, thiol groups and aromatic hydrophobicity in treated samples.

Furthermore, transglutaminase treatment resulted in samples with an increased water-holding capacity, fat adsorption and emulsion stability but also a slightly decreased emulsion activity. On addition of barley or soy to wheat flour, fat adsorption and emulsion stability decreased. However, these properties increased to the same levels as wheat flour alone on addition of transglutaminase to the flour blend (Ahn et al., 2005). The viscoelastic moduli of the rice batter were significantly modified by the action of transglutaminase and it was reported that viscoelasticity was increased by pea and soybean while egg albumen and whey protein decreased them (Marco and Rosell, 2008).

A protein network capable of retaining the gas produced during proofing was proposed to have been formed as a result of transglutaminase crosslinking. The rice bread produced had an acceptable specific volume and crumb strength due to covalent protein crosslinking (Gujral and Rosell, 2004). Onyango et al. (2010) reported that microbial transglutaminase crosslinking action on gluten-free bread prepared from pregelatinised cassava starch, sorghum and egg white produced bread that had a firmer crumb and amplified chewiness as enzyme concentration was increased. Renzetti et al. (2008) also observed the network forming potential of transglutaminase on flours from gluten-free cereals (brown rice, buckwheat, corn, oat, sorghum and tef). The supplementation of this enzyme resulted in desirable enhancements and improvements in terms of loaf volume, diminished crumb hardness, decreased chewiness in corn, buckwheat and rice bread. No noteworthy changes in bread from oat, sorghum and tef were observed (Renzetti et al., 2008).

Although transglutaminase can be used to modify gluten-free dough, the protein source is crucial when defining the changes resulting from enzyme crosslinking. It is necessary to select a protein high in lysine and glutamine. The exact effect of transglutaminase on protein modification of gluten-free flours still needs to be determined as most of the past research investigated the effect of enzyme together with protein and hydrocolloid addition on the dough and bread (Marco and Rosell, 2008; Gujral and Rosell, 2004; Moore et al., 2006).

2.8.2 Laccase

Laccases (E.C.10.3.2), *p*-diphenol dioxygen oxidoreductases, are multi-copper enzymes catalysing a one-electron oxidation of a variety of aromatic substrates while reducing oxygen to water at the same time (Acero et al., 2014). The substrates for laccases include the countless aromatic compounds which include substituted mono- and polyphenols, aromatic amines and thiols (Kudanga et al., 2011). Laccase can readily oxidise para-, meta- and ortho-diphenols (hydroquinone/1.4-benzenediol, resorcinol/1.3-benzenediol, and pyrocatechol/1.2-benzenediol, respectively) unlike tyrosinase (Selinheimo et al., 2008). The oxidation of these substrates results in the production of free radicals (Fig 2.8). These highly reactive radicals may further react and give a rise to cross-linking of monomers, degradation of polymers and ring cleavage of aromatics (Selinheimo et al., 2007). The reaction products occur randomly.

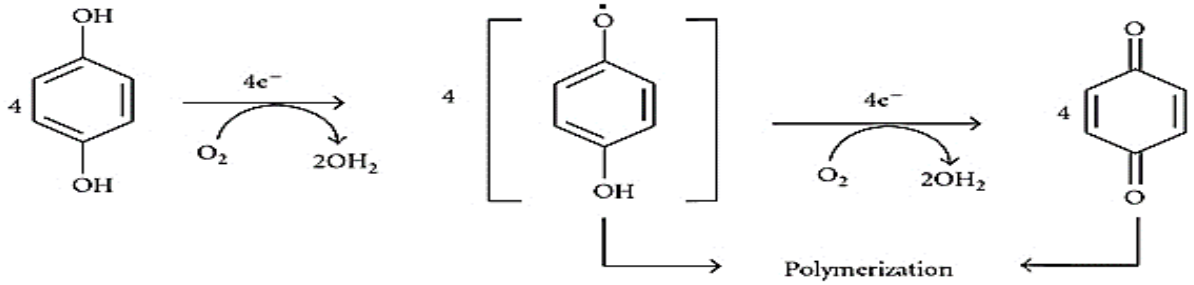


Figure 2.8: General reaction mechanism for phenol oxidation by laccase (Martínez-López et al., 2013)

In dough, laccase can oxidise ferulic acid into a phenoxy radical that may react non-enzymatically to produce di- and triFA and hence forms cross-links between feruloylated arabinoxylan of wheat

(Labat et al., 2000). Arabinoxylans are cereal non-starch polysaccharides which are made up of a linear backbone D-xylopyranosyl units linked by β-(1→4) bond and α-L arabinofuranosyl subunits are attached to the D-xylopyranosyl units. Ferulic acid (FA, 3-methoxy-4 hydroxycinnamic acid) is esterified to some of the arabinose residues. Crosslinking of ferulic acid results in a three-dimensional system with an increased water holding capacity and it exhibits no syneresis during storage (Martínez-López et al., 2013).

The formation of heteroconjugates between tyrosine, tyrosine-containing peptides or proteins and feruloylated arabinoxylan has been reported previously (Mattinen et al., 2005b). Piber and Koehler (2005) have also found evidence for a covalent cross-linking between arabinoxylan (AX) and protein. These heteroconjugates have negative effects on gluten structure and properties (Piber and Koehler, 2005).

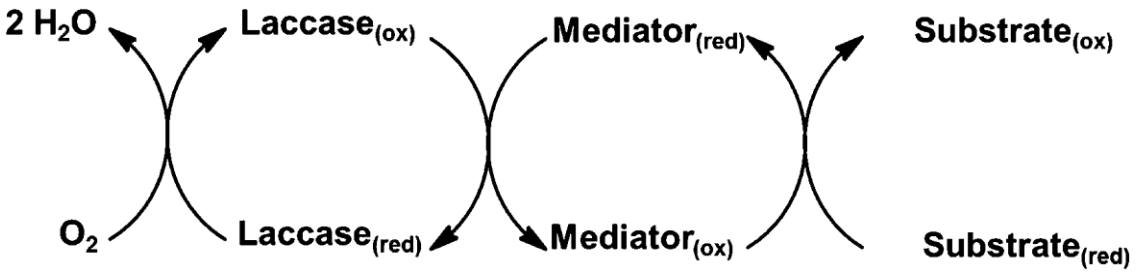


Figure 2.9: Laccase mediator reactions (Christopher et al., 2014).

Intermolecular cross-links were observed in alpha-casein due to ferulic acid-mediated laccase-catalysed oxidation and this included dityrosine formation (Steffensen et al., 2009). In the study by Labat et al. (2000), they observed that the -SH oxidation of wheat dough with laccase improved by 47%, when equated to control dough. In oat dough, laccase did not affect the water extractable arabinoxylan content of the dough (Renzetti et al., 2010). However, they detected the establishment of large protein aggregates, which could be visualized using a confocal laser scanning microscope and a significant decrease in the amount of the amino nitrogen which they could extract, which led to a conclusion that protein polymerization had occurred (Renzetti et al., 2010). The thiols of cysteine and glutathione may be oxidised into disulfides whilst they reduce the phenoxy radicals, formed by laccase, back to original ferulic acid (Labat et al., 2000.

The use of laccases in baking has been reported to result in increased strength, stability and reduced stickiness of dough therefore it improved dough machinability (Labat et al., 2000). Laccase at activity levels of 5–50 nkat/g flour has been reported to decrease dough extensibility (Selinheimo et al., 2006). At activity levels above 50 nkat/g flour, wheat dough was observed to soften and reduce its resistance to stretching, suggesting that the arabinoxylan chain was depolymerized (Selinheimo et al., 2006). Arabinoxylan treated with laccase has been reported to show poor stability properties which might be due to depolymerisation by laccase-generated radicals (Carvajal-Millan et al., 2005).

Laccase improved the specific volume of white wheat bread by 4–9% (Primo-Martin et al., 2003, Selinheimo et al., 2007). The softness of fresh white wheat bread was also improved by 17–19% (Primo-Martin & Martinez-Anaya, 2003; Selinheimo et al., 2007b). After 4 days storage the softness was 25% better than the control bread (Primo-Martin & Martinez-Anaya, 2003), whilst Selinheimo et al. (2007b) also observed that after 3 days storage, the softness of laccase modified bread and that of control wheat bread did not differ significantly.

There are limited studies on laccase applications in gluten-free bread. The specific volume and softness of fresh oat bread were also improved by 9% and 17%, respectively when compared to control oat bread (Renzetti et al., 2010). A high specific volume of oat bread containing *Trametes versicolor* laccase was also reported (Flander et al., 2011). The effect of laccase on amadumbe batter and bread has not been studied.

2.8.3 Tyrosinase

Tyrosinase is a copper-containing phenol oxidase which catalyses the oxidation of mono and diphenols with oxygen as a co-substrate (Nunes and Vogel, 2018). They catalyse two distinct reactions which are hydroxylation due to their monophenolase activity and oxidation by ortho-diphenolase activity (Lantto et al., 2007b). A single atom of oxygen is incorporated into the aromatic ring of the monophenolic substrate during hydroxylation, for example tyrosine, the other is reduced to water. Therefore, the initial oxidation results in the formation of an *o*-diphenol from a monophenol whilst the second reaction proceeds as the *o*-diphenol is further oxidised to a corresponding *o*-quinone (Thalmann and Lötzbeyer, 2002). Quinones are highly unstable and can

further react to form higher molecular weight polymers. In biological systems, tyrosinase is responsible for melanogenesis as it initiates the production by converting L-tyrosine to dopaquinone, which subsequently undergoes spontaneous reactions to yield melanin. The reaction mechanism of tyrosinase is shown in Fig 2.10.

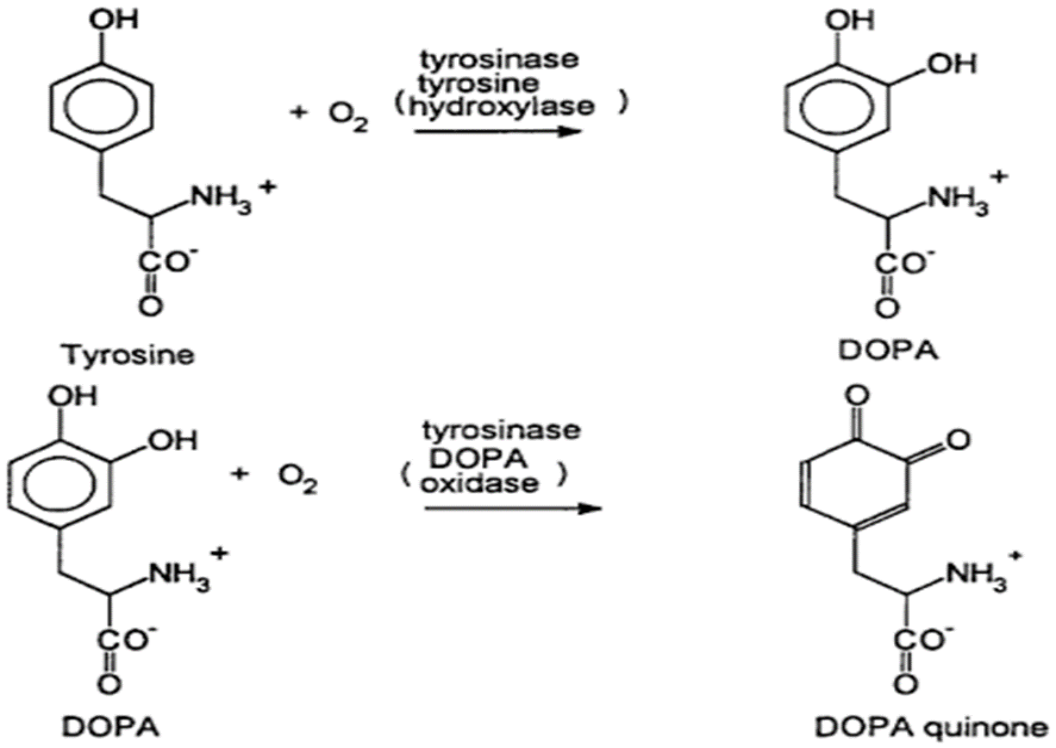


Figure 2.10: Hydroxylation and oxidation by tyrosinase (Thalmann and Lötzbeyer, 2002)

Fungal tyrosinase was observed to make wheat doughs stiffer and less extensible and this effect was observed to increase with an increase in enzyme concentration (Selinheimo et al., 2007). This was because of the proposed ability of tyrosinase to form stronger gluten networks through crosslinking of gluten proteins by dityrosine bond formation (Fig 2.11).

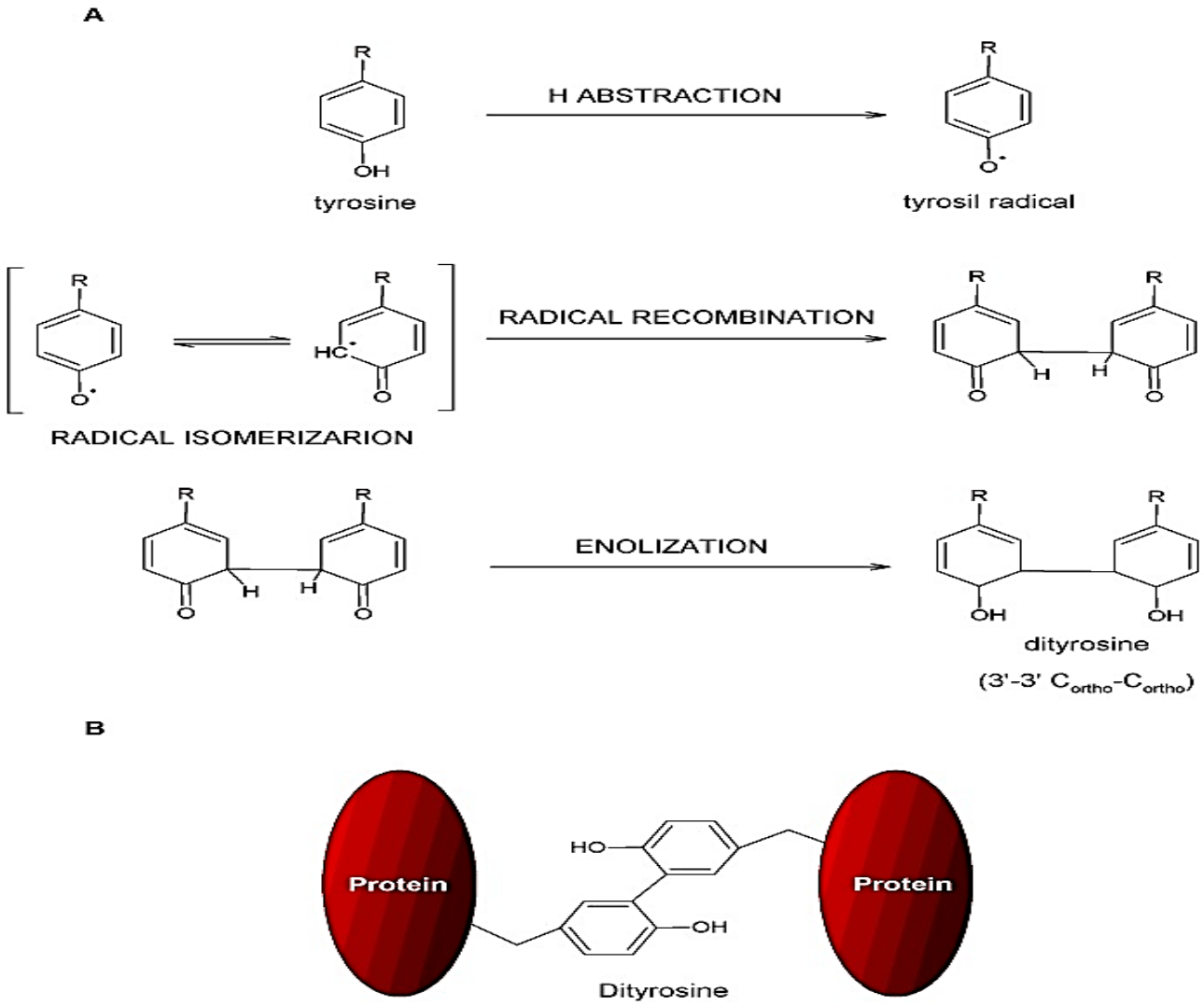


Figure 2.11: Di-tyrosine bond formation A) Reaction mechanism for the formation of dityrosine. (B) Intermolecular dityrosine cross-linking in proteins (Correia et al., 2012).

The resulting quinones from the oxidation of phenolics by tyrosinase have also been shown to couple to lysyl, tyrosyl, cysteinyl and histidinyl moieties resulting in the formation of products such as dityrosine, tyrosine-cysteine and tyrosine-lysine and hence cross-links in proteins (Land et al., 2007). However, the tyrosine-cysteine cross-linkages are not desirable as they hinder the formation of intra- and inter-chain disulphide linkages in the gluten proteins. Such hindrance weakens the gluten system and hence the ability of wheat dough to retain gas with effects such as poor loaf volume and irregular, the large pore size of bread.

Tyrosinase significantly increased gluten-free oat bread volume (Flander et al., 2011). The use of a higher enzyme activity (30 nkat/g) resulted in softer dough than lower activities. This was proposed to be due to side activities of hydrolyzing enzymes increasing as tyrosinase activity was increased and protein crosslinking reactions became slower. There are limited studies on the effect of tyrosinase on gluten-free bread. There are also no studies on the effect of tyrosinase on amadumbe dough and bread.

2.9 Conclusion

The use of hydrocolloids and enzymes has significantly improved gluten-free bread. However, it is still a challenge to produce gluten-free baked products by adding hydrocolloids or a single enzyme as gluten is so complex to mimic. Further research into novel hydrocolloids, enzymes and their combinations is still necessary to improve the acceptability of the products.

2.10 Aim, hypothesis and objectives

2.10.1 Aim

To modify the dough rheology of amadumbe flour using multiple enzymes for gluten-free bread production.

2.10.2 Hypothesis

The use of a combination of enzymes (transglutaminase, tyrosinase and laccase) supplemented with mucilage will produce a dough that is more viscoelastic and bread that has a higher specific volume compared to single enzymes or mucilages alone.

Formation of protein-protein bonds by transglutaminase (Onyango et al., 2010), di-sulphide bonds by laccase (Flander et al., 2011), di-tyrosine bonds by tyrosinase (Land et al., 2007), protein-oligosaccharide conjugates (Selinheimo et al., 2008) and oxidative gelation of ferulic acid esterified to arabinoxylan will improve dough viscoelasticity. The more viscoelastic dough will retain more carbon dioxide during proofing.

2.10.3 Objectives

- To determine the compositional, thermal and viscosity profile of amadumbe and cactus pear mucilages using HPLC, DSC and a rheometer, respectively.
- To model enzymatic reactions and determine the consequent effect of enzymes on amadumbe-based dough rheology.
- To optimise enzymatic modification of amadumbe-based dough using response surface methodology (RSM).
- To determine the physical and sensory characteristics of bread prepared from the optimised enzyme combination and hydrocolloid.

CHAPTER 3

3.0 Composition, thermal and rheological properties of polysaccharides from amadumbe (*Colocasia esculenta*) and cactus (*Opuntia* spp).

Abstract

The extensive application of hydrocolloids in the food industry, coupled with their short supply and shortcomings, has led to the ongoing search for alternative sources. In this study, the compositional, rheological and thermal properties of amadumbe and cactus mucilages were investigated. The mucilages had a similar qualitative composition of monosaccharides and amino acids, except for the absence of rhamnose in amadumbe mucilage. Fractionation of amadumbe and cactus mucilages on an anion-exchange column yielded four and three fractions, respectively. The fractions eluting with protein showed no β-elimination, suggesting stronger glycosylation bonds such as those in arabinogalactan proteins (AGPs). There was no evidence of thermal depolymerisation of the mucilages up to 195^{o}C. Cactus mucilage showed a pseudoplastic flow behaviour whilst amadumbe mucilage showed a Newtonian flow behaviour at up to 5% (w/v) concentrations. Amadumbe mucilage may be a potential emulsifier, whilst cactus mucilage can potentially be used as a thickening or emulsifying agent.

3.1 Introduction

The increasing usage of hydrocolloids in the food industry is attributed to a wide range of functional properties, which include thickening, gelling, emulsifying, stabilization and coating (Saha & Bhattacharya, 2010). Their use results mainly in texture and viscosity modifications, which greatly influence the sensory appeal of food (Milani & Maleki, 2012). There is a growing interest in the use of plant-based viscous polysaccharides as hydrocolloids due to their biocompatibility, safety and the ease of marketing products modified using these natural biopolymers (Ahmad, Mustafa, & Che Man, 2015). Furthermore, these plant viscous polysaccharides are dietary components of significant benefit in the management and prevention of diabetes, obesity, cardiovascular disease and metabolic syndrome (Lovegrove et al., 2017). Plant viscous polysaccharides such as guar gum, locust bean, tragacanth gum and gum arabic are

therefore commonly applied in the food industry. The choice of hydrocolloid for a particular application, however, depends on the unique properties exhibited which in turn are influenced by structure and composition.

The highly branched, compact structure and heterogeneous composition of gum arabic results in high solubility and low viscosity at high concentrations compared to most available hydrocolloids (Patel & Goyal, 2015; Saha & Bhattacharya, 2010). The structure of gum arabic is relatively complex, with the main chain of this polysaccharide consisting mainly of galactose and glucuronic acid units whilst the side branches may contain rhamnose, arabinose, galactose and glucuronic acid units (Nie et al., 2013). Anion exchange chromatography revealed that the gum is composed of five fractions with protein contents ranging from 0.31 to 2.8% (Osman et al., 1995). This unique association of the polysaccharide with a protein moiety is responsible for the multiple functionalities of the gum (Montenegro, Boiero, Valle, & Borsarelli, 2012), as an emulsifier and stabilizer especially in low viscosity-high solid applications such as flavour emulsions in the beverage, confectionery and pharmaceutical industries. This has resulted in an increase in demand for gum arabic. However, Sudan is the main world source (80%) of this exudate released by trees which take at least 6 years to establish (Diallo, Nielsen, Hansen, Ræbild, & Kjær, 2015). The supply is also uncertain due to climatic, economic and political conditions in the region (Yadav, Johnston, Hotchkiss, & Hicks, 2007), resulting in price fluctuations. An alternative hydrocolloid which totally or partially replaces gum arabic would benefit the beverage and confectionery industry.

Amadumbe (*Colocasia esculenta*) also known as taro, is a source of about 3 to 19% mucilage (Njintang et al., 2014), though not much attention has been given to it. Mucilages are viscous complex polymers composed mainly of branched polysaccharides and some glycoproteins (Sepúlveda, Sáenz, Aliaga, & Aceituno, 2007). Hence mucilages may be composed of many different polymeric units, and therefore are likely to show multiple functionalities. Taro mucilage is composed of mainly arabinose, xylose, mannose, galactose and glucose implying a highly branched structure (Andrade, Nunes, & Pereira, 2015; Jiang & Ramsden, 1999). The mucilage may also contain arabinogalactan proteins (AGPs) (Nguimbou et al., 2014; Njintang et al., 2014). However, for potential application of the mucilage in the food industry, further characterisation is

necessary to understand the molecular components of the mucilage and its viscosity properties. Such information will not only facilitate prediction of its behaviour during extraction and processing, but may also uncover opportunities for modifying the mucilages.

Therefore, the aim of this study was to characterise amadumbe mucilage in terms of composition, thermal and viscosity properties for potential food applications. Cactus cladode mucilage is also reported for comparative purposes. Although the plant originated from Europe, the Food and Agricultural Organization (FAO) revived the interest in cactus cultivation for agricultural purposes in developing countries in an attempt to minimize the risks of global climate change, land degradation and diminishing food security (Novoa, Le Roux, Robertson, Wilson, & Richardson, 2015). As a control measure, to prevent its invasiveness, the increase in utilisation of the cactus plant is necessary. In addition, the composition of cactus mucilage has been observed to vary based on geographical locations and growth conditions (Majdoub et al., 2001; Sepúlveda et al., 2007). Therefore mucilage from cactus cladodes grown in the semi-arid regions of Southern Africa is also characterised for potential food applications.

3.2 Materials and Methods

3.2.1 Preparation of amadumbe flour

Amadumbe corms were obtained from Jozini, KwaZulu Natal (KZN) province, South Africa. Amadumbe flour was prepared following a method previously described (Naidoo, Amonsou, & Oyeyinka, 2015). Freshly harvested amadumbe corms were rinsed, peeled and sliced to a thickness of 3 mm. The slices were dried at 50^{o}C for 48 h in a hot air oven (D-37520, Thermo Fisher Scientific, Germany). Dried slices were then milled into flour using a warring blender (8010S, Torrington, USA) and sieved (screen size 250 nm) to obtained fine flours, which were then stored at 4^{o}C until the mucilage was extracted.

3.2.2 Extraction of amadumbe mucilage from flour

Amadumbe mucilage extraction was done according to the method of Lin & Huang (1993). Amadumbe flour was dispersed in water at a flour/water ratio of 1:3 and stirred overnight at 4^{o}C. The slurry was centrifuged (Eppendorf 5810R Centrifuge, Germany) at 5000 × g for 20 min at 4^{o}C

and the supernatant collected. The extraction was repeated following the same procedure using the pellet and a second supernatant was obtained. The two supernatants were combined, filtered (Whatman 4 filter paper, Whatman, UK) and the mucilage was precipitated using three volumes of 95% ethanol to one volume of the supernatant. Mucilage was collected as the sediment by centrifugation at 4000 × g for 10 min at 4^{o}C. The precipitate was washed three times with 95% ethanol and three times with acetone, then freeze dried.

3.2.3 Extraction of cactus mucilage

Cactus cladodes were obtained from Durban, KZN, South Africa. Mucilage was extracted from the cladodes according to the method of Sepúlveda et al. (2007) with modifications. The cladodes were washed, diced and crushed in a blender with water added in the ratio 1:3 (w/v). The slurry was centrifuged and then filtered through a muslin cloth. Mucilage was precipitated from the supernatant as above and freeze-dried.

3.2.4 Purification of mucilage

Mucilage (0.2 g) was dissolved in 15 ml of deionised water and dialysed (10 kDa cut-off membrane) against deionized water for 48 h to remove unconjugated lower molecular weight proteins. The residue was collected and mucilage was precipitated using 95% ethanol. Mucilage was recovered as the sediment obtained by centrifugation at 4000 × g for 10 min at 4^{o}C and this was successively washed three times with ethanol and then acetone (Lin & Huang, 1993). The mucilage was then freeze-dried and pulverised to a powder.

3.2.5 Determination of chemical composition of mucilages

The soluble protein in mucilage was determined by the method of Bradford (1976) using a BSA standard. The total sugars in the mucilages were analysed using the phenol-sulphuric method with glucose as a standard (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The pH of 1% mucilage solutions was also measured at 25^{o}C. The free and bound phenolics were determined using a Folin-Ciocalteau method described by Nguimbou et al. (2014). The phenolic content was expressed as mg of ferulic acid equivalent per g of dry mucilage weight.

3.2.6 Monosaccharide analysis

The monosaccharides in amadumbe and cactus mucilage extracts were determined using the method of Muñoz Hernández (2012) with slight modifications. Briefly, mucilage extracts (25 mg) were hydrolysed by adding 3 ml of 1 M H_2SO_4 to previously homogenized samples, which were then kept at 100°C for 90 min. The hydrolysate was then cooled to room temperature (22-25°C) and the pH adjusted to between pH 3.0 and 4.0 by adding saturated Ba (OH) $_2$ to form a water-insoluble salt (instead of KOH which forms a water-soluble salt) and centrifuged at 5000 × g for 5 min. The supernatant was filtered through 0.22 µm Whatman microfilters (Sigma-Aldrich, South Africa) and freeze-dried. Hydrolysed mucilage (1 mg) was dissolved in 1 ml of deionized water and the quantitative analysis of the monosaccharides was achieved using high-performance liquid chromatography (HPLC). The Shimadzu UHPLC system (Shimadzu, Kyoto, Japan) used was equipped with a Biorad Aminex HPX-87H column (300 × 7.8 mm). Isocratic separation was performed at 50°C using water as a mobile phase at a flow rate of 0.5 ml/min for 15 min with an injection volume of 10 µl. The elution was monitored with a Shimadzu Evaporative Light Scattering Detector (ELSD) (Shimadzu, Kyoto, Japan). The quantification of monosaccharides was performed by comparing the peak areas of the samples to a standard calibration curve.

3.2.7 Amino acid analysis

Amino acid analysis was done according to the method of Njitang et al. (2014). Mucilage (40 mg) was weighed out into digestion tubes containing 6 N HCl in 1% phenol. The tubes were then flushed with nitrogen, sealed and incubated for hydrolysis at 150°C for 60 min. Hydrolysed samples were centrifuged at 5000 × g for 5 min at 20°C. The supernatant was filtered into vials through a 0.22 µm Whatman microfilter (Sigma-Aldrich, South Africa). The pre-column derivatisation used phenylisothiocyanate (PITC) and the separation was achieved using a reverse phase column PTC RP-18 (2, 1*220 mm) (Applied Biosystems Corp, Foster City, CA, USA) on a Shimadzu UHPLC (Shimadzu, Kyoto, Japan). The elution was carried out using 45 mM sodium acetate buffer (pH 5.90) (A) and 30% of 100 mM sodium acetate buffer (pH 4.60) combined with 70% acetonitrile (B), and the amino acid derivatives were detected at 254 nm. The level of amino acid was expressed as $mg.g^{-1}$ mucilage.

3.2.8 Fractionation and composition of mucilage fractions

Samples of mucilage solutions (10 mg/ml) were centrifuged at 5000 × g for 5 min at 20^{o}C and filtered through a 0.22 μm Whatman microfilter (Sigma-Aldrich, South Africa), then loaded onto a HiTrap^{TM} Capto DEAE (GE Healthcare, Sweden) anion-exchange column previously equilibrated with 20 mM Tris-HCl buffer A (pH 7.8). Elution was achieved with a linear gradient from 0-100% of buffer B (20 mM Tris-HCl buffer pH 7.8 + 1 M NaCl) at a flow rate of 1 ml/min. Fractions (1 ml) were collected using an AKTA Purifier system (GE Healthcare, Sweden) and elution was monitored at 201 nm for polysaccharides and 280 nm for proteins. Elution was also monitored at 490 nm using the phenol-sulphuric method. Peak fractions were pooled, desalted by dialysis (10 kDa cut-off membrane) against water for 48 h at 4^{o}C and freeze-dried. The fractions were analysed for total sugars using the phenol-sulphuric method (Dubois et al., 1956) and proteins using the method of Bradford (1976).

3.2.9 Analysis of the carbohydrate-peptide linkage

The carbohydrate-peptide linkage was analysed on fractions eluting with protein by the β-elimination reaction. Mucilage solutions (5 mg/ml) were incubated with 0.2 mol/l NaOH containing 1.0 mol/l $NaBH_4$ for 16 h at 45^{o}C and scanned from 200 nm to 260 nm using a UV-Vis spectrophotometer (Shi et al., 2017). A sample solution without the addition of NaOH served as the control.

3.2.10 ATR–FTIR spectroscopy

Infrared spectra were collected on a Perkin Elmer model Spectrum 400 FTIR spectrometer equipped with a diamond crystal ATR (Waltham, MA, USA). The spectra of the mucilage powders were collected from the scanning range 400–4000 cm^{-1} with a resolution of 1 cm^{-1}. After the crystal area was cleaned, the solid material was placed onto the small crystal area; the pressure arm was positioned over the crystal area. Force was applied to the sample, pushing it onto the diamond surface and the spectrum was collected.

3.2.11 Thermal analysis of mucilages

Differential scanning calorimeter (DSC) technique was used to analyse the thermal stability of amadumbe and cactus mucilages. The moisture content of both mucilages was approximately 7%

as determined by the AOAC method (1990). The measurements were performed using Q2000 Differential scanning calorimeter V24.10 (TA Instruments, USA) in a nitrogen atmosphere (25 ml/min) and a temperature range from 20 to 290^{o}C at a heating rate of 10^{o}C/min. Sample weights between 3 and 5 mg were placed in an aluminium pan.

3.2.12 Steady-shear viscosity of mucilages

The rheological properties of the mucilage were determined at 25^{o}C in a Physica MCR501 rheometer (Anton Paar Co). To evaluate the steady-shear viscosity as a function of shear rate, the shear stress was measured with the application of a given shear rate from 1 s^{-1} to 300 s^{-1} using a unidirectional steady-shear flow. Data were fitted to the Power law model as follows:

$$\tau = k\gamma^{n}$$

where τ is the shear stress (Pa), k is the consistency coefficient,(Pa s)^{n}, γ is the shear rate (s^{-1}) and n is the flow behaviour index.

3.2.13 Statistical analysis

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

3.3 Results and discussion

3.3.1 Yield, composition and pH of amadumbe and cactus mucilages

The yield, composition and pH of amadumbe mucilage and cactus mucilage showed considerable differences (Table 3.1). Although both mucilages were mainly composed of sugars, cactus mucilage had a significantly higher total sugar content than amadumbe mucilage. Similar total sugar contents have been reported for amadumbe (Jiang & Ramsden, 1999; Njintang et al., 2014) and cactus mucilage (Sepúlveda et al., 2007). Amadumbe mucilage was also associated with more protein compared with cactus mucilage. However, the protein content of amadumbe mucilage was much lower than previously reported (Njintang et al., 2014). This may be due to the purification process which resulted in the removal of unconjugated proteins. The protein content of cactus

mucilage was comparable to that previously reported (Sepúlveda et al., 2007). Some phenolic compounds in the mucilages were esterified (bound phenolics). In dicots, such as cactus, phenolics are associated with pectic polysaccharides via ester linkages to galactose and arabinose residues. In monocots, such as amadumbe, they are mainly linked by ester bonds to the carboxyl group or to arabinose residues (Ou & Sun, 2014). The presence of esterified phenolic compounds may enable enzymatic modification of mucilages by polyphenol oxidases such as laccase, resulting in improved functionality.

The monosaccharide compositions of the hydrolysed mucilages are also shown in Table 3.1. Generally, the qualitative neutral sugar profiles of both mucilages were similar, except for the presence of rhamnose in cactus mucilage. Galactose and glucose were the main monosaccharides in amadumbe mucilage, while galactose and galacturonic acid were the main monosaccharides in cactus mucilage. The presence of galacturonic acid in appreciable amounts and some rhamnose in cactus mucilage, suggests that it is a pectic polysaccharide (Ognyanov et al., 2016). The molar ratio of arabinose to galactose (1:2.15) in cactus mucilage and (1:6.6) in amadumbe mucilage, coupled with the presence of proteins associated to the mucilages, also strongly suggest the presence of AGPs in the mucilages (Jiang & Ramsden, 1999; Nguimbou et al., 2014).

Table 3.1. Yield, composition and pH of amadumbe and cactus mucilages

Parameters	Mucilage	
	Amadumbe (AM)	**Cactus (CM)**
Yield (g/100g)	4.44 ± 0.16	3.86 ± 0.35
Total sugars (g/100g)	68.50 [b] ± 1.27	74.50 [a] ± 0.16
Protein (g/100g)	6.53 [a] ± 0.58	3.12 [b] ± 0.64
pH	6.35 [a] ± 0.01	5.54 [b] ± 0.02
Bound phenolics (mg/g)	24.01 [a] ± 0.88	24.89 [a] ± 0.46
[*]Monosaccharides		
Glucose (%)	28.2 [a] ± 0.8	5.2 [b] ± 0.1
Galactose (%)	36.7 [b] ± 0.3	25.6 [a] ± 0.8
Arabinose (%)	4.7 [b] ± 0.0	10.1 [a] ± 0.5
Rhamnose (%)	0.0	9.8 ± 0.2
Xylose (%)	2.1 [b] ± 0.2	8.4 [a] ± 0.1
Mannose (%)	11.8 [b] ± 0.2	12.1 [a] ± 0.3
Glucuronic (%)	8.3 ± 0.3	0.0
Galacturonic (%)	0.0	18.5 ± 0.2

Means ± SD; n=3; values with different letters within the same row differ significantly ($p<0.05$).

[*]Monosaccharide compositions are expressed as % of the total sugars.

3.3.2 Amino acid composition

Amadumbe mucilage had a generally higher amino acid content than cactus mucilage (Table 3.2). The amino acid profile of amadumbe mucilage compares favourably to literature and is similar to that of the flour (Njintang et al., 2014). To our knowledge, no reports have been published on the amino acid content of cactus mucilage. Appreciable amounts of proline, serine, alanine and threonine in the mucilages suggest the presence of AGPs (Tan et al., 2012). These AGPs were proposed to be responsible for the emulsifying properties of taro mucilage (Andrade et al., 2015). This may be due to the presence of both hydrophobic and hydrophilic amino acids. Amino acids with reactive functional groups such as lysine and glutamine may also be substrates of cross-linking enzymes such as transglutaminase, enabling the modification of these mucilages to improve functionality.

Table 3.2. Comparative amino acid profiles of amadumbe and cactus mucilages expressed as mg. g^{-1} mucilage

	Mucilage	
Amino acids	**Amadumbe**	**Cactus**
Histidine	4.15 [a] ± 0.07	1.50 [b] ± 0.03
Serine	12.00 [a] ± 0.02	5.05 [b] ± 0.09
Arginine	17.65 [a] ± 0.15	5.00 [b] ± 0.05
Glycine	13.20 [a] ± 0.11	6.05 [b] ± 0.06
Aspartic acid	31.90 [a] ± 0.05	11.45 [b] ± 0.17
Glutamic acid/glutamine	19.95 [a] ± 0.04	13.05 [b] ± 0.21
Threonine	10.35 [a] ± 0.18	4.70 [b] ± 0.09
Alanine	8.95 [a] ± 0.03	7.00 [b] ± 0.08
Proline	10.30 [a] ± 0.19	5.05 [b] ± 0.08
Lysine	8.65 [b] ± 0.03	9.60 [a] ± 0.15
Tyrosine	12.65 [a] ± 0.16	4.90 [b] ± 0
Methionine	4.05 [a] ± 0.06	3.05 [b] ± 0.02
Valine	10.55 [a] ± 0.15	6.55 [b] ± 0.06
Isoleucine	19.95 [a] ± 0.02	8.20 [b] ± 0.06
Leucine	19.25 [a] ± 0.32	9.25 [b] ± 0.06
Phenylalanine	11.55 [a] ± 0.11	4.60 [b] ± 0.02
Total polar amino acids	104.65 [a] ± 0.54	50.35 [b] ± 0.11
Total non-polar amino acids	110.45 [a] ± 1.15	54.65 [b] ± 0.44

Means ± SD; n=2; values with different letters within the same row differ significantly ($p<0.05$).

3.3.3 Fractionation of mucilages by anion exchange chromatography

Anion-exchange chromatography of amadumbe mucilage revealed 4 peak fractions and the water-soluble cactus mucilage showed 3 fractions (Fig. 3.1) separated according to their differences in charge density. The protein contents of the fractions eluting with proteins ranged from 6 to 10%. The more acidic fractions (F4 of AM, F3 of CM) of both mucilages eluted with more protein compared to the less acidic fractions (F3 and F2 of AM, F2 of CM) (Fig. 3.2). This may be due to the increase in protein attracted to the negatively charged carboxyl groups of uronic acids. As proposed, AGPs are made of a positive protein core surrounded by negatively charged polysaccharides (Nguimbou et al., 2014). Cactus mucilage was mainly composed of acidic than neutral fractions and this may be due to the high galacturonic acid content in the mucilage. Amadumbe and cactus mucilage are all composed of neutral glycan, acidic glycan and possibly

acidic glycoproteins. Flaxseed mucilage was also fractionated into a rhamnogalacturonan, arabinoxylan, AGPs and glucans (Ray et al., 2013). Each fraction may have a unique functional property, for example, AGPs or proteoglycans exhibit emulsifying properties whilst other glycans are potential viscosity modifiers or gelling agents.

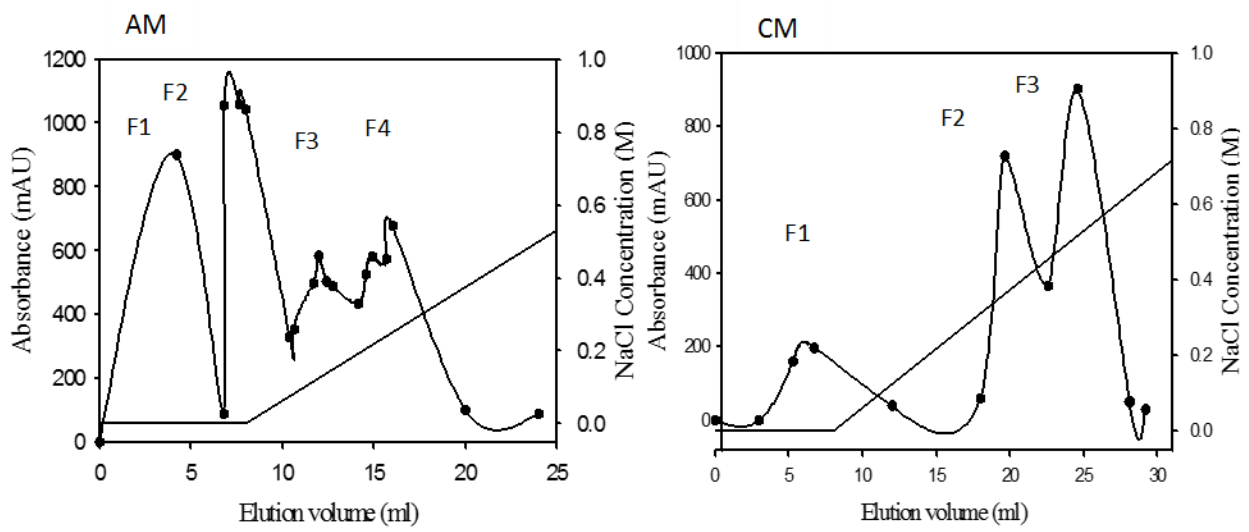


Figure 3.1. Fractionation of water-soluble amadumbe (AM) and cactus mucilage (CM) on a HiTrapTM Capto DEAE column.

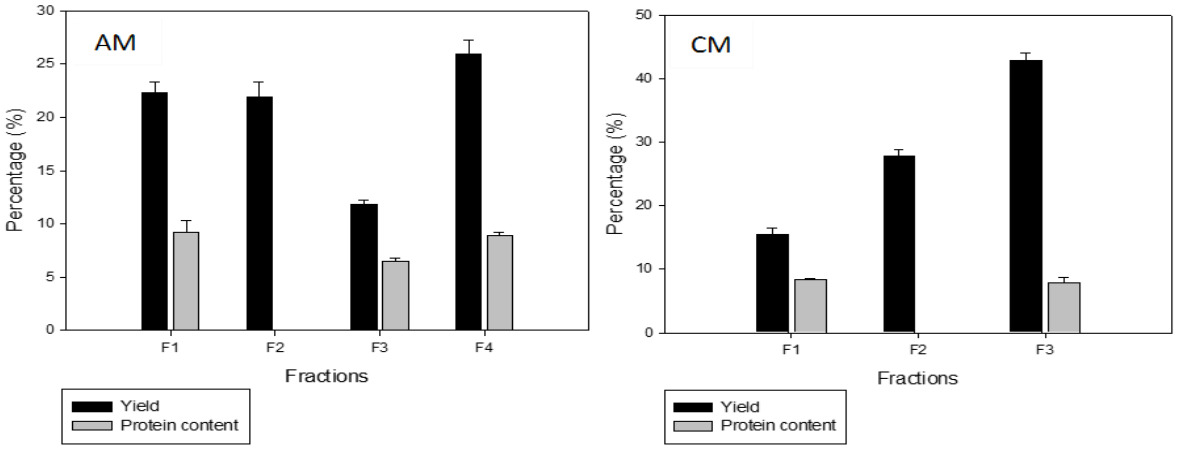


Figure 3.2. Yield and protein contents of each fraction of amadumbe (AM) and cactus mucilage (CM) from a Capto DEAE column

3.3.4 Analysis of the carbohydrate-peptide linkage

The linkages of possible glycoproteins in fractions where polysaccharides eluted with protein, were also analysed (please refer to supplementary data Tables S1 and S2). No β-elimination was observed, implying the absence of *O*-glycosidic linkages. The β-elimination reaction is known to remove sugars *O*-linked to serine or threonine. N-Linked glycans are not cleaved under the mild conditions (Dai et al., 2016). Arabinogalactans in AGPs are Hyp-*O*-linked glycans which are stable to β-elimination (Tan, Qiu, Lamport, & Kieliszewski, 2004). Similar observations were made when *Arabidopsis* cell wall proteoglycan which consisted of pectin and arabinoxylan was subjected to mild alkaline conditions (Tan et al., 2013).

3.3.5 FTIR of amadumbe and cactus mucilage

The FTIR spectra (Fig. 3.3) of the mucilages were typical of polysaccharides. A major band in the region between 3000–3600 cm^{-1}, resulting from the axial deformation of hydroxyl groups with intermolecular hydrogen bonding (Njintang et al., 2014), was observed in both mucilages. The amide I band observed between 1700 and 1600 cm^{-1}, mainly due to the C=O stretching of the peptide groups, also implies the presence of proteins in the mucilages (Andrade, 2015). Similar bands have been observed in taro mucilages (Njintang et al., 2014; Lin & Huang, 1993).

Another peak at 1411 cm^{-1}, which may be due to COO^- symmetric stretching was also observed in cactus mucilage (Njintang et al., 2014). Both mucilages had a band between 1320 and 1210 cm^{-1}, which is characteristic of the C-O stretching of carboxylic acids (Njintang et al., 2014). This suggests that both mucilages contain uronic acids. Bands between 1200 and 1000 cm^{-1} may result from strong vibration of –C-O-C- and –OH of polysaccharides (Barka, Abdennouri, El Makhfouk, & Qourzal, 2013). The band was more intense in cactus mucilage confirming that there are more uronic acids in cactus mucilage. The peak at 895 cm^{-1} in cactus mucilage is thought to be characteristic of β-anomeric carbon, implying that cactus mucilage contains mainly β-type glycosidic linkages as observed in *Opuntia cochenillifera* mucilage (Monrroy, García, Ríos, & García, 2017). This confirmed previous suggestions that cactus mucilage is a linear chain of β-D-galacturonic acid (McGravie & Parolis, 1981). From the spectra, it can also be observed that both

mucilages are mainly polysaccharides with associated proteins. The C-O, OH as well as the C = O groups confirm the carbohydrate nature of mucilage.

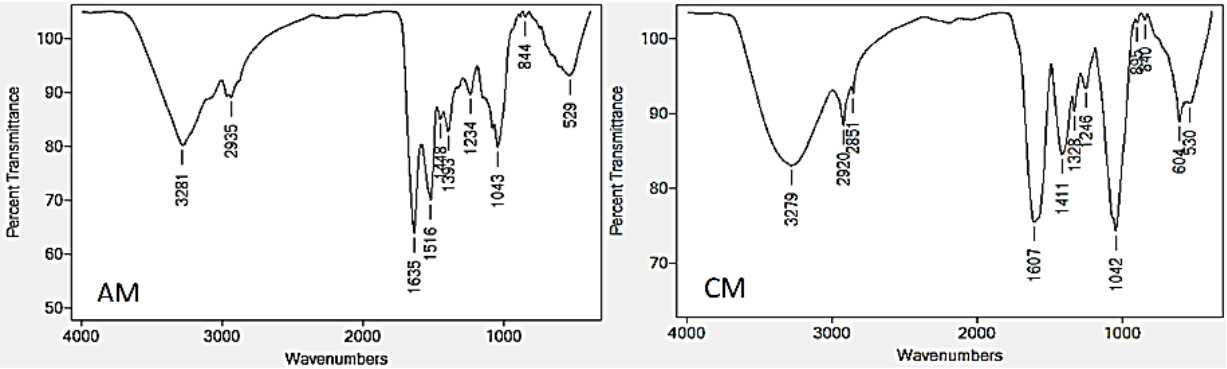


Figure 3.3. FT-IR spectra of amadumbe (AM) and cactus (CM) mucilages

3.3.6 Thermal profiles of amadumbe and cactus mucilages

The DSC thermal profile of amadumbe mucilage showed a broad endothermic transition with an onset temperature of 44ºC and a peak of 111ºC (Fig. 3.4). Cactus mucilage also showed a similar thermal profile with an onset temperature of 48ºC and a peak of 98ºC (Fig. 4). Cactus mucilage with 7.2% moisture showed a similar thermal profile (León-Martínez, Mendez-Lagunas & Rodriguez-Ramirez, 2010). The broad peaks observed are possibly due to vapourisation of water and successive thermal events (Otálora, Carriazo, Iturriaga, Nazareno, & Osorio, 2015). Similar broad peaks have previously been reported for cactus mucilage (Otálora, Carriazo, Iturriaga, Nazareno, & Osorio, 2015). This is because mucilages are heterogeneous hydrophilic carbohydrates which may contain mostly amorphous and some crystalline polysaccharides. DSC data compared favourably to those of chia mucilage (Velázquez-Gutiérrez et al., 2015) and tamarind mucilage (Alpizar-Reyes et al., 2017) incubated at temperatures from 25ºC to 40ºC, with moisture at water activities varying from 0.11 to 0.7. There was no evidence of depolymerisation of amadumbe mucilages up to 195ºC since degradation is exothermic. Similar DSC profiles have also been reported for gum arabic and cashew gum (Mothé & Rao, 2000). The delayed degradation at very high temperatures shows that the mucilages are thermally stable and may be used in food processed at high temperatures such as baked products.

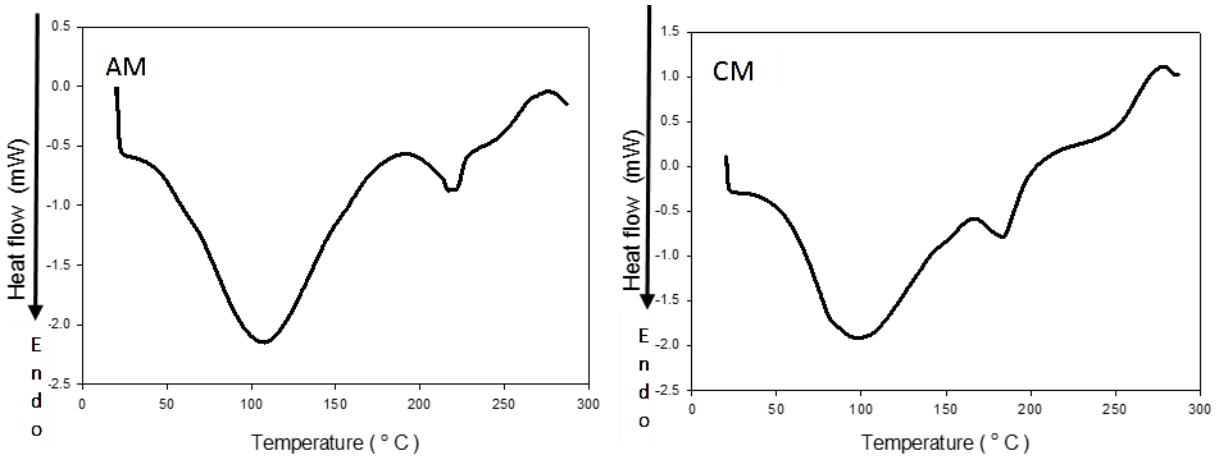


Figure 3.4. DSC thermograms of amadumbe (AM) and cactus (CM) mucilages

3.3.7 Mucilage steady-shear viscosity profiles

The viscosity curves of amadumbe and cactus mucilages are shown in Fig. 5. Gum arabic was used as a standard for the flow behaviour of low viscosity hydrocolloids. Amadumbe mucilage was less viscous than cactus mucilage at any given concentration. At low concentrations (below 10%), amadumbe mucilage had a Newtonian flow behaviour. Gum arabic exhibited Newtonian flow behaviour at up to 10% concentration, similar to a previous report (Massey, MacNaughtan, Williams, Wolf, & Iqbal, 2017). This makes amadumbe mucilage important considering the economic importance of gum arabic. Cactus mucilage was shear thinning (pseudoplastic) even at 1% concentration. This is comparable to previous reports in literature (Gebresamuel & Gebre-Mariam, 2011; Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez, & Katthain, 2000).

The mucilage flow behaviour fitted well within experimental error by the power law model as shown by $r^2 > 0.9$ (Table 3.3). Pseudoplasticity is shown by $n < 1$, whilst $n = 1$ shows Newtonian flow behaviour. An increase pseudoplasticity of both mucilages was also observed with an increase in concentration. The consistency coefficient, k, showed that increasing mucilage concentrations increased viscosity at any given shear rate. A similar behaviour has been reported for chia mucilage (Capitani et al., 2015). The flow behaviour of amadumbe mucilage shows that it may have limited applications as a viscosity modifier of food, but is important in beverages, where emulsions between food flavour oils and water are stabilised without changes in viscosity (Cunningham,

2011; Saha & Bhattacharya, 2010). The viscosity and flow characteristics of cactus mucilage indicate that it is a potential food thickener and stabiliser.

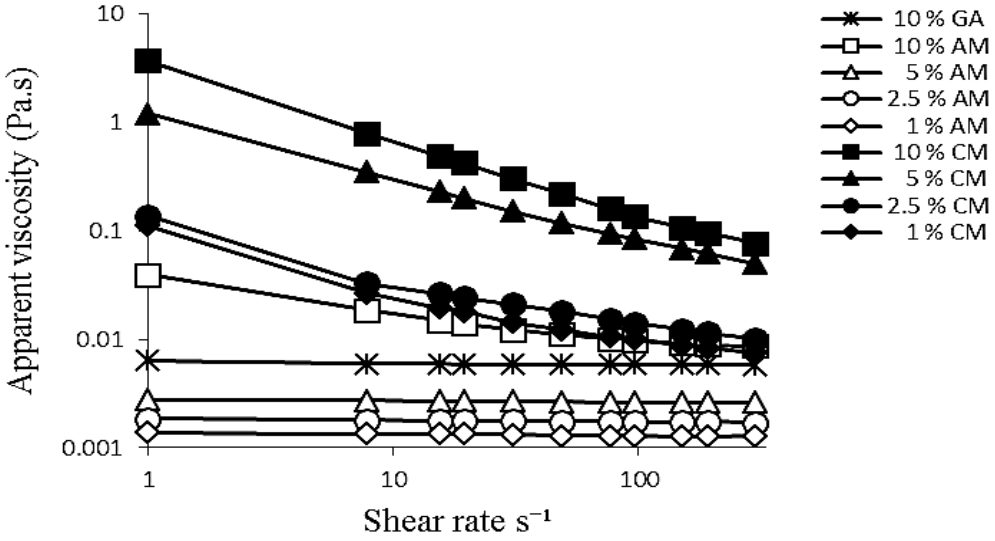


Figure 3.5. Viscous flow curves of amadumbe (AM) and cactus mucilage (CM) of different concentrations compared to a gum arabic (GA) standard at 25°C.

Table 3.3. Power-Law model coefficients of amadumbe (AM), cactus (CM) and a gum arabic (GA) standard.

Mucilage	**k (Pa.s)**	**n**	**r^2**
10% GA	0.00591 ± 0.0	0.99759 ± 0.0	1
10% AM	0.01829 ± 0.0	0.86682 ± 0.0	0.99848
5% AM	0.00271 ± 0.0	0.99417 ± 0.0	0.99999
2.5% AM	0.00191 ± 0.0	0.98069 ± 0.0	0.99919
1% AM	0.00125 ± 0.0	1.00651 ± 0.0	0.9998
10% CM	2.89144 ± 0.4	0.35081 ± 0.0	0.93414
5% CM	0.88394 ± 0.1	0.49392 ± 0.0	0.99021
2.5% CM	0.06183 ± 0.0	0.68025 ± 0.0	0.99669
1% CM	0.03716 ± 0.0	0.71738 ± 0.0	0.98707

Mean ± SD. k: Consistency index, n: flow behaviour index, r^2: correlation coefficient.

3.4 Conclusion

Amadumbe and cactus mucilages consist of polysaccharides with associated proteins and phenolics. Cactus mucilage contains rhamnose which is absent in amadumbe mucilage. The acidic sugar composition is different for the two mucilages; galacturonic acid is present in cactus while glucuronic acid is associated with amadumbe mucilage. Both mucilages are thermally stable. Amadumbe mucilage exhibits a Newtonian flow behaviour at low concentrations whilst cactus mucilage displays a pseudoplastic behaviour. Both mucilages show a shear thinning behaviour with an increase in concentration. Amadumbe mucilage may be a potential emulsifier in low viscosity applications such as beverages and jelly candy, as a substitute for gum arabic. Cactus mucilage can potentially be used as a thickening and emulsifying agent in food applications such as in gluten-free dough and mayonnaise ingredient formulations.

CHAPTER FOUR

4.0 Laccase-mediated crosslinking of gluten-free amadumbe flour improves rheological properties

Abstract

The absence of gluten in gluten-free flours presents a challenge to their application in baking. Enzymatic modification of the protein and polysaccharides may result in a network that mimics gluten. In the current study, the effects of laccase on the rheological properties of amadumbe dough were investigated. Thiol and total phenolic contents of dough decreased by up to 28% and 93%, respectively, as laccase activity was increased (0-3 U/g flour). Both G' and G" of laccase-treated dough increased significantly due to laccase-catalysed cross-linking of proteins and polysaccharides esterified with phenolics, as demonstrated by relevant model reactions. Tan δ decreased with increase in laccase activity indicating an increase in the elastic character of the dough. The improvement in dough viscoelasticity may enable the retention of adequate carbon dioxide during proofing and production of more acceptable gluten-free bread.

4.1 Introduction

There is a growing demand for gluten-free products in many countries. This is associated with some consumers experiencing gluten intolerance or suffering from celiac disease. Celiac disease is an inflammatory autoimmune disease in genetically susceptible individuals and can be managed by a total exclusion of gluten from the diet. Although celiac disease affects about 1% of the Western population and 0.33% of black Africans (Badenhorst, 2014), health-conscious consumers are also opting for gluten-free foods. However, the exclusion of gluten from baked products poses a number of technological challenges. In general, gluten-free dough lacks cohesive and elastic properties, resembling cake batters in its viscosity. Furthermore, the resulting bread has a crumbling texture, poor volume, is less tasty and stales faster (Onyango, Mutungi, Unbehend, & Lindhauer, 2010). The network formed by gluten is essential for adequate water absorption, dough strength, extensibility, elasticity and viscosity (Renzetti, Dal Bello, & Arendt, 2008) which enable

carbon dioxide retention during proofing (Sly, Taylor, & Taylor, 2014). Disulphide bonds, dityrosine bonds and other weaker bonds such as hydrogen bonds are proposed to confer gluten its properties, resulting in the desirable dough and bread (Shewry, Halford, Belton, & Tatham, 2002).

Diverse ingredients are used in the formulation of gluten-free bread. These include gluten-free cereal flours and starch from maize and rice, with hydrocolloids and proteins added to improve the viscoelastic properties of dough (Matos & Rosell, 2015). However, the increase in the world population has resulted in a sharp rise in demand for these cereal crops whilst the production is falling behind (Reynolds et al., 2016). This creates an opportunity to explore alternative crops in gluten-free applications.

Amadumbe *(Colocasia esculenta*) also known as Taro may potentially be applied in gluten-free bread making since it is gluten-free. These starchy corms also contain appreciable amounts of mucilage, ranging from 3 to 19% (Nguimbou et al., 2014; Njintang et al., 2014), which may make it unnecessary to amend it with additional hydrocolloids. The mucilage has been shown to improve wheat bread softness and acceptability (Nagata, Andrade, & Pereira, 2015). However, amadumbe flour has not been extensively used in bread making because of its poor viscoelastic properties. It is low in protein and hence the formulation of gluten-free amadumbe bread requires protein supplementation. The complexity of mimicking bonds occurring in hydrated gluten has also led to the use of cross-linking enzymes in gluten-free dough systems. Cross-linking enzymes such as transglutaminase (Marcoa & Rosell, 2008) tyrosinase (Flander, Holopainen, Kruus, & Buchert, 2011) and laccase (Flander et al., 2011; Flander et al., 2008) have been applied to modify gluten-free rice or oat dough. Of these, laccase has a greater potential due to its wider substrate range which includes diphenols, methoxy-substituted monophenols, and aromatic and aliphatic amines (Kudanga, Nyanhongo, Guebitz, & Burton, 2011).

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper enzymes catalysing one-electron oxidation reactions of a wide range of substrates while simultaneously reducing molecular oxygen to water (Kudanga, Nemadziva, & Le Roes-Hill, 2017). Generally, mucilages are rich in proteins and phenolic compounds are esterified to the polysaccharide chain (Chen, Harris, Sims, Zujovic, & Melton, 2017; Nguimbou et al., 2014). Since potential laccase substrates

are present in amadumbe, laccase-catalysed modification may improve the rheological properties of amadumbe dough.

Therefore, the aim of this study was to investigate, for the first time, laccase-mediated crosslinking of amadumbe dough for potential gluten-free applications. The mechanistic evidence of crosslinking by *Trametes versicolor* laccase is provided using model substrates followed by an investigation of the effect of the enzyme on amadumbe flour.

4.2. Materials and methods

4.2.1 Materials, model compounds, reagents and enzymes

T. versicolor laccase was purchased from Sigma Aldrich, South Africa (SA) and the manufacturer specification of its activity was 0.5 U/g. Ferulic acid, reduced glutathione, cysteine, tyrosine, Folin Ciocalteu and Ellman's Reagent (5, 5'-dithio-bis-[2-nitrobenzoic acid]) were also purchased from Sigma Aldrich (SA). Soy protein isolate was purchased from Lionheart Chemicals, Durban, (SA). The moisture, protein and ash content of soy protein were 6.5%, 90.2% and 1.8%, respectively. Amadumbe corms were purchased from Jozini, KwaZulu Natal province (SA). The moisture, protein and ash contents of amadumbe flour were 4.9%, 3.2% and 3.1%, respectively, determined according to the methods of the Association of Official Analytical Chemists (AOAC, 1990).

4.2.2 Model reactions

Reactions were carried out in an aqueous solution to predict possible reactions that would occur in amadumbe flour when *T. versicolor* laccase is added. The oxidation of glutathione and cysteine (5 mM each, final concentration), was carried out in the presence of 5 mM ferulic acid, 5 mM tyrosine or both at 25°C in 50 mM ammonium acetate buffer (pH 5.5). The reaction was initiated by adding 2.5 U of *T. versicolor* laccase. The reactions were incubated for 1 h and the enzyme was precipitated by adding an equal volume of ice-cold methanol.

4.2.3 LC-MS of reaction products

The samples were clarified through 0.22 µm Whatman microfilters (Sigma-Aldrich, South Africa) and 1.5 ml aliquots were transferred to clean vials. HPLC was carried out on a Shimadzu UHPLC system coupled to a Shimadzu ESI mass spectrophotometer (Shimadzu, Kyoto, Japan). Separation of the reaction products was done on a Sunfire C18 reversed phase column (Waters, Johannesburg, South Africa) using a gradient elution consisting of 0.1% formic acid (A) and acetonitrile (B). The gradient setup was as follows: 98% A - 0% A (20 min); 0% A - 98% A (20–21 min); 98% A (21–23 min). MS spectra were acquired in negative mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 l min^{-1} with a temperature of 350°C and nebulizer gas pressure was set to 35 psi. Peaks were analysed using Shimadzu LabSolutions software.

4.2.4 Dough preparation

The dough was prepared manually by adding 5% (w/w) soy protein isolate to amadumbe flour, a level pre-determined by baking trials to achieve the minimum protein content of 8% necessary for acceptable bread, then adding 90% (v/w) water to make a batter consistency. Laccase at different activities was added and the batter was incubated for 35 min at ± 25°C. The enzyme reaction was terminated by immediately placing samples in a freezer at -80°C before lyophilisation.

4.2.5 Effect of enzymes on dough free thiols

Changes in thiol groups were determined using the Ellman's reagent according to the method of Steffolani, Ribotta, Pérez, & León (2010). The modified and lyophilised dough (200 mg) was suspended in 1 ml GuHCl/Tris-Glycine solution and vortexed for 10 min. The sample was then centrifuged for 10 min at 10,000 × g (Eppendorf 5810R Centrifuge, Germany). To 0.1 ml of the clear supernatant, 0.15 ml of GuHCl/Tris-Glycine solution and 0.05 ml of Ellman's reagent were added and the absorbance was read at 412 nm using a Cary 100 CONC UV-VIS spectrophotometer (Varian Inc, USA). Results were calculated against a cysteine standard curve.

4.2.6 Effect of laccase on total phenols

The total phenols in freeze-dried dough samples were determined using Folin–Ciocalteu reagent, according to the method of Nguimbou et al, (2012). To 50 µl of flour suspension (30 mg/ml), 75 µl of Folin–Ciocalteu reagent was added and mixed thoroughly. After 3 min, 750 µl of Na_2CO_3

solution (20% w/v) was added. The mixture was allowed to stand for 1 h with intermittent shaking. The absorbance was measured at 760 nm using a UV-VIS spectrophotometer. The total phenolic content was expressed as mg of ferulic acid equivalent per g of flour, using an equation obtained from the standard ferulic acid calibration curve.

4.2.7 Effect of laccase on dough colour

Colour measurement of the freeze-dried dough was carried out using a Colour Flex EZ spectrophotometer (Hunterlab, Virginia, USA) on the basis of (L*), (a*) and (b*) values. L* measures lightness from black to white (0-100); a* indicates red (+) to green (–); while b* measures yellow (+) to blue (–). Amadumbe flour without enzyme (control) was used as a reference (L*= 87.72 a*=0.93 and b*=11.09). The instrument was calibrated against white and black colour tiles before colour measurement. The total colour difference (ΔE) was calculated as shown below:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$ where

ΔL = Colour difference is calculated as the sample L value minus standard.

Δa = Colour difference is calculated as the sample a value minus standard.

Δb = Colour difference is calculated as the sample b value minus standard.

4.2.8 Rheological properties of dough

Dynamic rheological measurements of the dough were determined using an Anton Paar MCR501 Rheometer with RHEOPLUS/32 V3.41 software (TA Instruments, New Castle, Delaware) as described previously by Marco & Rosell (2008). The measuring system consisted of parallel plate geometry (40 mm diameter, 1 mm gap). The dough was placed between the plates within 1 h of mixing, and the test was started after the dough had rested for 5 min. The rim of the sample was coated with a thin layer of paraffin oil to prevent evaporation during measurement. Measurements were performed at 30°C. The linear viscoelastic zone was determined by stress sweeps at a constant frequency of 1 Hz. Frequency sweep tests were performed from 0.01 to 15.00 Hz to determine the storage modulus (G').

4.2.9 Statistical analysis

Except where stated, all experiments were carried out in triplicate. Data were analysed using analysis of variance (ANOVA) and means were compared using Fischer's Least Significant Differences Test ($p < 0.05$ and $p < 0.01$).

4.3. Results and discussion

4.3.1 Laccase model reactions

Glutathione was used as a model peptide with sulfhydryl groups. In the absence of laccase, no products were formed by mixing tyrosine, ferulic acid and reduced glutathione as expected. However, laccase-catalysed reactions showed a number of products which were analysed by mass spectrometry (MS).

4.3.1.1 Analysis of products of ferulic acid oxidation by MS

MS analyses, in negative ion mode, showed ferulic acid dimers *m/z* $[M-H]^-$ = 385 (Exact mass [M] = 386) (Fig. 4.1) at retention times A (t_R = 13.407) and B (t_R = 14.542). To characterise the structure, the in-source fragmentation spectrum of the ions was analysed. The fragment ions at *m/z* = 341 and *m/z* = 297 could be explained by the loss of one CO_2 and two CO_2 groups from the parent ion (*m/z* = 385), respectively. Surprisingly, the two dimers (Fig.4.1A and B) had a similar fragmentation pattern. Previous reports have shown the oxidation of ferulic acid resulting in two dimers (β-5 and β-β) with different fragmentation patterns (Adelakun et al., 2012). In the β-β configuration the carboxylic groups are not available for ionization. Therefore the fragmentation of the dimers obtained in this study indicates that the two dimers formed have covalent links elsewhere, other than being β-β dimers, due to the ease of loss of CO_2. The β-dimers have been shown to be preferred, with several possibilities existing due to a large number of mesomeric forms resulting from resonance stabilization of the ferulic acid radical. Coupling of a similar molecule, coniferyl alcohol, also resulted in the formation of β–β, β-O-4 and β-5 dimers only (Vanholme, Demedts, Morreel, Ralph, & Boerjan, 2010). Furthermore, lignin synthesis has been observed to be due to the formation of predominantly β-O-4 linkages (Kandanarachchi, Autrey, & Franz, 2002). By also factoring in the polarity of the molecules, it can be deduced that the compounds

formed are most likely the β-5 (A) and β-O-4 (B) dimers. Similar fragmentation patterns have been reported for the β-5 (Adelakun et al., 2012) and β-O-4 dimers (Chiremba, Rooney, & Beta, 2012).

The ability of laccase to oxidise ferulic acid resulting in dimerisation is important in dough systems. The use of laccase in wheat dough has been found to increase the dough strength (Flander et al., 2011; Selinheimo, Kruus, Buchert, Hopia, & Autio, 2006) and stability (Labat, Morel, & Rouau, 2000). The resultant bread had an increased specific volume and decreased hardness. This has been attributed to the cross-linking of ferulic acid esterified to cell wall polysaccharides forming a gel with a higher water absorption capacity as well as a matrix which mimics gluten.

4.3.1.2 Analysis of thiol oxidation products by MS

Laccase-mediated reactions resulted in thiol oxidation, but only in the presence of tyrosine and ferulic acid. This was due to the inability of laccase to directly oxidise thiols. Therefore phenolic molecules such as ferulic acid acted as mediators. In a previous study, no oxygen was consumed when laccase was added to cysteine and glutathione in the absence of phenolic compounds implying that no reaction had occurred (Figueroa-Espinoza, Morel, & Rouau, 1998).

In the presence of tyrosine and/or ferulic acid, laccase addition resulted in disulphide bond formation between cysteine molecules (*m/z* 239; exact mass 240; t_R = 3.124) (Fig. 4.1C). Furthermore, disulphide bonds were also formed between glutathione-cysteine (*m/z* 425; exact mass 426; t_R = 3.428) (Fig. 4.1D). Glutathione was also readily oxidised in the presence of phenolic radicals formed by laccase, resulting in disulphide crosslinks of glutathione *m/z* 611 (exact mass 612) as proposed in Fig. 4.1E.

Laccase addition may therefore result in protein cross-linking in the dough due to disulphide bond formation. The high molecular weight (HMW) subunit of glutenin has been proposed to confer dough elasticity and cohesiveness to wheat gluten. This protein is stabilized by interchain disulphide bonds which are considered to form the 'elastic backbone' of gluten. The amounts of these HMW subunits positively correlated with dough strength (Shewry et al., 2002). The formation of disulphide bonds may increase gluten-free dough strength and elasticity, which may enhance the ability of the dough to expand and retain carbon dioxide released during proofing.

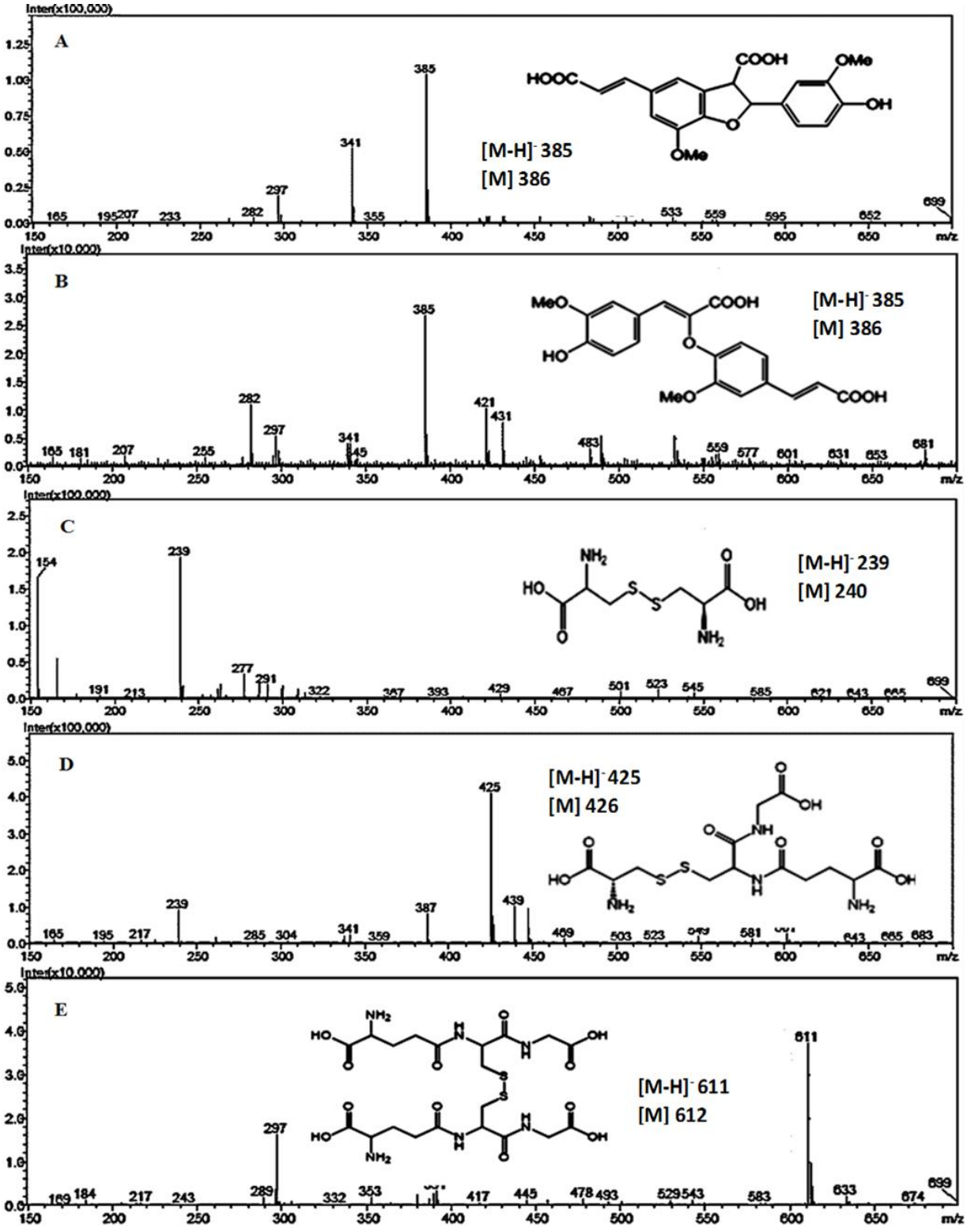


Figure 4.1. Mass spectra and proposed structures of ferulic acid dimers (A), (B); products of laccase-mediated oxidation of thiols in the presence of tyrosine and/or ferulic acid; cystine (C), cysteine-glutathione conjugate (D) and glutathione-glutathione conjugate (E).

4.3.1.3 MS analysis of other homo- and hetero-conjugates formed due to oxidation reactions

Tyrosyl radicals formed by laccase oxidation also formed conjugates with thiols as shown in Fig 4.2. Conjugates were formed with cysteine (m/z 299 exact mass 300; t_R =10.531) (Fig. 2A) and glutathione (m/z 485 exact mass 486; t_R = 15.325) (Fig. 2B).). Formation of these conjugates in the dough may also result in protein crosslinking, thereby increasing dough strength.

Ferulic acid also formed conjugates with cysteine (*m/z* 313 exact mass 314; t_R = 18.520) (Fig. 4.2C), glutathione (*m/z* 501 exact mass 502; t_R = 19.491) (Fig. 4.2D) and tyrosine (*m/z* 375 exact mass 376; t_R = 18.033) (Fig. 4.2E), as a result of laccase-mediated oxidation reactions. The addition of thiols and tyrosine to a feruloyl radical may involve the activated double bond as shown in the proposed structures. Laccase has been reported to form heteroconjugates between tyrosine, tyrosine-containing peptides or proteins and feruloylated arabinoxylan (Mattinen et al., 2005). The formation of these conjugates in the dough may also lead to increased dough strength. Heteroconjugates may also act as emulsifiers in the dough due to the presence of a hydrophilic polysaccharide chain and hydrophobic amino acids.

Laccase-mediated reactions did not result in detectable dityrosine bonds in the presence or absence of ferulic acid. Previously, it was reported that only a small amount of the available tyrosine (< 0.2%) formed dityrosine upon laccase-catalysed oxidation in the absence of ferulic acid, whilst no detectable dityrosine was formed in the presence of ferulic acid (Steffensen et al., 2009). This showed that ferulic acid was preferentially oxidized by *T. versicolor* laccase.

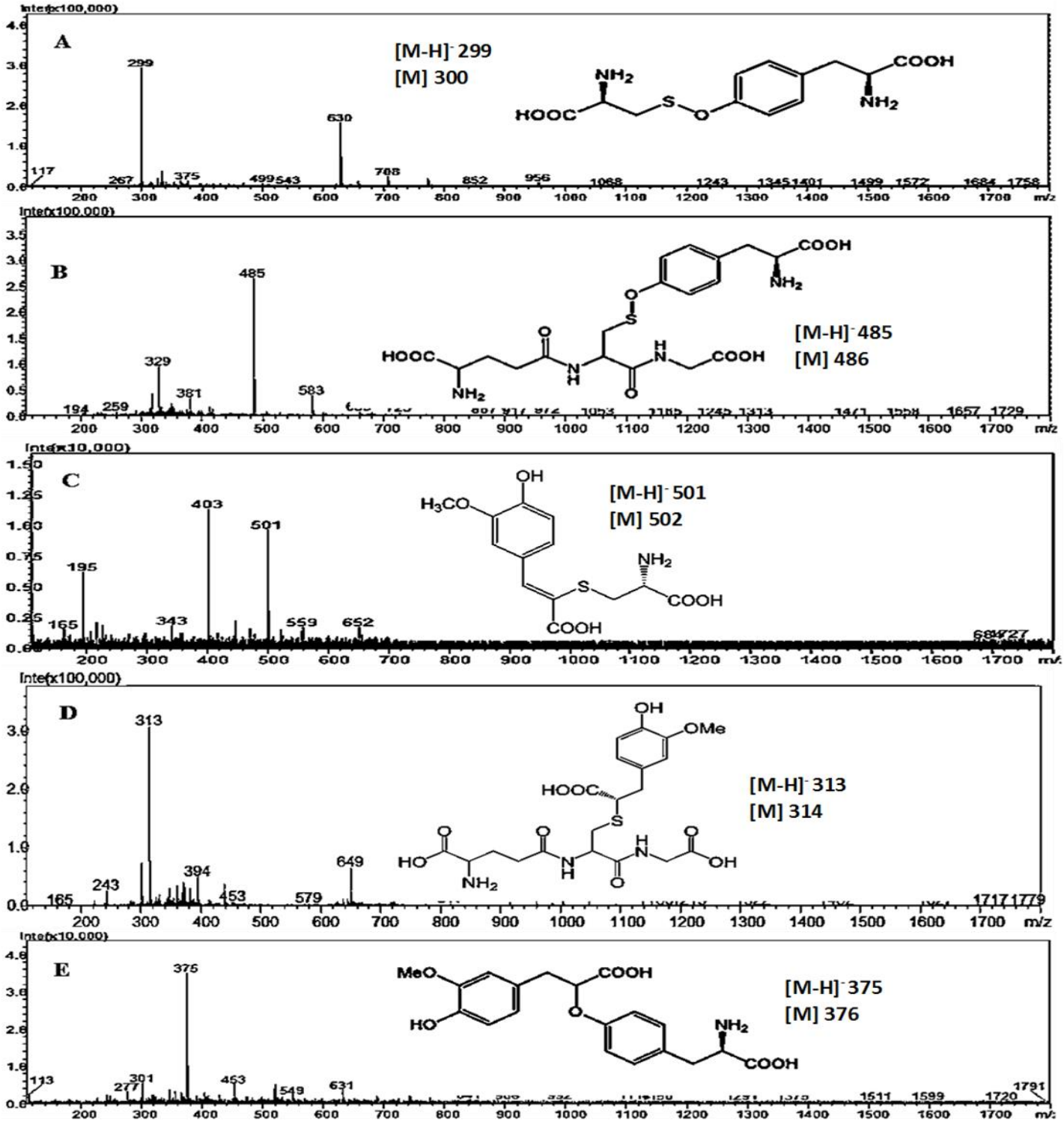


Figure 4.2. Mass spectra and proposed structures of tyrosine conjugates with cysteine (A) and glutathione (B); ferulic acid conjugates with cysteine(C),glutathione (D) and tyrosine (E), formed during laccase-mediated oxidation reactions.

4.3.2 Effect of laccase on dough thiol and phenolic content

The effect of laccase on modified and freeze-dried amadumbe dough supplemented with 5% soy protein was also studied by quantifying the free sulfhydryl content. Soy protein was added to increase the amino acid content of the dough since amadumbe flour is low in protein. Furthermore, soy protein isolate has potential substrates of laccase oxidation such as tyrosine and cysteine. The addition of laccase to amadumbe dough resulted in up to 28% decrease in the dough free sulfhydryl content (Fig. 4.3A). The decrease of the thiols confirmed the possible disulphide bond or thiol-phenolic conjugate formation in the dough. The model reactions also confirmed that feruloyl and tyrosyl radicals may also result in thiol oxidation. Increasing the enzyme activity from 0.5 to 2.5 U/g flour had no statistically significant effect ($p > 0.05$) on the thiol content as only a slight decrease was observed. Previous studies report a 55% decrease in the -SH groups in oat dough and the decrease was attributed to the presence of free ferulic acid which was oxidised to phenoxy radicals in the dough and led to disulphide bond formation (Flander et al., 2008). Amadumbe flour contains mucilage which may also gel due to laccase-mediated dimerisation of ferulic acid esterified to its arabinogalactan moiety or the formation of protein-phenolic conjugates such as those formed due to ferulic acid-tyrosine covalent links, hindering extensive protein aggregation and limiting the extent of disulphide bond formation.

A significant decrease ($p < 0.05$) in dough phenolics (up to 93%) was observed with increase in laccase activity (Fig. 4.3B). This was due to cross-linking and possibly polymerisation of phenolic compounds. Laccases are widely used in bioremediation because of their ability to reduce the total phenolic content. Laccase significantly decreased olive oil waste phenolic content by up to 90% (Martirani, Giardina, Marzullo, & Sannia, 1996). It has been demonstrated in this study that dimers and phenolic conjugates may be formed. The decrease in the total phenolic content confirmed the cross-linking of phenolics in amadumbe dough when laccase was added.

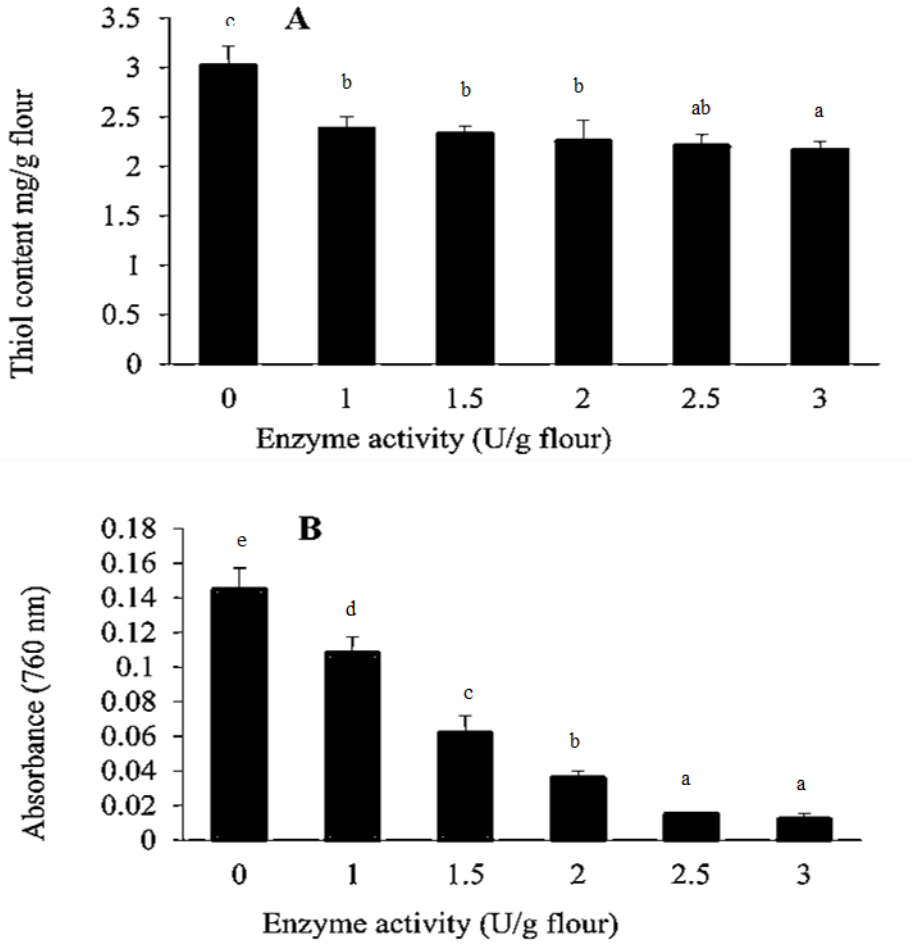


Figure 4.3. Effect of laccase activity on dough thiol content (A) and total phenols (B). Means ± SD, n=3; Bars with different letters differ significantly ($p<0.05$).

4.3.3 Effect of laccase on freeze-dried dough colour

The optical properties of freeze-dried dough were determined. Generally, there was an activity-dependent change in dough colour (Table 4.1). The whiteness index (L*) decreased, showing that the dough darkened as a result of oxidation reactions. However, further increases in enzyme activity from 1.5 U to 3 U did not significantly ($p < 0.01$) decrease the whiteness of the dough. There was a significant ($p < 0.01$) increase in redness (a*) and yellowness (b*), shown by the positive chromatic coordinates, as enzyme activity was increased. Polymerisation of ferulic acid has been shown to increase redness of a solution (Aljawish et al., 2014). The increase in redness may therefore be due to ferulic acid oxidation and dimerisation resulting in pigments in the dough. The darkening of the dough may be attributed to the formation of polymeric phenolic compounds

and pigments by laccase-mediated reactions. However, the change in colour was limited since phenol dimerization and poymerisation seems to be preceded by thiol oxidation. The presence of thiols has been similarly reported to reduce the rate of phenol polymerization (Figueroa-Espinoza et al., 1998).

Table 4.1: Effect of laccase on dough colour

Laccase activity (U/g flour)	**L***	**a***	**b***	**ΔE**
0	$87.72^{a} \pm 0.503$	$0.93^{f} \pm 0.05$	$11.09^{c} \pm 0.37$	0.00^{d}
1	$87.86^{a} \pm 0.12$	$1.21^{e} \pm 0.02$	$11.88^{b} \pm 0.07$	0.85^{c}
1.5	$85.65^{b} \pm 0.14$	$1.71^{d} \pm 0.04$	$11.69^{b} \pm 0.09$	2.29^{b}
2	$85.83^{b} \pm 0.02$	$1.90^{c} \pm 0.05$	$11.72^{b} \pm 0.09$	2.22^{b}
2.5	$86.11^{b} \pm 0.19$	$2.07^{b} \pm 0.06$	$12.94^{a} \pm 0.10$	2.70^{a}
3	$85.62^{b} \pm 0.09$	$2.18^{a} \pm 0.03$	$12.65^{a} \pm 0.10$	2.90^{a}

Mean ± SD (n=3). Values along the column followed by different superscripts are significantly different ($p < 0.01$).

4.3.4 Effect of laccase on amadumbe dough dynamic rheological properties

An oscillatory test with a frequency sweep from 0.01 to 10 Hz was conducted to determine the effect of laccase on the dynamic viscoelastic properties of amadumbe flour supplemented with soy protein (Fig. 4.4). The dynamic moduli were all frequency-dependent. Generally, the elastic or storage modulus (G') was higher than the viscous or loss modulus (G") at any given frequency, indicating that the resulting dough had a solid, elastic-like structure (Marco & Rosell, 2008). There was a significant increase ($p < 0.05$) in the viscous and elastic moduli when laccase activity was increased from 0 to 1.5 U/g flour. However, further increases in laccase activity beyond 1.5% did not result in a significant increase ($p > 0.05$) in the viscous modulus but increased the elastic modulus. The improvement in dough properties may be attributed to modification of proteins in the dough by disulphide bond formation, oxidative gelation of mucilage as well as hetero-crosslinking between proteins and mucilage, which may strengthen the dough. The formation of large protein aggregates visualized using a confocal laser scanning microscope coupled with a significant decrease in the amount of extractable amino nitrogen was observed when laccase was added to oat dough, implying that protein polymerization had occurred (Renzetti, Courtin,

Delcour, & Arendt, 2010). Furthermore, laccase has been reported to increase dough strength by cross-linking ferulic acid esterified to cell wall polysaccharides (Flander et al., 2011; Selinheimo et al., 2006). Amadumbe flour contains mucilage which also contains phenolic compounds. Oxidative gelation of the mucilage may therefore result in the formation of a more viscous dough.

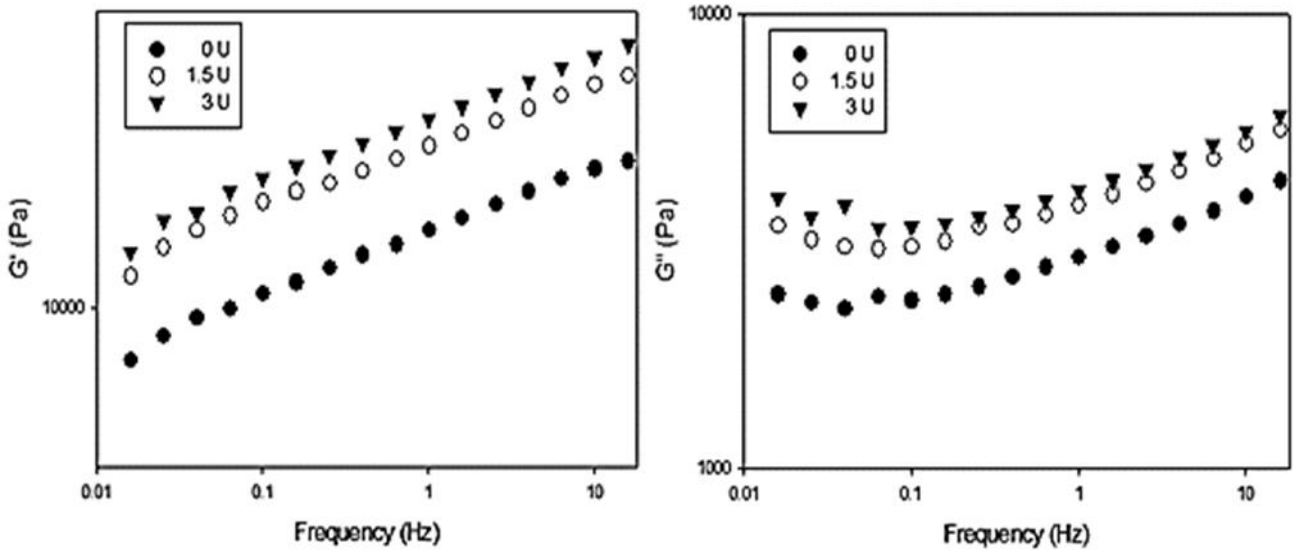


Figure 4.4. Effect of laccase activity on the viscous and storage modulus of amadumbe dough.

Tan δ was less than 1 at all frequencies indicating that the dough had a relative elastic behaviour (Table 4.2). The complex modulus (G*) increased while Tan δ decreased as the enzyme activity was increased (Table 4.2). The increase in the complex modulus shows the general increase in the elastic, viscous characteristics and the overall strength of the dough (Marco & Rosell, 2008). The decrease in Tan δ in the presence of enzyme suggests an increase in the relative elastic character of the dough (Lahiji, Mohammadi, Moslemy, & Aminigram, 2013). Disulphide bonds have been proposed to provide a backbone in the protein structure for elasticity (Shewry et al., 2002). This study has also demonstrated disulphide bond formation using model reactions and this could also be implied from the decrease in thiol functional groups. Furthermore, oxidative gelation of ferulic acid esterified to cell wall polysaccharides results in a viscoelastic gel with a higher water holding capacity (Carvajal-Millan et al., 2005; Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003). This

increases dough viscoelasticity. Increased dough strength has also been reported for oat dough modified by laccase (Flander et al., 2008). The use of laccase in baking has also been reported to result in increased strength, stability and reduced stickiness of wheat dough (Labat et al., 2000). Increased dough strength may therefore result in a dough with better carbon dioxide retention during proofing and may result in bread that has improved characteristics.

Table 4.2: Effect of laccase on the dynamic moduli and loss factor of amadumbe dough

Laccase activity (U/g flour)	**Viscous modulus**(G") Pa	**Elastic modulus** (G') Pa	**Complex modulus** G* Pa	**Phase angle** Tan δ
0	$4300^{a} \pm 370$	$16100^{a} \pm 600$	$16700^{a} \pm 100$	$0.267^{b} \pm 0.001$
1.5	$5580^{b} \pm 330$	$21200^{b} \pm 400$	$22000^{b} \pm 700$	$0.263^{b} \pm 0.006$
3	$5960^{b} \pm 140$	$23400^{c} \pm 300$	$24100^{c} \pm 600$	$0.255^{a} \pm 0.004$

Values at 10 Hz. Mean ± SD (n=2). Values followed by different letters in the same column are significantly different ($p < 0.05$).

4.4 Conclusion

T. versicolor laccase was able to crosslink phenolics and thiols producing a wide range of homo- and hetero-conjugates, as demonstrated by model reactions. Laccase-mediated oxidation of amadumbe dough resulted in a decrease in dough phenolic and thiol groups, confirming crosslinking reactions. The reduction in Tan δ indicates an improvement in the elastic properties of the laccase-treated dough. Therefore, laccase can potentially be used to improve the dough-forming properties of gluten-free amadumbe flour.

CHAPTER FIVE

5.0 Transglutaminase and tyrosinase as potential crosslinking tools for the improvement of rheological properties of gluten-free amadumbe dough

Abstract

Gluten-free bread remains of poor quality despite efforts to amend gluten-free flours with ingredients such as hydrocolloids, and proteins. Enzymatic modification of the proteins in dough may result in polymers that mimic gluten. This research investigated the effects of transglutaminase and tyrosinase on the rheological properties of amadumbe dough. Tyrosinase oxidation resulted in a 7.7 – 39.4% decrease in dough free amine and a 16.8 – 46.3% decrease in the dough thiol content as activity was increased from 0 to 80 U/g flour. Transglutaminase treatment decreased the dough free amino groups by 10 – 38.1% as activity was increased from 0 to 2 U/g flour. Evidence of tyrosinase and transglutaminase-mediated crosslinking was provided by relevant model reactions monitored by mass spectrometry. An increase in dough G' and G", showed that both transglutaminase and tyrosinase improved dough viscoelasticity. The increase in dough viscoelasticity may enable increased retention of carbon dioxide during proofing.

5.1 Introduction

The demand for gluten-free products has recently increased due to the increase in diagnosed celiac disease, gluten sensitivity and wheat allergy which necessitate a total exclusion of gluten from the diet (Southgate et al., 2017, Jnawali et al., 2016). However, exclusion of gluten especially in bread making is still a challenge. The bread is of poor volume, texture and acceptability. This is because gluten confers wheat dough its viscoelastic properties. It is therefore necessary to form a viscoelastic matrix which mimics gluten in gluten-free dough so that the dough may retain carbon dioxide during proofing and prevent the bread from collapsing during baking (Marcoa and Rosell, 2008).

Starches of different origins, flours from non-gluten cereals, pseudocereals and pulses, dairy proteins, legume proteins and hydrocolloids have been used as ingredients in gluten-free bread

making. However, increasing demand and poor quality of existing products have led to an increased interest in alternative and novel gluten-free flours (Nitcheu Ngemakwe et al., 2014). Amadumbe flour may be an interesting alternative in gluten-free breadmaking as it already contains mucilage (hydrocolloid) which may be a functional substitute for gluten.

Amadumbe *(Colocasia esculenta)*, also known as taro or cocoyam, are gluten-free starchy corms mainly grown for subsistence in many tropical countries as well as sub-tropical areas. It is estimated that 60% of amadumbe produced globally is from Africa and 40% is from Asia, with an average yield of 5.314 mt/ha (Owusu-Darko et al., 2014). Amadumbe flour is rich in carbohydrates of which about 80% is starch (Mawoyo et al., 2017). The flour also has a bland taste and contains about 7% mucilage (Njitang et al., 2014). Therefore, it is a potential flour for gluten-free bread making. However, amadumbe flour is low in proteins and it is necessary to supplement it with protein. Previous studies have shown that addition of protein to rice resulted in improved dough and bread specific volume (Marcoa and Rosell, 2008). However, covalent crosslinking of gluten-free dough proteins and polysaccharides may result in a stronger network which may further improve the dough and the resulting bread quality. Crosslinking may be achieved by chemical and enzymatic methods, with enzymes preferred since they are generally regarded as safe (GRAS) and are more acceptable to health-conscious consumers. Enzymes such as transglutaminase (Moore et al., 2006; Smerdel et al., 2012) and tyrosinase (Flander et al., 2011) have shown potential for protein crosslinking in gluten-free bread making.

Transglutaminase (Protein-glutamine γ-glutamyltransferase, EC 2.3.2.13), is an enzyme that catalyses acyl transfer reaction between a γ-carboxy amide of a peptide or protein-bound glutamine and a primary amine. It catalyses three reactions resulting in deamidation, crosslinking and acyl transfer. Network formation by transglutaminase resulted in improved bread from different gluten-free cereals (brown rice, buckwheat, corn, oat, sorghum and tef) (Renzettia et al., 2008). Marco and Rosell (2008) also observed improvements in rice bread characteristics when transglutaminase and legume protein isolates were added. Transglutaminase crosslinking may improve amadumbe dough viscoelasticity. However, the specificity of transglutaminase reduces the crosslinking efficiency when lysine is limiting. Another less specific protein crosslinking enzyme, tyrosinase, may be of greater potential in amadumbe dough.

Tyrosinase is a copper-containing phenol oxidase which catalyses the oxidation of mono and diphenols with oxygen as a co-substrate. It catalyses two distinct reactions which are hydroxylation due to their monophenolase activity and oxidation by ortho-diphenolase activity (Lantto et al., 2007b). Therefore, in the first oxidation an *o*-diphenol is formed from a monophenol and in the second reaction *o*-diphenol is further oxidised to a corresponding *o*-quinone (Thalmann and Lötzbeyer, 2002). Quinones are highly unstable and can further react to form higher molecular weight polymers. Tyrosinase was also observed to increase the specific volume of gluten-free oat bread (Flander et al., 2011).

The ability of these enzymes to crosslink dough proteins suggest the possibility of structural modification of amadumbe mucilage through its covalently linked protein chains (Chapter 3) and other dough proteins, which may increase the amount of higher molecular weight polymers. This may improve dough viscoelasticity. Therefore, the aim of this research was to investigate the effect of protein crosslinking on amadumbe dough rheology. The mechanistic evidence of crosslinking by transglutaminase and tyrosinase is provided for the first time using model substrates and mass spectrometry, followed by an investigation of the effect of the enzymes on amadumbe flour.

5.2 Materials and methods

5.2.1 Materials, model compounds, reagents and enzymes

Tyrosinase was purchased from Sigma Aldrich (South Africa) and the manufacturer specification of its activity was 1000 U/mg. Transglutaminase (Activa WM, 100 U/g) was kindly donated by Maccallum & Associates (Durban, South Africa; representing Ajinomoto). Glutathione, cysteine, tyrosine, histidine, asparagine, lysine, OPA and *Ellman's Reagent* (5, 5'-dithio-bis-[2-nitrobenzoic acid]) were also purchased from Sigma Aldrich (SA). Soy protein isolate was purchased from Lionheart Chemicals, Durban, South Africa (SA). The moisture, protein and ash contents of soy protein were 6.5%, 90.2% and 1.8%, respectively. Amadumbe corms were purchased from Jozini, KwaZulu-Natal province, South Africa. The moisture, protein and ash contents of amadumbe flour were 4.9%, 3.2% and 3.1%, respectively, determined according to the methods of the Association of Official Analytical Chemists (A.O.A.C, 2000)

5.2.2 Flour amino acid analysis

Amino acid analysis of amadumbe flour and soy protein isolate was carried out according to the method of Njitang et al. (2014). Flour (40 mg) was weighed out into digestion tubes containing 6 N HCl in 1% phenol. The tubes were then flushed with nitrogen, sealed and incubated for hydrolysis at 150^{o}C for 60 min. Hydrolysed samples were centrifuged at 5000 × g for 5 min at 20^{o}C. The supernatant was filtered into vials through a 0.22 µm Whatman microfilter (Sigma-Aldrich, South Africa). The pre-column derivatisation used phenylisothiocyanate (PITC) and the separation was achieved using a reversed phase column PTC RP-18 (2, 1*220 mm) (Applied Biosystems Corp, Foster City, CA, USA) on a Shimadzu UHPLC (Shimadzu, Kyoto, Japan). The elution was carried out using 45 mM sodium acetate buffer (pH 5.90) (A) and 30% of 100 mM sodium acetate buffer (pH 4.60) combined with 70% acetonitrile (B), and the amino acid derivatives were detected at 254 nm. Amino acids were expressed as mg/g flour.

5.2.3 Model reactions

Reactions were carried out using model amino acids in order to predict possible reactions that would occur in amadumbe flour supplemented with soy protein when transglutaminase or tyrosinase was added. Glutamine (5 mM) was mixed with 5 mM lysine, cysteine, asparagine or histidine in 50 mM ammonium acetate buffer (pH 5.5). Transglutaminase (2 U) was added to the mixture in order to initiate the reaction which was run for 1 h at 25°C. The tyrosinase reaction mixture contained 5 mM tyrosine and 5 mM glutathione, cysteine, lysine or histidine in 50 mM ammonium acetate buffer (pH 5.5). The reaction was initiated by adding tyrosinase (20 U) and was run for 1 h at 25°C. Thereafter an equal volume of ice-cold methanol was added to each reaction and the mixtures were kept on ice for 20 min in order to precipitate out the enzyme.

5.2.4 LC-MS of reaction products

The samples were clarified through 0.22 µm Whatman microfilters (Sigma-Aldrich, South Africa) and 1.5 ml aliquots were transferred to clean vials. HPLC was carried out on a Shimadzu UHPLC system coupled to a Shimadzu ESI mass spectrophotometer (Shimadzu, Kyoto, Japan). Separation of the reaction products was carried out on a Sunfire C18 reversed phase column (Waters, Johannesburg, South Africa) using a gradient elution consisting of 0.1% formic acid (A) and acetonitrile (B). The gradient setup was as follows: 98% A to 0% A (20 min); 0% A to 98% A

(20–21 min); 98% A (21–23 min). MS spectra were acquired in negative mode and electrospray voltage was set to +3500 V. Dry gas flow was set to 9 l min^{-1} with a temperature of 350°C and nebulizer gas pressure was set to 35 psi. Peaks were analysed using Shimadzu LabSolutions software.

5.2.5 Dough preparation

The dough was prepared manually by adding 10% (w/w) soy protein isolate to amadumbe flour, to achieve the minimum protein content of about 12% as in wheat flour, then adding 90% (v/w) water to make a batter consistency. Tyrosinase (0-80 U/g flour) or transglutaminase (0-2 U/g flour) was added and the batter was incubated for 35 min at ± 25°C. The enzyme reaction was terminated by immediately placing samples in a freezer at -80°C before lyophilisation.

5.2.6 Effect of enzymes on dough free thiols

Changes in thiol groups were determined using the Ellman's reagent according to the method of Steffolani, Ribotta, Pérez, & León (2010). The modified and lyophilised dough (200 mg) was suspended in 1 ml GuHCl/Tris-Glycine solution and vortexed for 10 min. The sample was then centrifuged for 10 min at 10 000 × g (Eppendorf 5810R Centrifuge, Germany). To 0.1 ml of the clear supernatant, 0.15 ml of GuHCl/Tris-Glycine solution and 0.05 ml of Ellman's reagent were added and the absorbance was read at 412 nm using a Cary 100 conc UV-vis spectrophotometer (Varian Inc, North Carolina, USA). Results were calculated against a cysteine standard curve.

5.2.7 Effect of enzymes on dough free amino groups

Changes in free amino groups in dough were determined using ortho- phthaldialdehyde according to a previously reported method (Marcoa and Rosell, 2008). Amadumbe freeze dried dough samples (0.2 g) were suspended in 2 ml of 0.1 M HCl (pH 1.0), vortexed for 10 min and then centrifuged for 10 min at 10 000 × g (Eppendorf 5810R Centrifuge, Germany). OPA reagent (2.5 ml) was added to 0.1 ml of the clear supernatant. The mixture was allowed to stand for 2 min and the absorbance read at 340 nm using a Cary 100 conc UV-vis spectrophotometer (Varian Inc, North Carolina, USA). Results were calculated against a serine curve.

5.2.8 Protein solubility

The protein solubility of the freeze-dried samples was determined at pH 4 and pH 6 using a method by Babiker (2000) with slight modifications. Samples (0.2%, w/v) were suspended in 0.05 M acetate buffer (pH 4) or 0.05 M phosphate buffer (pH 6), vortexed for 10 min and centrifuged for 2 min at 3 000 × g (Eppendorf 5810R Centrifuge, Germany). The turbidity of the supernatant was measured at 500 nm using a Cary 100 conc UV-vis spectrophotometer (Varian Inc, North Carolina, USA). Four replicates of each measurement were made.

5.2.9 Rheological properties of dough

Dynamic rheological measurements of the dough were determined using an Anton Paar MCR501 Rheometer with RHEOPLUS/32 V3.41 software (TA Instruments, New Castle, Delaware) as described previously (Demirkesen et al., 2010). The measuring system consisted of parallel plate geometry (40 mm diameter, 1 mm gap). The dough was placed between the plates within 1 h of mixing, and the test was started after the dough had rested for 5 min. The rim of the sample was coated with a thin layer of paraffin oil to prevent evaporation during measurement. Measurements were performed at 30°C. The linear viscoelastic zone was determined by stress sweeps at a constant frequency of 1 Hz. Frequency sweep tests were performed from 0.01 to 15.00 Hz to determine the storage modulus (G').

5.2.10 Statistical analysis

Except where stated, all experiments were carried out in triplicate. Data were analysed using analysis of variance (ANOVA) and means were 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 analysis of amadumbe flour and soy protein isolate

Amino acid analysis showed that soy protein isolate had more amino acids per gram sample compared to amadumbe flour, as expected (Table 5.1). Generally, both amadumbe flour and soy protein isolate had a higher content of the acidic amino acids with aspartic acid being the major amino acid in amadumbe flour whilst glutamic acid was the highest in soy protein isolate. This

amino acid profile compared favourably with that previously reported for taro (Njintang et al., 2014) and for soy protein (Friedman and Brandon, 2001). Addition of soy protein isolate to amadumbe flour would improve the composite's amino acid content. Transglutaminase has been proposed to form crosslinks by catalysing an acyl transfer reaction between a γ-carboxy amide of the peptide or protein-bound glutamine and a primary amine (Babiker et al., 1998, Dłużewska et al., 2015). The presence of glutamine and lysine in the flours confirms them as potential sources of substrates for modification. Asparagine has also been proposed to be an acyl donor whilst arginine and histidine have been proposed to be acyl acceptors during transglutaminase catalysed reactions (Rachel and Pelletier, 2013). Tyrosinase has been proposed to form di-tyrosine crosslinks in proteins as well as crosslinks between tyrosine and thiol groups (Isaschar-Ovdat and Fishman, 2017). The presence of tyrosine and cysteine in both flours suggests that they are potential sources of substrates for tyrosinase-catalysed reactions.

Table 5.1: Amino acid contents of amadumbe flour and soy protein isolate expressed as mg/g sample

Amino acid	**Amadumbe flour**	**Soy protein isolate**
Histidine	1.2 ± 0.1	13.4 ± 0.6
Serine	3.3 ± 0.0	42.6 ± 0.1
Arginine	3.4 ± 0.1	16.6 ± 0.3
Glycine	2.9 ± 0.0	14.8 ± 1.2
Aspartic acid/Asparagine	7.5 ± 0.7	96.2 ± 3.7
Glutamic acid/ Glutamine	5.5 ± 0.2	149.6 ± 2.3
Threonine	2.4 ± 0.1	62.7 ± 0.4
Alanine	2.4 ± 0.0	41.5 ± 1.1
Proline	2.2 ± 0.4	50.7 ± 0.8
Lysine	2.4 ± 0.1	76.1 ± 2.1
Tyrosine	2.0 ± 0.1	25.6 ± 0.3
Isoleucine	1.8 ± 0.0	54.9 ± 0.1
Phenylalanine	3.2 ± 0.1	24.8 ± 1.1
Valine	2.7 ± 0.2	51.7 ± 0.7
Cysteine	3.2 ± 0.0	34.7 ± 0.8
Leucine	4.5 ± 0.4	88.7 ± 3.1

Means ± SD; n=2

5.3.2 Model reactions

The effect of adding transglutaminase or tyrosinase to solutions containing model amino acids was studied and the products were analysed by mass spectrometry (MS).

5.3.2.1 Characterisation of products of tyrosine oxidation by MS

MS analyses, in negative ion mode, showed the formation of a dopaquinone-dopachrome conjugate (Fig 5.1A), *m/z* $[M-H]^-$ = 383 (exact mass [M] = 384.06), a tyrosine-tyrosine conjugate (Fig 5.1B), *m/z* $[M-H]^-$ = 375 (exact mass [M] = 376.13), di-dopaquinone (Fig 5.1C) *m/z* $[M-H]^-$ = 387 (exact mass [M] = 388.09) and dopaquinone-lysine conjugate (Fig 5.1D) *m/z* $[M-H]^-$ = 337 (exact mass [M] = 339.14). The reaction mechanism of tyrosinase oxidation entails firstly hydroxylation of a monophenol, in this case tyrosine, to form L -3, 4-dihydroxyphenylalanine (L-DOPA). L-DOPA is further oxidized to dopaquinone (Agarwal et al., 2016). Quinones, being highly reactive species, can undergo nonenzymatic reactions with tyrosines, cysteines, lysines or histidines in the same or a different molecule (Flander et al., 2011, Ercili-Cura et al., 2015). In this study, the quinones formed dimers as expected. However, only lysine was observed to react with a quinone. Formation of these conjugates leads to possible inter- and/or intramolecular cross-links, consequently modifying protein structure and functionality. Since amadumbe mucilage also contains tyrosine, tyrosinase may also modify the cell wall polysaccharide resulting in a gel. Tyrosinase was previously reported to form heteroconjugates between α-casein and xylan (Selinheimo et al., 2008).

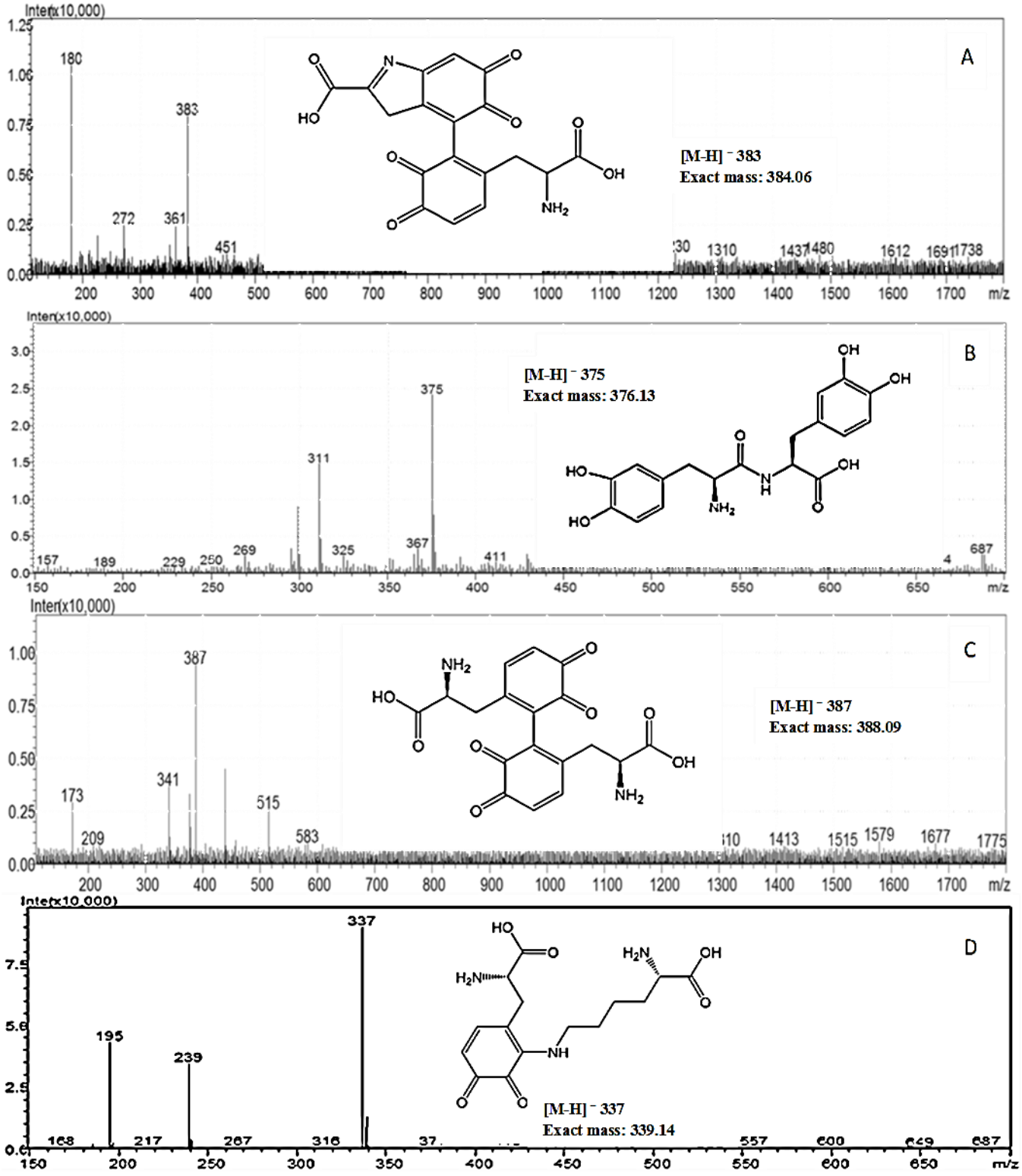


Figure 5.1. Mass spectra and proposed structures of products resulting from tyrosine oxidation by tyrosinase: (A) dopaquinone-dopachrome conjugate, (B) tyrosine-tyrosine conjugate, (C) di-dopaquinone and (D) dopaquinone-lysine conjugate.

5.3.2.2 Characterisation of products of thiol oxidation by tyrosinase using MS

Tyrosinase-mediated oxidation reactions also resulted in thiol oxidation. Disulphide bond formation between cysteine molecules resulted in the formation of cystine, *m/z* $[M-H]^-$ = 239 (exact mass [M] = 240.02) (Fig 5.2A). Furthermore, disulphide conjugates were formed between glutathione molecules, *m/z* $[M-H]^-$ = 611 (exact mass [M] = 612.15), as proposed in Fig 5.2B. It is plausible that tyrosine is a mediator in the tyrosinase-catalysed oxidation of sulphydryl groups in cysteine and glutathione. Tyrosinase oxidation in the dough may therefore also result in protein cross-linking by disulphide bond formation, resulting in higher molecular weight proteins stabilized by disulphide bonds. This may mimic the high molecular weight (HMW) subunit of glutenin which has been proposed to confer dough elasticity and cohesiveness to wheat gluten (Shewry et al., 2002). The formation of disulphide bonds could therefore possibly increase gluten-free dough strength and elasticity, enhancing the ability of the dough to expand and retain carbon dioxide released during proofing.

5.3.2.3 Characterisation of products of transglutaminase crosslinking by MS

Transglutaminase catalysis formed a ε-(γ-glutamyl) lysine-isopeptide bond between glutamine and lysine. The proposed structure of the resultant isopeptide [*m/z* $[M-H]^-$ = 273 (exact mass [M] = 275.15)] is shown in Fig 5.2C. This confirms the ability of transglutaminase to catalyse cross-linking of proteins containing these amino acids. This also leads to an increase in molecular weight of the proteins. The acyl donor capacity of asparagine or the acyl acceptor capability of arginine and histidine were not observed. This may be due to glutamine and lysine being the most preferred substrates for transglutaminase catalysis.

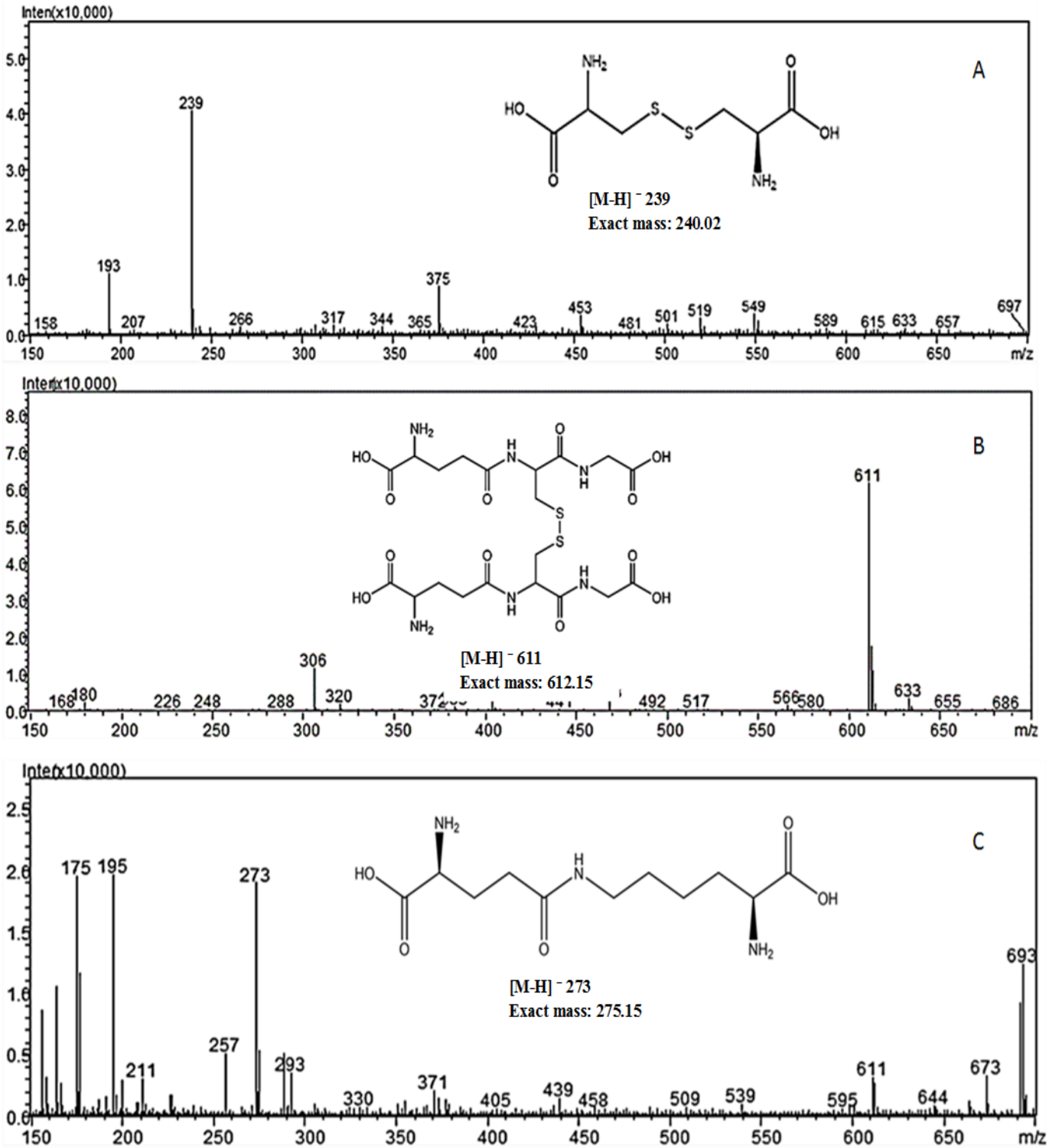


Figure 5.2A and 5.2B. Mass spectra and proposed structures of products resulting from disulphide bond formation by tyrosinase oxidation: (A) cystine and (B) oxidised glutathione. Fig 5.2C. The proposed structure of glutamyl-lysine isopeptide formed by transglutaminase-mediated crosslinking of glutamine with lysine.

5.3.3 Effect of tyrosinase on dough free thiols

The effect of tyrosinase on modified and freeze-dried amadumbe dough supplemented with 10% soy protein was also studied by quantifying the free thiol content. Tyrosinase action on amadumbe dough resulted in a 16.8 – 46.3% decrease in the dough thiol content (Fig 5.3). Increasing enzyme activity from 0 to 80 U/g resulted in a statistically significant ($p < 0.05$) decrease in dough thiol content. This confirmed the possible disulphide bond formation in the dough as proposed by the model reactions.

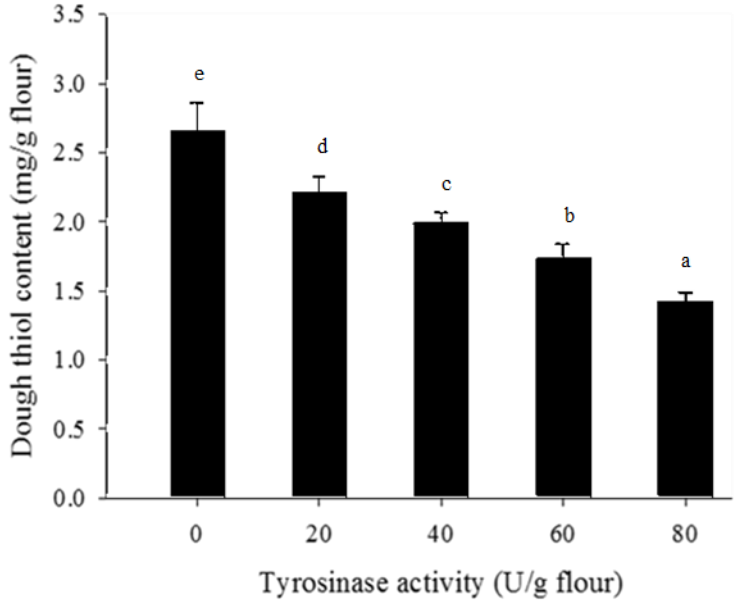


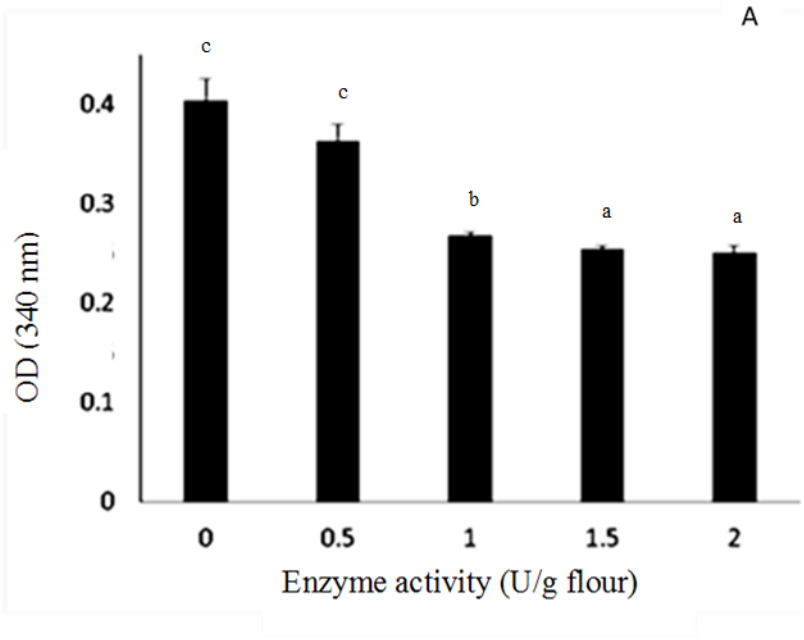
Fig. 5.3. Effect of tyrosinase on dough thiol content. Means ± SD, n=3;Bars with different letters differ significantly ($p<0.05$).

5.3.4 Effect of tyrosinase and transglutaminase on dough free amino groups

Both transglutaminase and tyrosinase treatments on amadumbe dough resulted in a decrease in free amines, confirming protein crosslinking (Fig 5.4). Transglutaminase crosslinking action in amadumbe dough resulted in a 10 – 38.1% decrease in the dough free amino content (Fig 5.4A). Increasing enzyme activity from 0 to 0.5 U/g resulted in a statistically insignificant ($p > 0.05$) decrease in dough free amino content. However, a further increase in enzyme activity from 0.5 to 1.5 U/g flour resulted in a statistically significant ($p < 0.05$) decrease in dough free amino content. Increasing enzyme activity beyond 1.5 U/g flour did not significantly decrease dough free amine

content. This may be due to lysine being the limiting amino acid and may have been exhausted in protein chains of close proximity to those with the peptide or protein-bound glutamine. Similar results have been observed when transglutaminase was used to modify rice, soybean and their composite flours (Marcoa and Rosell, 2008). Transglutaminase treatment also reduced wheat dough free amino groups, confirming crosslinking reactions (Bonet et al., 2005).

Tyrosinase also reduced the dough free amine content by 7.7 – 39.4% (Fig 5.4B). Increase in tyrosinase activity from 0 to 40 U/g flour did not result in a statistically significant ($p > 0.05$) decrease in dough free amino groups. However, a further increase in tyrosinase activity from 40 to 80 U/g flour led to a statistically significant ($p < 0.05$) reduction in dough amino groups. The decrease in dough free amine content confirms the possibility of the formation of tyrosine and lysine crosslinks, as proposed by the model reaction. Increasing tyrosinase in the dough was observed to continually reduce the free amine content unlike what was observed with transglutaminase. This may be due to the crosslinking of proteins through tyrosine moieties, resulting in protein aggregation and hence bringing glutamine and lysine residues of different protein chains into close proximity. Tyrosinase was similarly reported to form large protein aggregates of higher molecular weight in oat dough and the crosslinking efficiency was increased as tyrosinase activity was increased (Flander et al., 2011).



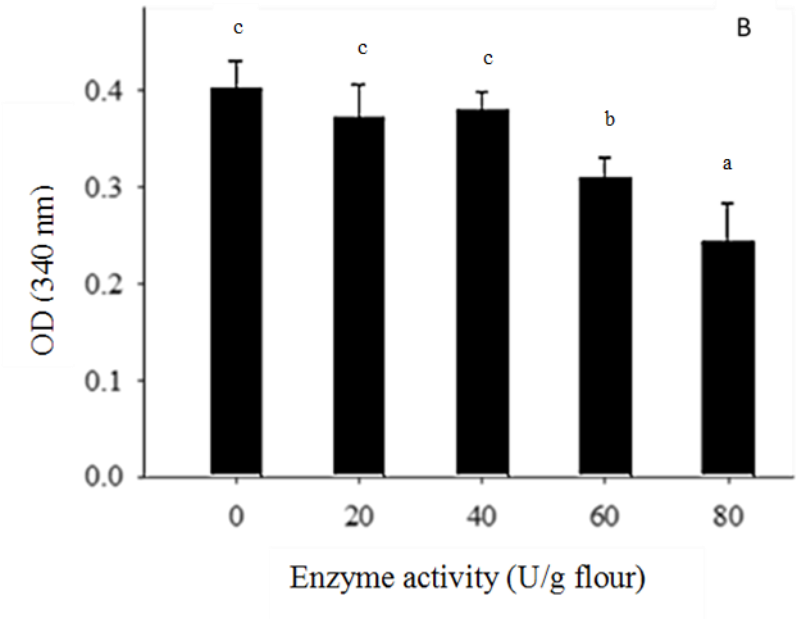


Figure 5.4A and 5.4B. Effect of enzymes on dough free amine content: (A) Effect of transglutaminase activity and (B) Effect of tyrosinase activity. Means ± SD, n=3; Bars with different letters differ significantly ($p<0.05$).

5.3.5 Effect of tyrosinase and transglutaminase on dough protein solubility.

The effect of enzyme activity on the turbidity of the supernatant obtained from dough was studied in order to determine if protein crosslinking had an effect on protein solubility. The optical density (OD) or turbidity of the solution was observed to decrease with increase in either transglutaminase or tyrosinase activity at pH 4 and pH 6 (Fig 5.5). This implies an increase in protein solubility with protein crosslinking by the enzymes. An increase in protein solubility has also been observed when transglutaminase was applied to rice dough which was supplemented with soybean protein (Marcoa and Rosell, 2008) and on soybean protein isolate (Babiker et al., 1998). A high optical density has been linked to lower protein solubility or a tendency of the proteins to form aggregates (Babiker et al., 1998).

Tyrosinase led to a better improvement in protein solubility compared to transglutaminase. This may be due to tyrosinase having a higher crosslinking efficiency due to a wider substrate range resulting from the reactive quinones formed during monophenol oxidation. Besides tyrosine conjugates, the model reactions also predicted disulphide bond formation. Transglutaminase and tyrosinase may also result in heteroconjugate formation between amadumbe mucilage and dough proteins. Conjugation of galactomannan to soy protein isolate by transglutaminase increased soy protein solubility even at pH 4, where it is normally sparingly soluble (Babiker et al., 1998). Conjugation of gum arabic to porcine myofibrillar protein also increased solubility (Davaatseren and Hong, 2014). However, crosslinking by both enzymes may also result in higher molecular weight polymers which sediment together with the flour during centrifugation and hence reducing the turbidity of the solutions. Soluble proteins are known to distribute more evenly in the liquid phase by linear aggregation which results in a stronger network during baking, unlike the random aggregations formed by insoluble proteins (Horstmann et al., 2017). Hence the increase in protein solubility may also increase dough strength.

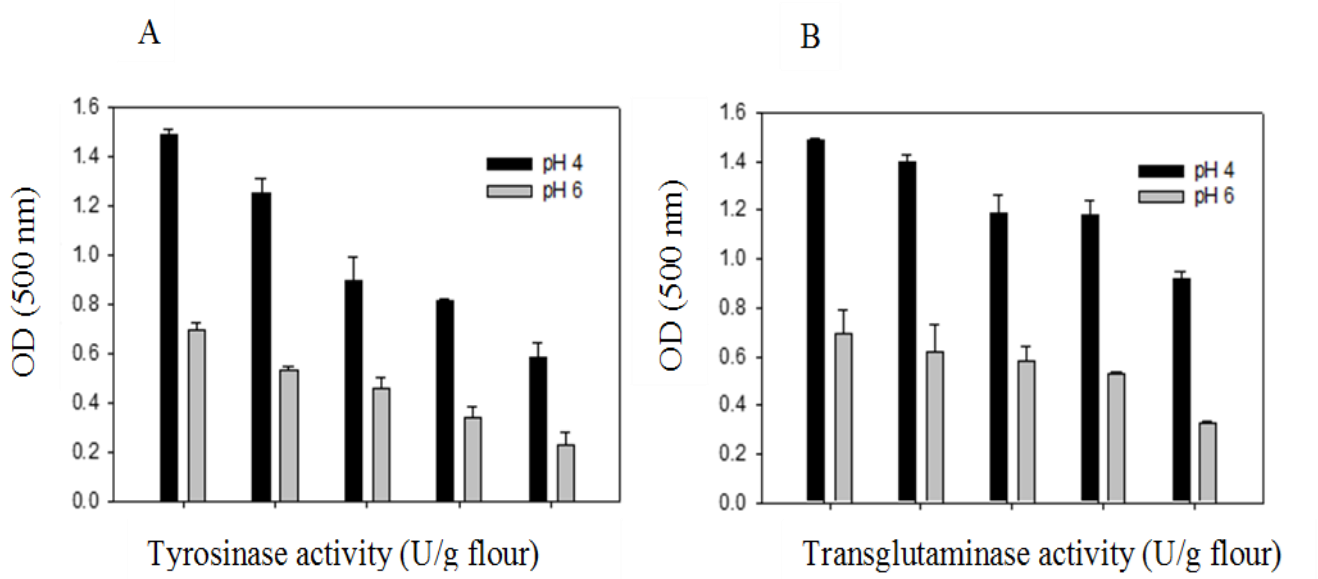


Figure 5.5. Effect of tyrosinase (A) and transglutaminase (B) on dough protein solubility. Means ± SD, n=3.

5.3.6 Effect of tyrosinase and transglutaminase on dough dynamic rheological properties.

The effect of transglutaminase and tyrosinase on the viscoelastic behaviour of amadumbe dough was analysed in terms of storage modulus (G′), loss modulus (G″), and tan δ value. At any frequency, G' was higher than G", indicating that the resulting dough had a solid, elastic-like structure (Fig 5.6). G' and G'' increased as transglutaminase and tyrosinase activities were increased. Increasing transglutaminase activity from 0 to 0.5 U/g flour resulted in a statistically insignificant ($p > 0.05$) increase in G' and G". However, further increasing the activity to 2 U/g flour resulted in a statistically significant ($p < 0.05$) increase in the dynamic moduli. Transglutaminase has been previously reported to increase G' and G" of rice dough (Gujral and Rosell, 2004, Marcoa and Rosell, 2008). The increase in G' and G" as a result of tyrosinase oxidation was statistically significant at all activities added.

Tan δ was less than 1 at all frequencies for the control and modified dough. This shows that the dough was more of elastic than viscous systems. Modification of amadumbe dough with transglutaminase and tyrosinase led to a decrease in tan δ although it was not statistically significant. Similarly, transglutaminase modification of rice dough resulted in a statistically insignificant decrease in tan δ (Marcoa and Rosell, 2008). The improvement in dough

viscoelasticity may be due to modification of proteins in the dough by disulphide bond formation, tyrosine conjugation and possible hetero-crosslinking between proteins and mucilage, which may strengthen the dough. This may therefore enhance the ability of amadumbe dough to retain carbon dioxide released during proofing.

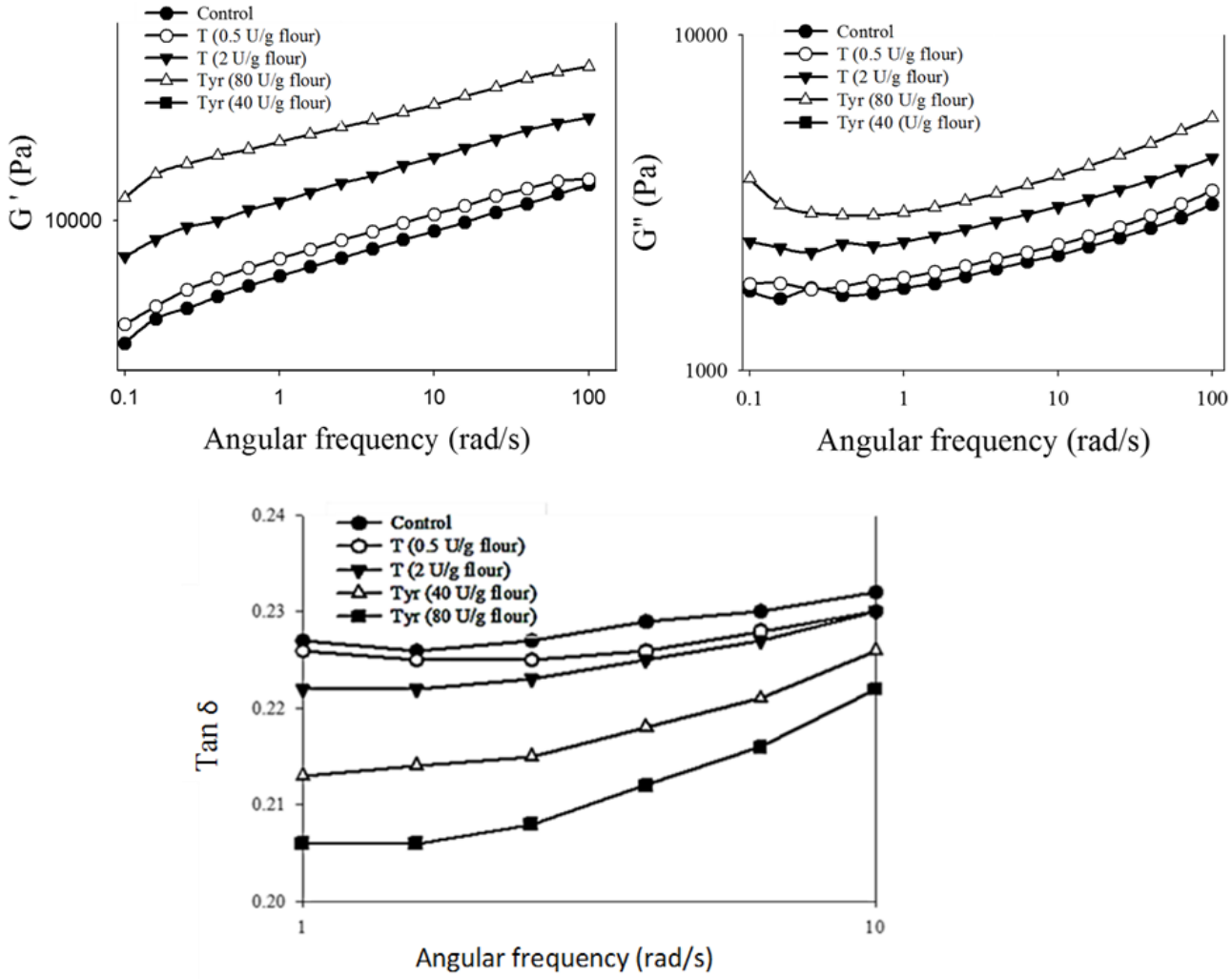


Figure 5.6. Effect of transglutaminase (T) and tyrosinase (Tyr) on dough viscous modulus (G"), storage modulus (G') and tan δ.

5.4 Conclusion

Tyrosinase mediated the crosslinking of tyrosine and thiols producing homo- and hetero-conjugates. Transglutaminase crosslinking resulted in the formation of glutamyl-lysine isopeptide.

Tyrosinase oxidation of amadumbe dough resulted in a decrease in dough thiol content and dough free amino groups, confirming the crosslinking predicted by model reactions. Transglutaminase crosslinking also led to a reduction in dough amino groups. Application of both enzymes in amadumbe dough increased protein solubility and dough viscoelasticity. Transglutaminase and tyrosinase crosslinking may therefore be used to improve gluten-free bread characteristics.

CHAPTER SIX

6.0 Effect of multiple crosslinking enzyme systems and hydrocolloids on gluten-free amadumbe dough rheology and bread quality

Abstract

Gluten-free dough improvement remains a technological challenge. A combination of multiple enzymes and hydrocolloid may result in dough with improved viscoelasticity and more acceptable bread. In this study, G', G" and Tan δ of amadumbe dough were optimised using a central composite design (CCD) and response surface methodology (RSM). The combination of enzymes resulted in amadumbe dough that had a higher G' and G", higher specific volume and lower crumb hardness compared to dough without enzymes or with a single enzyme. Addition of cactus and amadumbe mucilage to the dough modified with an optimum enzyme combination resulted in improved bread specific volume. The combination of enzymes and cactus mucilage improved bread texture, appearance and overall acceptability but did not significantly affect bread aroma and taste. The combined effect of multiple enzymes and a cactus mucilage resulted in more acceptable gluten-free amadumbe bread.

6.1 Introduction

The increase in the number of celiac patients globally has triggered an interest in exploring gluten-free products. Celiac disease (CD) is a digestive disorder which damages the villi in the small intestine compromising their ability to absorb nutrients due to an immunological reaction to dietary gluten (Bao, Green, & Bhagat, 2012). The only remedy available at present is a total exclusion of gluten from the diet. However, Gluten is responsible for the viscoelastic behaviour which is important in retaining gas released during baking, and obtaining the desired texture and volume in bread (Hüttner & Arendt, 2010).

Gluten is a protein occurring in wheat and other related cereals. Glutenin and prolamin are the major fractions of the gluten. Prolamins are responsible for viscosity and extensibility in a dough system, while the glutenin fraction is responsible for elastic and cohesive properties of dough

(Gujral & Rosell, 2004). When gluten is excluded, the bread has poorly developed structure, crumbling texture, colour and quality defects (Gallagher, Gormley, & Arendt, 2004).

In order to imitate gluten functionality in gluten-free bread, the use of hydrocolloids such as carboxymethyl cellulose, alginate and hydroxypropyl methylcellulose (HPMC) (Demirkesen, Mert, Sumnu, & Sahin, 2010), legume proteins (Marcoa & Rosell, 2008) and starch sources such as rice (Yano, 2010), have been proposed for improved gluten-free bread. However, these additives do not form a network similar to that formed by gluten therefore other options need to be explored. We have recently investigated the use of crosslinking enzymes such as laccase in gluten-free amadumbe dough. Laccase significantly improved the viscoelasticity of amadumbe bread due to various crosslinking reactions that were demonstrated by model reactions (Chapter 4). However, the crosslinking did not result in a network that has functionality similar to gluten.

Since the gluten network is proposed to be stabilized by intra-chain and inter-chain disulphide bonds (Shewry, Halford, Belton, & Tatham, 2002), non-covalent bonds such as hydrogen bonds, ionic bonds and hydrophobic interactions (Wieser, 2007), the combined use of hydrocolloids and multiple enzymes may improve the characteristics of gluten free bread. The use of a hydrocolloid and a single enzyme was shown to improve gluten free bread properties (Onyango, Mutungi, Unbehend, & Lindhauer, 2010). A combination of laccase and xylanase resulted in a synergistic effect with xylanase counteracting dough hardness from laccase on wheat dough (Flander et al., 2008; Selinheimo, Kruus, Buchert, Hopia, & Autio, 2006). It would be interesting to investigate the effects of an enzyme combination and hydrocolloid in gluten free bread.

Therefore, the aim of this work was to investigate for the first time, the effect of a combination of crosslinking enzymes transglutaminase, tyrosinase and laccase on gluten-free amadumbe dough, as well as to optimise the enzyme combination using response surface methodology (RSM). The effect of different hydrocolloids on the quality of bread prepared using dough modified with the optimum enzyme combination, was also studied.

6.2 Materials and Methods

6.2.1 Source of materials

T. versicolor laccase (LAC) was purchased from Sigma Aldrich (South Africa) and the manufacturer specification of its activity was 0.5 U/g. Mushroom tyrosinase (TYR) was purchased from Sigma Aldrich (South Africa) and the manufacturer specification of its activity was 1000 U/mg. Microbial transglutaminase (TG) (Activa WM, 100 U/g) was kindly donated by Maccallum & Associates (Durban, South Africa; representing Ajinomoto). Soy protein isolate was purchased from Lionheart Chemicals**,** Durban, South Africa (SA). The moisture, protein and ash content of soy protein was 6.5%, 90.2% and 1.8%, respectively. Amadumbe corms were purchased from Jozini, KwaZulu Natal. Amadumbe flour was prepared as previously described (Chapter 3). The moisture, protein and ash content of amadumbe flour was 4.9%, 3.2% and 3.1%, respectively, determined according to the methods of the Association of Official Analytical Chemists (A.O.A.C, 2000) Instant dry yeast, sugar, and salt were purchased from a local supermarket. All ingredients used in this study were of food grade.

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6.2.2 Experimental design for optimisation and statistical analysis

The effects of enzyme addition on dough properties were determined by Response Surface Methodology (RSM) using a central composite design (CCD). The independent variables were TG (0-2 U/g flour), TYR (0-80 U/g flour) and LAC (0-3 U/g flour). The levels of these enzymes were determined from our previous experiments. The dependent variables were G', G" and Tan δ. The outline of the experimental design (17 runs) with the coded levels as summarised in Table 6.1. These were generated by the statistical software Design Expert version 10 (StatEase Inc., U.S.A.). Each design point was performed in duplicates with the centre in three replicates. The experiment was carried out in a randomized order. The system behaviour was described by a quadratic polynomial model regression given by:

$$Y = \beta_0 + \Sigma^3_{k=1} \beta_i X_i + \Sigma^3_{k=1} \beta_{ii} X_i X_i + \varepsilon \quad \text{.....Equation (1)}$$

where Y is the response variable, β_0 is a constant; β_i and β_{ii} are the linear and interactive coefficients, respectively; X_i is the level of the ingredient and ε is the random error. The optimisation objective was to maximise G'and G" whilst minimising Tan δ of amadumbe dough.

Design Expert 10 (StatEase Inc., U.S.A) was used to estimate desirability, an objective function that ranges from zero outside of the limits to one at the goal. The numerical optimisation found a point that maximizes the desirability function. Analyses of variance (ANOVA) was used to check the significant differences in responses ($p < 0.05$) and to examine the statistical significance of the regression equation used.

Table 6.1: Process variables used in the central composite design for amadumbe dough preparation

Factors (U/g flour)	Coded (X_i)				
	$-\alpha$	-1	0	+1	α
LAC (X_1)	0	0.68	1.68	2.67	3.35
TYR (X_2)	0	20	60	100	127.3
TG (X_3)	0	0.16	0.5	1	1.84

LAC: laccase, TYR: tyrosinase; TG: transglutaminase

6.2.3 Dough preparation

The dough was prepared manually by adding 10% (w/w) soy protein isolate to amadumbe flour, a level aimed at achieving a total protein content of about 12% similar to wheat, then adding 100% (v/w) water to make a batter consistency. Enzymes at different activities were added according to the experimental design (Table 1) and the batter was incubated for 35 min at ± 25°C before being analysed.

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6.2.4 Rheological properties of dough

Dynamic rheological measurements of the dough were determined using an Anton Paar MCR501 Rheometer with RHEOPLUS/32 V3.41 software (TA Instruments, New Castle, Delaware), as previously described (Demirkesen et al., 2010). The measuring system consisted of parallel plate geometry (40 mm diameter, 1 mm gap). The dough, modified with enzymes according to the experimental design, was placed between the plates within 1 h of mixing, and the test was started after the dough had rested for 5 min. The rim of the sample was coated with a thin layer of paraffin

oil to prevent evaporation during measurement. Measurements were performed at 30°C. The linear viscoelastic zone was determined by stress sweeps at a constant frequency of 1 Hz. Frequency sweep tests were performed from 0.1 to 100 rad/s to determine the storage modulus (G'), loss modulus (G") and Tan δ. Values of dynamic moduli obtained at a frequency of 10 rad/s were used to detect significant differences among enzyme treatments (Caballero, Gómeza, & Rosell, 2007).

6.2.5 Verification of optimal amadumbe dough rheology

The storage modulus (G'), loss modulus (G") and Tan δ of the optimum amadumbe dough were compared to the prediction interval at 95% confidence level.

6.2.6 Effect of enzymes and selected hydrocolloids on the rheology of optimum amadumbe dough

The effect of addition of transglutaminase (2 U/g flour), laccase (2 U/g flour), tyrosinase (80 U/g flour), xanthan gum, amadumbe mucilage or cactus mucilage (0, 1 or 2% (w/w) together with the optimum enzyme combination (previously determined), on the dynamic rheological properties of amadumbe dough was determined as described above (section 6.2.4). Frequency sweep tests were performed from 0.1 to 100 rad/s to determine the storage modulus (G'), loss modulus (G") and Tan δ.

6.2.7 Bread making

Bread was made using the optimum dough formulation, with a hydrocolloid added at the different levels stated above. The basic recipe consisted of: amadumbe flour (100 g), soy protein isolate (10 g), salt (2 g), sugar (8.5 g), fat (2.5 g), water (90 g), yeast (2.25 g). The enzyme combination for optimum dough rheology (LAC, 1.78 U/g flour; TYR, 79 U/g flour and TG, 1.97 U/g flour), and the hydrocolloid were then added to the batter. The batter was subjected to a first proof of 30 min at 35°C without yeast, mixed again after yeast addition and then put in greased pans (measuring 120 x 50 mm). The batter was proofed again for 45 min at ± 25°C and then baked at 200°C for 30 min. After baking, the loaf was removed from the pan, cooled on the rack for 2 h at room temperature and analysed.

6.2.8 Bread analysis

The bread with single enzymes, a combination of enzymes or a combination of enzymes with added hydrocolloids was analysed.

6.2.8.1 Bake loss and specific volume

Loaves were weighed and loaf volume was measured by rapeseed displacement method (Ngemakwe et al., 2016). A 1000 ml cylinder was filled with 500 ml rapeseed seeds. The 500 ml rapeseed seeds from the measuring cylinder were poured over the bread loaf in another cylinder and then the difference in height from the original volume (500 ml) was expressed as the volume of the loaf. The specific loaf volume of the bread was calculated as the loaf volume per weight of the loaf (ml/g).

6.2.8.2 Crumb moisture loss

To determine crumb moisture loss, a 10 g portion of the bread was removed from the centre of the bread. The weight of the breadcrumb portions was recorded 4 times at 24 h intervals and the differences noted.

6.2.8.3 Sensory evaluation

Freshly baked bread prepared from dough modified with the optimum combination of enzymes and hydrocolloid, and amadumbe bread with no enzymes or hydrocolloid, were submitted for an

acceptance test using a 9 point hedonic scale and 45 consumers (untrained) panellists. The panellists were asked to score the samples based on overall acceptability, taste, texture, aroma and appearance on a scale from 0 to 9 (0 dislike extremely, 9 like extremely).

6.2.9 Statistical analysis

Except where stated, all experiments were carried out in triplicate. Data were analysed using analysis of variance (ANOVA) and means were compared using Fischer's Least Significant Differences Test ($p < 0.05$ and $p < 0.01$).

6.3 Results and Discussion

6.3.1 Effects of laccase, transglutaminase and tyrosinase on amadumbe dough dynamic rheological properties: Model analysis

The effects of the enzymes on the dough rheological properties are were analysed. The quadratic model for G', G" and the linear model regression coefficients for the effect on Tan δ are presented in Table 6.2. The significance of the model parameters was tested using ANOVA. Many model terms were significant ($p < 0.05$) for all the responses evaluated. The model p-value ranged from <0.0006 to 0.02 indicating that all the models were significant ($p < 0.05$). The lack of fit for all models was not significant, showing that the models were adequate to navigate the design space. Analysis of second-order interactive effects of design factors on G" revealed significant ($p < 0.05$) interactions between LAC and TYR as well as LAC and TG (Table 6.2). Significant ($p < 0.05$) second-order interactive effects of LAC and TYR on G' were also observed. In Chapter 5, we also observed that TG and TYR crosslinking increased the G' and G" of amadumbe dough but did not significantly affect Tan δ. This is also observed as only LAC had a significant ($p < 0.05$) effect on Tan δ.

Table 6.2: Regression coefficients of a quadratic model for dynamic rheological properties of amadumbe dough

Coefficients	Y_1	Y_2	Y_3
Intercept	2927.58	13960.52	0.22
A-LAC	16.86	550.05	**0.013**
B-TYR	281.46	1169.85	1.611E-003
C-TG	159.91	214.61	0.011
AB	**-105.75**	**-507.27**	
AC	**-2078.07**	-7798.14	
BC	69.06	316.71	
A^2	**-465.18**	**-2057.59**	
B^2	71.53	-163.52	
C^2	-384.09	-2000.40	
R^2	0.89	0.88	0.50

A: laccase, B: tyrosinase, C: transglutaminase. Responses Y1: G”; Y2: G’; Y3: Tan δ. Values in bold font are significant ($p < 0.05$).

The effect of TYR and LAC on G” and G’ are shown on the 3-dimensional surface plots in Fig 6.1. LAC, TYR and TG addition to amadumbe dough resulted in an increase in G’ and G’. This is as previously observed in Chapter 4, that LAC crosslinking significantly increased G’ and G” whilst Tan δ significantly decreased. In Chapter 5, TG and TYR crosslinking also increased the G’ and G” of amadumbe dough. This shows that structural modification occurred in the dough.

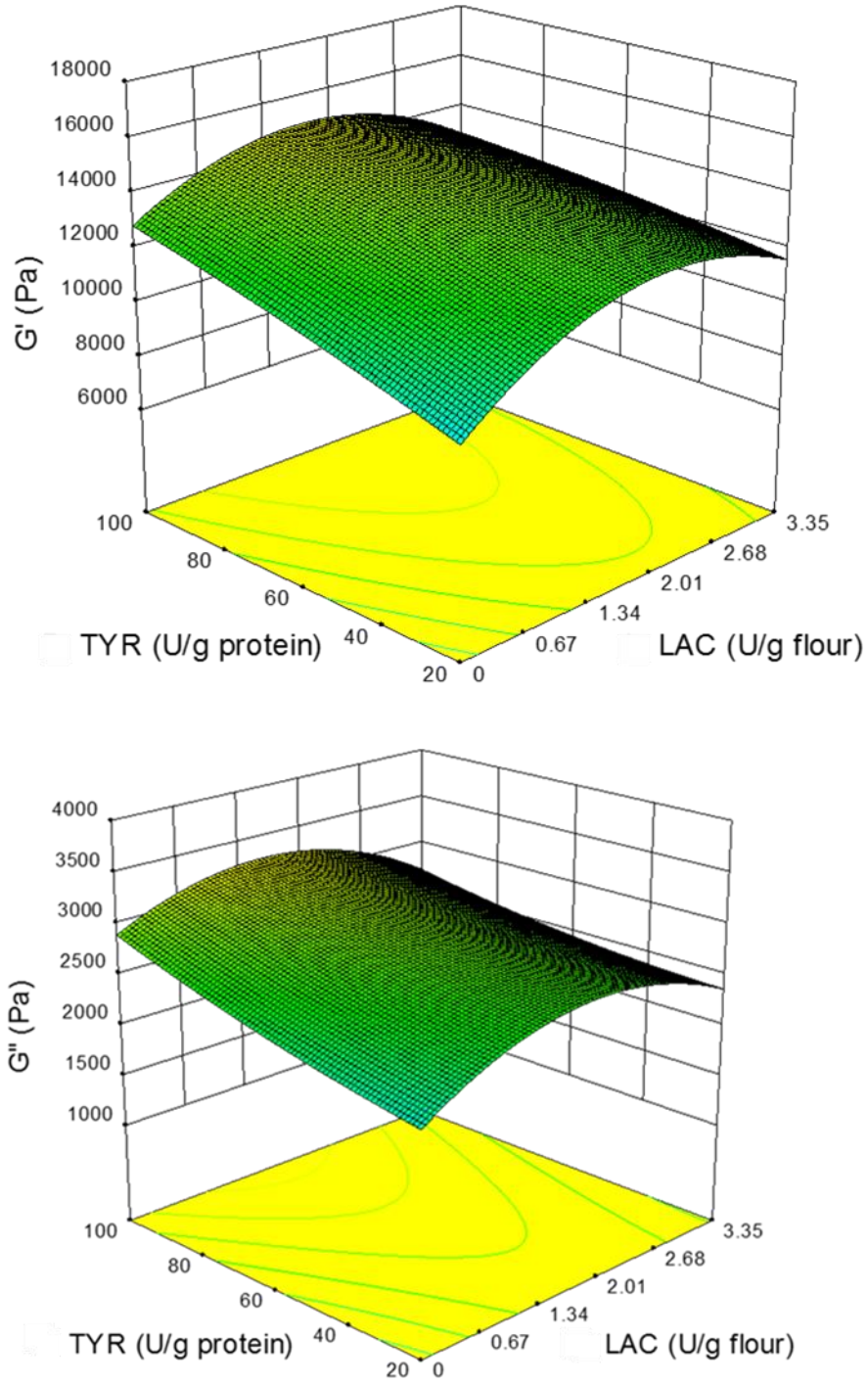

Figure 6.1: Effect of tyrosinase (TYR) and laccase (LAC) on the viscosity modulus (G") and elasticity modulus (G') of amadumbe dough.

6.3.2 Optimisation and verification

The optimisation goal was to maximize G" and G', while minimising Tan δ. The predicted optimal enzyme combinations were: LAC (1.78 U/g flour), TYR (79 U/g flour) and TG (1.97 U/g flour) with a desirability of 0.87. The model predicts that this formulation would result in a maximum G' of 25152.5, G" of 5653.9 and minimum Tan δ of 0.25. The verification shows that the dough had a G' of 21500 Pa, G" of 4940 Pa and Tan δ of 0.23. This is close to the predicted values and therefore confirms that the model adequately predicted G', G" and Tan δ.

6.3.3 Effect of enzymes and hydrocolloids on amadumbe dough rheology

All batters showed that the elastic modulus (G') was higher than the viscous modulus (G"), indicating that the batters had a solid, elastic-like behaviour (Fig 6.2). A similar behaviour has been observed with oat dough (Renzetti et al., 2010). Control dough had the lowest viscous and elastic modulus. The use of either LAC, TYR or TG increased G' and G" of amadumbe dough as previously observed (Chapter 4 and 5). Addition of the optimum enzyme combination significantly increased G' and G" compared to the use of single enzymes (Fig 6.2). This may be due to the various crosslinking reactions occurring simultaneously, which result in the formation of protein aggregates and possibly protein-polysaccharide conjugates of higher molecular weight and viscosity. The possibility of formation of phenolic-thiol conjugates, thiol-thiol conjugates and phenolic-phenolic conjugates by LAC catalysis (Chapter 4), thiol conjugates and tyrosine-tyrosine conjugates by TYR (Chapter 5) and glutamyl-lysine conjugates by TG (Chapter 5) was demonstrated using model reactions. LAC and TYR have also been reported to form protein-protein and protein-polysaccharide conjugates (Selinheimo et al., 2008). TG has been reported to form higher molecular weight proteins but the presence of glycoproteins (mucilage) in amadumbe dough (Chapter 2) may also result in protein-polysaccharide crosslinking through the protein moiety. Addition of a hydrocolloid also increased G' and G" of the optimum amadumbe dough. Similar effects have previously been reported when different hydrocolloids were added to gluten-free batter based on rice flour, corn starch and sodium caseinate (Lazaridou et al., 2007b). Xanthan gum addition resulted in the highest dough G' and G", followed by cactus mucilage and finally amadumbe mucilage. This may be due to the different viscosities of different gums at similar concentrations, with xanthan gum having the highest viscosity. Addition of xanthan gum above 1% and cactus mucilage above 2% resulted in dough hardening.

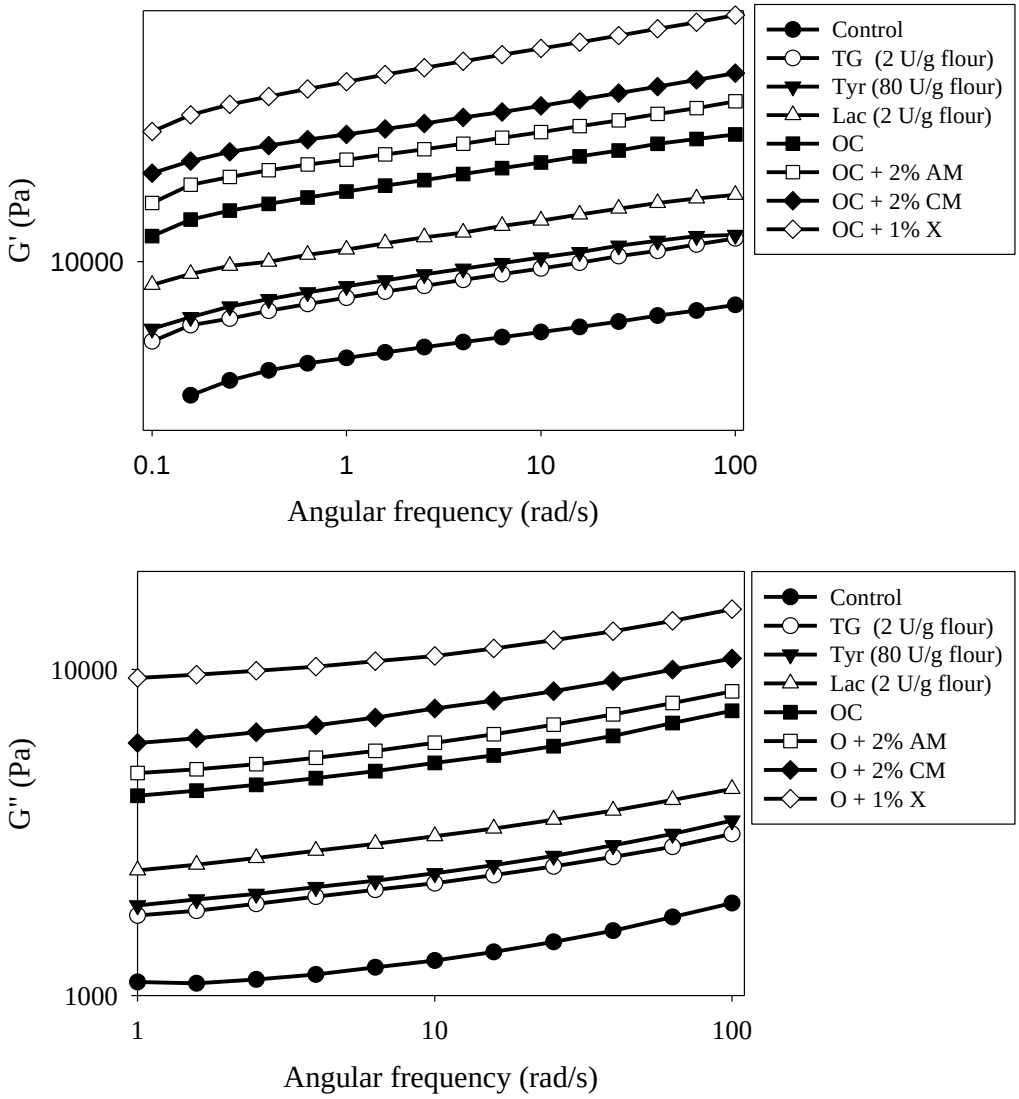

Figure 6.2: Effect of enzymes and hydrocolloids on amadumbe dough dynamic rheological properties G' and G". Control: bread with no enzymes, Lac: laccase, Tyr: tyrosinase; TG: transglutaminase, OC: bread from dough modified with optimum enzyme combination, AM: amadumbe mucilage, CM: cactus mucilage and X: xanthan gum.

6.3.4 Effect of enzymes and hydrocolloid on the specific volume of amadumbe bread

The use of TG, TYR or LAC significantly ($p < 0.05$) increased amadumbe bread specific volume (Fig 6.3). Addition of a combination of enzymes also significantly ($p < 0.05$) increased amadumbe bread specific volume compared to the use of one enzyme only (Fig 6.3). This may be due to the effect of various enzymatic crosslinking reactions, as demonstrated in Chapter 4 and 5. Addition

of amadumbe mucilage at 1% or 2% to the dough formulation containing a combination of enzymes did not have a significant ($p > 0.05$) effect on the specific volume of the bread. This may be ascribed to the low viscosity of amadumbe mucilage which only becomes a thickener at high concentrations above 5% (Chapter 2). Addition of cactus mucilage (1%) to the dough formulation with an optimum enzyme combination significantly ($p < 0.05$) increased the bread specific volume. Increasing cactus mucilage to a concentration of 2% further improved the bread specific volume. This may be due to the high viscosity of cactus mucilage, a pectic polysaccharide (Chapter 2). Addition of cactus mucilage increases the viscosity of amadumbe dough, which in turn increases the ability of the dough to retain gas released during proofing. However, increasing the percentage to 3% lowered the loaf specific volume. Similarly, pectin (1% and 2% w/w) improved the specific volume of gluten-free bread based on rice flour, corn starch and sodium caseinate (Lazaridou et al., 2007b). Furthermore, mucilage contains substrates for the enzymes and may have been modified resulting in an improved network which retains carbon dioxide more efficiently. The effect of the enzymes and cactus mucilage on the bread crumb structure and height is shown in Fig 6.4. Addition of 1% xanthan gum to the dough together with an optimum enzyme combination significantly ($p <0.05$) increased bread specific volume. However, further increasing the concentration of xanthan gum to 2% decreased the loaf specific volume. This may be due to xanthan gum making the dough too rigid, thus reducing its ability to rise during proofing. A similar effect of xanthan gum has been observed on gluten-free bread based on rice flour, corn starch, and sodium caseinate (Lazaridou et al., 2007b) and on bread with formulations based on corn starch and rice flour (Sabanis and Tzia, 2011).

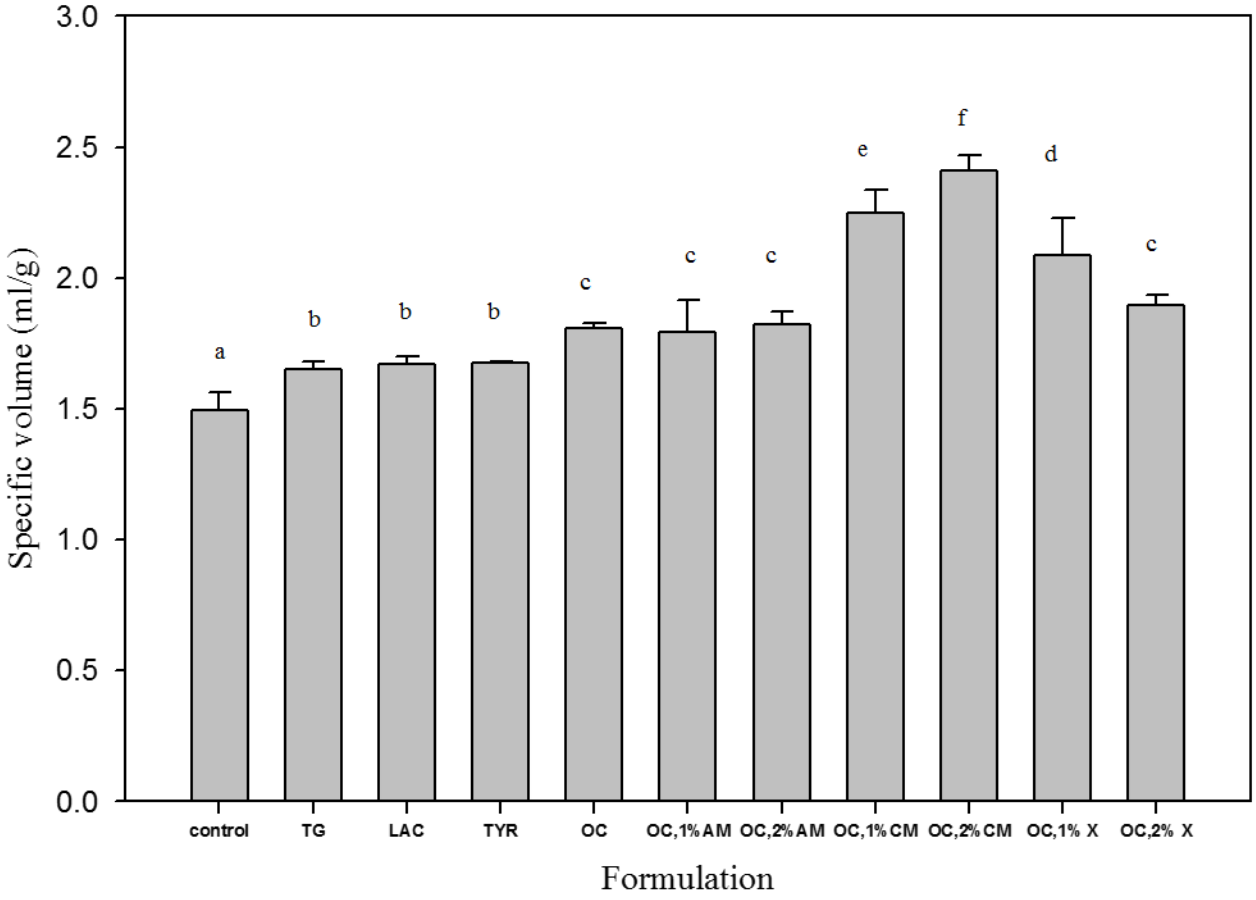

Figure 6.3: Effect of enzymes and hydrocolloids on the specific volume of amadumbe bread. Control: bread with no enzymes, LAC: laccase (2 U/g flour), TYR: tyrosinase (80 U/g flour; TG transglutaminase (2 U/g flour), OC: bread from dough modified with optimum enzyme combination, AM: amadumbe mucilage, CM: cactus mucilage, and X: xanthan gum. Means ± SD, n=3; Bars with different letters differ significantly ($p<0.05$).

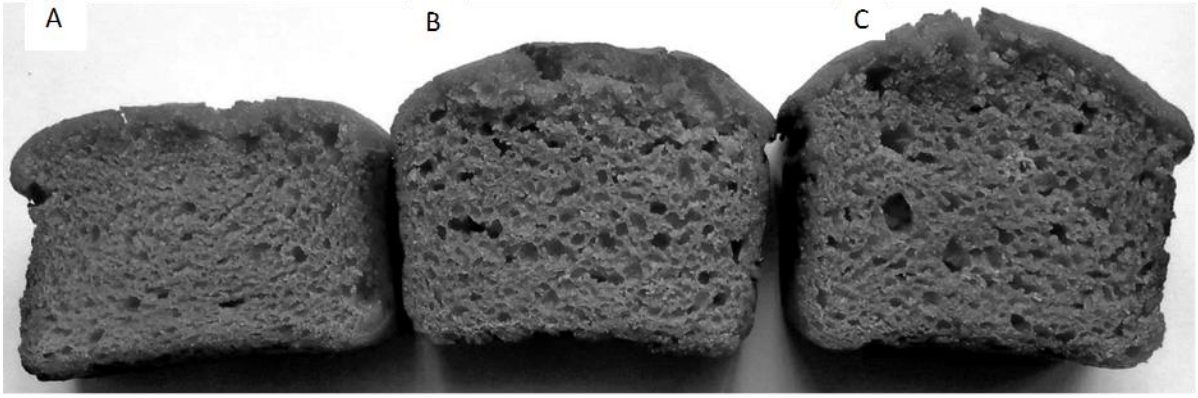

Figure 6.4: Effect of enzymes and cactus mucilage on amadumbe bread. A: control, B: bread from dough modified with optimum enzyme combination, C: bread from dough modified with optimum enzyme combination + 2% cactus mucilage.

6.3.5 Effect of hydrocolloids on the bake loss of amadumbe bread

The use of a combination of enzymes in amadumbe dough resulted in a significant decrease in bake loss (Fig 6.5). Bake loss occurs due to loss of moisture during baking. The enzymes may have crosslinked dough proteins and polysaccharides resulting in molecules with a higher water holding capacity. Oxidative gelation of feruloylated arabinoxylan by laccase has been reported to for a hydrophilic polysaccharide (Figueroa-Espinoza, Morel, & Rouau, 1998). Furthermore, transglutaminase crosslinking may improve protein water holding capacity (Ahn, Kim, & Ng, 2005). Tyrosinase has also been reported to increase myofibrillar protein water holding capacity (Lantto, Puolanne, Kruus, Buchert, & Autio, 2007). Combined use of enzymes together with amadumbe mucilage at 1% or 2% did not significantly ($p > 0.05$) decrease the bake loss. This may be due to the low water holding capacity of the mucilage. Cactus mucilage and xanthan gum at 1% did not significantly reduce the bake loss. However, at 2% concentration of xanthan gum or cactus mucilage, bake loss was significantly reduced. It was observed that xanthan gum at 2% resulted in the least bake loss. This may be attributed to its high affinity for water.

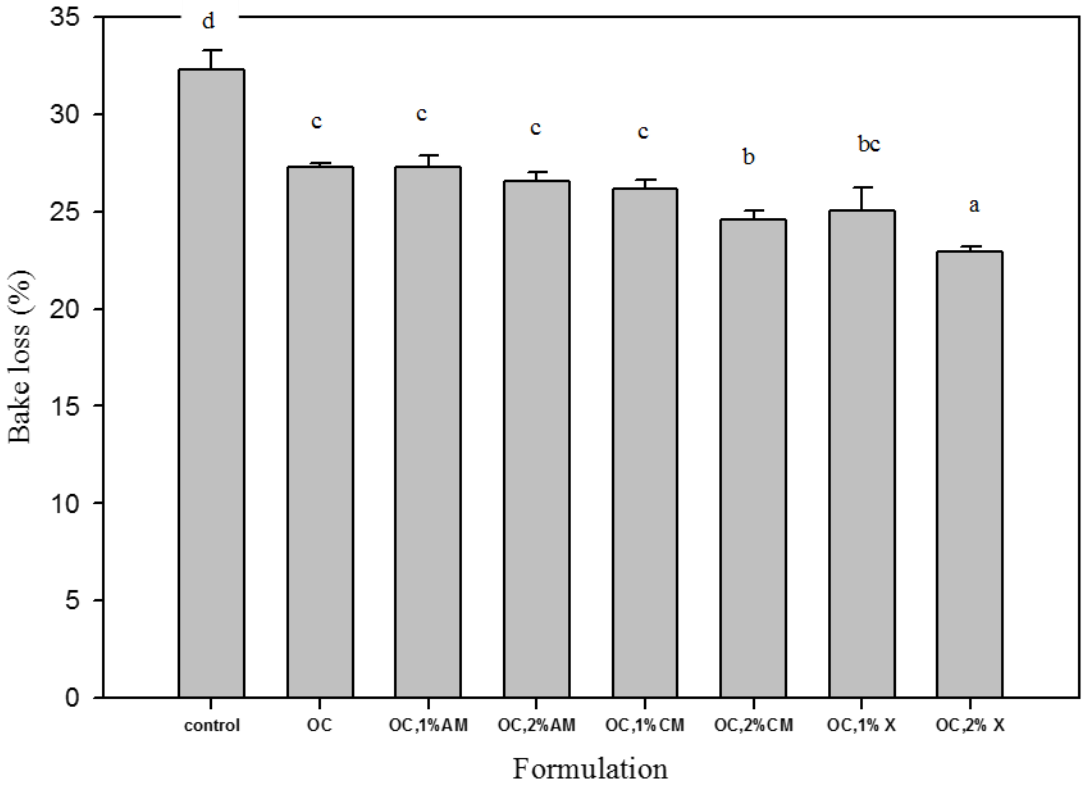

Figure 6.5: Effect of enzymes and hydrocolloids on the bake loss of amadumbe bread**.** Control: bread with no enzymes, OC: bread from dough modified with optimum enzyme combination, AM: amadumbe mucilage, CM: cactus mucilage and X: xanthan gum. Means ± SD, n=3; Bars with different letters differ significantly ($p<0.05$).

6.3.6 Effect hydrocolloids on bread moisture loss during storage

Moisture loss during storage is an indication of bread staling. Moisture was lost from the bread throughout storage and moisture loss increased with time. The use of a combination of enzymes in amadumbe dough increased the bread stability during storage by reducing moisture loss (Fig 6. 6). This may be due to increase in water holding capacity the dough. Amadumbe mucilage was only effective in reducing moisture loss at 2%. However, cactus mucilage and xanthan gum significantly ($p < 0.05$) decreased the moisture loss at all concentrations. Xanthan gum application resulted in the least moisture loss. This may be due to its high water holding capacity, increasing the dough water holding capacity (Collar, Andreu, Martınez, & Armero, 1999) and delaying amylopectin recrystalisation (Bárcenas & Rosell, 2007).

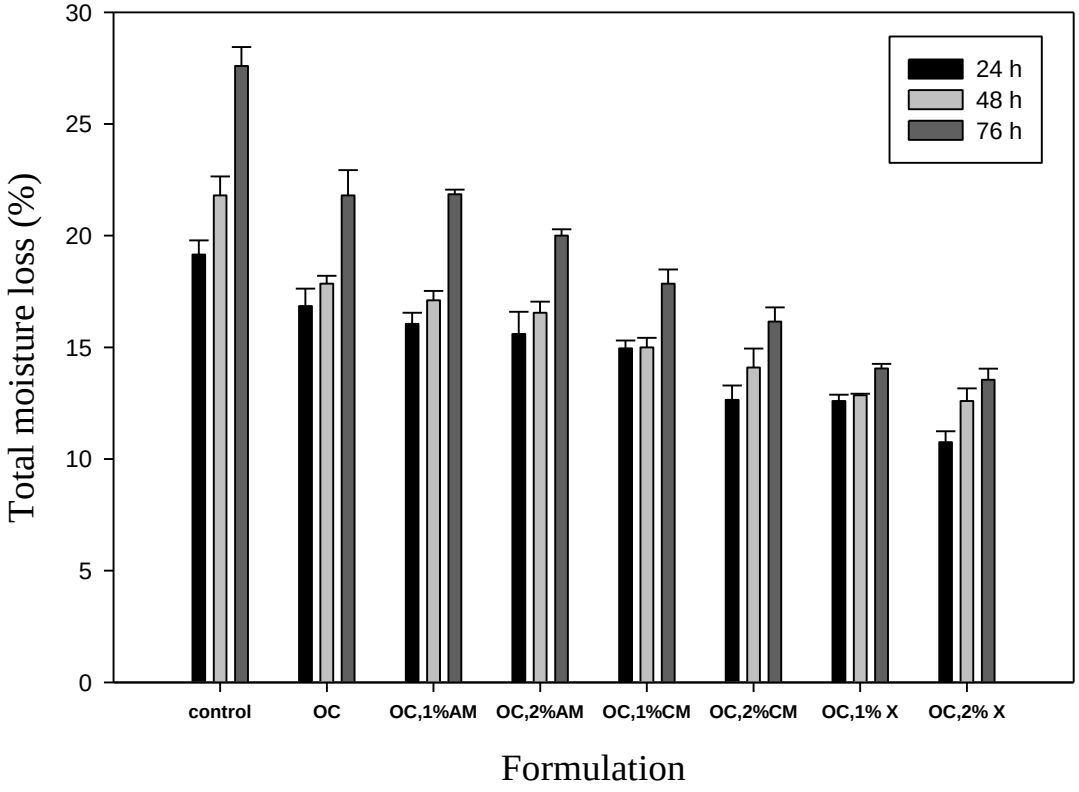

Figure 6.6: Effect of enzymes and hydrocolloids on the total moisture loss of amadumbe bread. Control: bread with no enzymes, OC: bread from dough modified with optimum enzyme combination, AM: amadumbe mucilage, CM: cactus mucilage and X: xanthan gum. Means ± SD, n=3.

6.3.7 Effect of enzymes and hydrocolloids on the sensory properties of amadumbe bread

Sensory evaluation was carried out on the best formulation with enzymes and cactus mucilage. Bread with no enzymes was used as a control and bread with a combination of enzymes but without a hydrocolloid was used for comparison purposes. Amadumbe bread was generally rated higher for its aroma, followed by taste (Fig 6.7). The use of a combination of enzymes significantly ($p < 0.05$) improved the texture, appearance and overall acceptability of amadumbe bread. This is due to the improvement in the bread specific volume. A higher specific volume has been linked to better bread texture and less hardness (Gambuś, Sikora, & Ziobro, 2007; Hardeep Singh Gujral, Guardiola, Carbonell, & Rosell, 2003). A combination of cactus mucilage and enzymes slightly

improved amadumbe bead texture, appearance and overall acceptability. There was no significant ($p > 0.05$) effect of treatment on amadumbe bread taste and aroma.

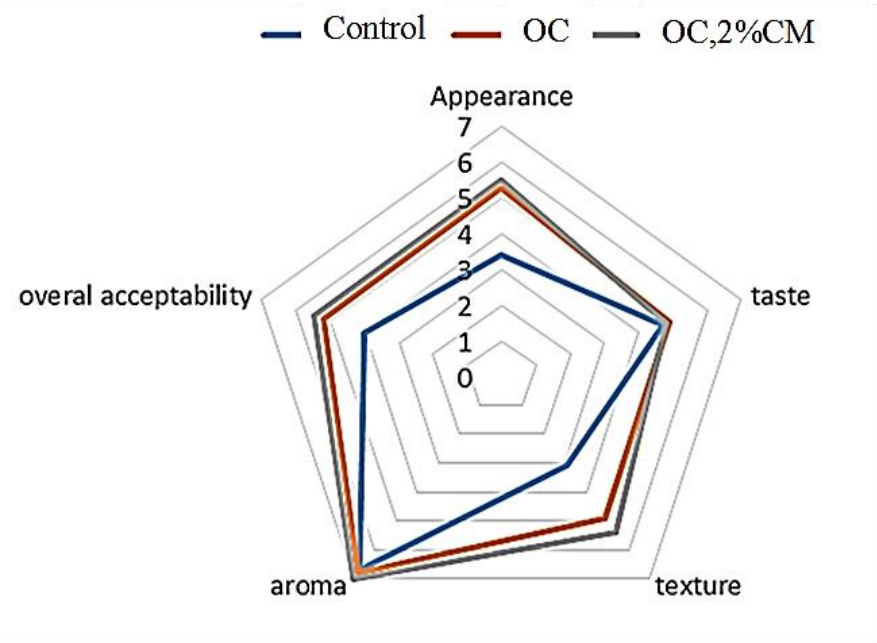

Figure 6.7: Effect of hydrocolloids and enzymes on the sensory properties of amadumbe bread. Control: bread with no enzymes, OC: bread from dough modified with optimum enzyme combination, CM: cactus mucilage

6.4 Conclusion

An optimised combination of enzymes resulted in improved dough rheological, physical and sensory properties. Laccase had an additive effect on dough G", with either transglutaminase or tyrosinase. Cactus mucilage addition at 2% w/w to the formulation with optimum enzyme activities resulted in an increased G', G" and best bread which had a high specific volume, low moisture loss and better sensory acceptability.

CHAPTER SEVEN

7.0 General Discussion

In this thesis, laccase, tyrosinase and transglutaminase were used (separately and combined) to modify amadumbe dough supplemented with soy protein and mucilage. The first part of this section discusses the composition and rheology of mucilages extracted from amadumbe and cactus plants (Chapters 3). The second part focuses on the influence of laccase, transglutaminase and tyrosinase on amadumbe dough rheological properties (Chapters 4 and 5). The third part discusses the effect of the application of a combination of the above-mentioned enzymes (optimised using response surface methodology, RSM) and mucilage on amadumbe dough and bread quality (Chapter 6).

The composition and rheology of amadumbe and cactus mucilages were determined as potential modifiers of amadumbe dough. Both mucilages were highly heterogeneous (Chapter 3). The mucilages had polysaccharide fractions which were associated with a protein. It was observed that the amino acid profile of amadumbe mucilage resembled that of the flour. Both mucilages contained amino acids that are potential substrates of the crosslinking enzymes. For example, glutamine and lysine which are substrates for transglutaminase-mediated crosslinking, and tyrosine and cysteine which are substrates of tyrosinase and laccase. Phenolic compounds were also present.

The importance of oxidative gelation of arabinoxylan in gluten-free bread making cannot be overemphasized. Since mucilages contain potential substrates for oxidative enzymes, gelation of mucilages could occur leading to an improvement in dough rheology, bread specific volume and delayed staling. Gelation of water-soluble polysaccharides has been previously proposed to improve gluten-free bread characteristics (Rosell, 2009, Sciarini et al., 2012). The mucilages also contained both hydrophilic and hydrophobic groups, indicating that they are potential multifunctional hydrocolloids in gluten-free dough systems.

It may also be worth noting that amadumbe mucilage is a low viscosity hydrocolloid containing both hydrophilic (polysaccharide and protein) and hydrophobic (protein) moieties (Chapter 2), making it a potential emulsifier. Currently in the food industry, the most commonly used hydrocolloids are mainly viscosity modifiers. These include xanthan gum, guar gum and pectin. In beverages, a hydrocolloid that has a low viscosity and emulsifying properties is necessary to stabilise the flavouring oils and prevent them from coalescing. However, gum arabic has been the only hydrocolloid with such multifunctionality. Amadumbe mucilage may therefore be a potential hydrocolloid in such applications. In some instances, a hydrocolloid that increases viscosity and is also an emulsifier is necessary in food applications. Such situations include the production of mayonnaise, an oil-in-water emulsion, and in baking. Since the commonly available gums lack multi-functionality, an emulsifier has to be added. However, cactus mucilage is a high viscosity hydrocolloid with potential emulsifying properties (Chapter 2) and may potentially serve both purposes. The properties of the mucilages indicate their potential in gluten-free dough application as hydrocolloids and substrates for crosslinking enzymes.

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The effects of laccase on the rheological properties of amadumbe dough were also investigated (Chapter 4). Model reactions showed that laccase-catalysed crosslinking could be a result of many different reactions which include dimerisation of phenolics, thiol phenolic conjugation and thiol-thiol conjugation through the formation of disulphide linkages. Dimerisation was previously proposed to influence the oxidative gelation of feruloylated arabinoxylan but they were not confirmed (Figueroa-Espinoza et al., 1998). The model reactions demonstrated the vast possibilities of reactions which have been proposed to occur in the dough system due to laccase oxidation reactions (Chapter 4). Formation of thiol-thiol conjugates or disulphide bond formation results in protein crosslinking whilst thiol-phenolic conjugation results in polysaccharide-protein crosslinking. Laccase was previously also reported to polymerise tyrosine-containing proteins (Mattinen et al., 2005a) but in this research, it was observed to preferentially oxidise ferulic acid (Chapter 4). Laccase has also been reported to form protein-polysaccharide conjugates (Selinheimo et al., 2008). The polysaccharide that could be involved in this reaction is the mucilage in amadumbe flour. These reactions result in higher molecular weight polymers with modified functionality. Since amadumbe mucilage already contains glycoproteins, crosslinking of the

protein moiety of one chain to another protein moiety of the other chain may result in modification of mucilage functionality. Crosslinking of phenolics esterified to polysaccharides with reactive amino acids may also result on inter- and/or intrachain linkages. Strong circumstantial evidence of crosslinking in the dough was shown by the decrease in dough free thiol and total phenolic contents. Both G' and G" of laccase-treated dough increased significantly due to laccase-catalysed cross-linking of proteins and polysaccharides esterified with phenolics, as demonstrated by relevant model reactions. Tan δ decreased with increase in laccase activity indicating an increase in the elastic character of the dough.

The model reactions gave evidence of formation of tyrosine dimers, tyrosine-lysine heteroconjugates as well as disulphide bond linkages (most likely involving oxidised tyrosine as a mediator). The decrease in dough thiols and free amines confirmed crosslinking. Tyrosinase has been reported to oxidise tyrosine side chains in proteins and lysyl, tyrosyl, cysteinyl, and histidinyl moieties, which may react further with tyrosine-oxidized tyrosine residues (Buchert et al., 2007; Lantto et al., 2007). This increases the molecular weight of proteins and modifying protein functionality. In this study, no evidence of tyrosine-histidine or tyrosine-cysteine conjugate formation was observed. Tyrosinase crosslinking increased amadumbe dough G' and G". This may be due to the formation of higher molecular weight polymers.

Transglutaminase treatment decreased the dough free amino groups by up to 38.1% as activity increased to 2 U/g flour. This was due to the formation of an isopeptide bond between glutamine and lysine, which was also demonstrated by mass spectrometry using a model reaction. The presence of glutamine and lysine in the mucilage (Chapter 2) may have resulted in inter and intra crosslinking of the glycoprotein chains resulting in modified functionality. Transglutaminase has also been previously noted to modify gum arabic, a similar molecule (Flanagan and Singh, 2006). An increase in dough G' and G", showed that both transglutaminase and tyrosinase improved dough viscoelasticity. This was consistent with what has been observed for rice dough (Marco and Rosell, 2008)

The combination of enzymes was finally optimised by response surface methodology (RSM) using a central composite design (CCD) (Chapter 6). The predicted optimal enzyme activities for

preparing amadumbe dough were: laccase (1.78 U/g flour), tyrosinase (79 U/g flour) and transglutaminase (1.97 U/g flour) with a desirability of 0.87. The optimum dough had a higher G' and G" (21500 Pa and 4940 Pa, respectively) than the control dough with no enzymes (G' and G" 5230 Pa and 1090 Pa, respectively) and dough with single enzyme systems, (transglutaminase (2 U/g flour): G' 8070 Pa, G" 1820 Pa; tyrosinase (80 U/g flour): G' 8750 Pa, G" 1970 Pa and laccase (2 U/g flour) : G' 11 400 Pa, G" 2520 Pa, as expected. The possibility of formation of phenolic-thiol conjugates, thiol-thiol conjugates and phenolic-phenolic conjugates by laccase catalysis (Chapter 4), thiol conjugates and tyrosine-tyrosine conjugates by tyrosinase (Chapter 5) and glutamyl-lysine conjugates by transglutaminase (Chapter 5) was demonstrated using model reactions. The higher G' and G" may be due to the effects of the combination of enzymes resulting in the formation of multiple covalent linkages. The combination of enzymes resulted in bread with a higher specific volume and lower crumb hardness compared to bread without any enzymes or modified with single enzymes. Specific volume and crumb hardness are well known to be inversely related. The high specific volume may be due to the formation of dough with improved viscoelasticity and capacity to retain carbon dioxide released during proofing. Transglutaminase use resulted in rice bread that had an acceptable specific volume and crumb hardness due to protein crosslinking which resulted in a protein network capable of holding the gas produced during proofing (Gujral and Rosell, 2004). Tyrosinase was also observed to increase the specific volume of gluten-free oat bread significantly (Flander et al, 2011). The specific volume and softness of fresh oat bread were improved by 9% and 17%, respectively when compared to control oat bread (Renzetti et al., 2010), due to laccase-mediated crosslinking. This study reports for the first time improvement in specific volume and texture as a result of modification of gluten-free amadumbe flour using an optimized combination of enzymes.

It was observed that xanthan gum led to the highest G' and G". However, cactus mucilage addition led to bread with the highest specific volume. Xanthan gum hardened the dough, preventing it from expanding during proofing. This effect is reduced by monitoring dough consistency before baking (Marco and Rosell, 2008). Sensory evaluation revealed that enzymes and cactus mucilage led to improved bread texture appearance and overall acceptability but did not significantly affect bread aroma and taste. Cactus mucilage being composed of polysaccharides and glycoproteins

may have increased dough viscosity (Chapter 3) and also acted as an emulsifier (Chapter 3), hence the improved bread.

In comparison with single enzyme systems, the optimised enzyme combination better improved rhelogical properties resulting in bread with lower hardness and higher specific volume. In addition, an optimised combination of enzymes improved sensory properties of gluten-free amadumbe bread

CHAPTER EIGHT

8.0 Conclusion and recommendations

8.1 General conclusion

Enzymatic modification is a promising approach for improving the quality of gluten-free products. Most approaches have used single enzyme systems. This study investigated an optimised multiple enzyme systems together with a hydrocolloid, and provided evidence of enzymatic reactions through relevant model reactions. The aim of the work was to modify the dough rheology of amadumbe flour using multiple enzymes for gluten-free bread production.

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The specific objectives were as follows:

- To determine the compositional, thermal and viscosity profile of amadumbe and cactus pear mucilages using HPLC, DSC and a rheometer, respectively.
- To model enzymatic reactions and determine the consequent effect of enzymes on amadumbe-based dough rheology.
- To optimise enzymatic modification of amadumbe-based dough using response surface methodology (RSM).
- To determine the physical and sensory characteristics of bread prepared from the optimised enzyme combination and hydrocolloid.

Below is a brief summary of the key findings.

Amadumbe and cactus mucilages consist of polysaccharides with associated proteins and phenolics. Cactus mucilage contains rhamnose, which is absent in amadumbe mucilage. Galacturonic is present in cactus while glucuronic acid is associated with amadumbe mucilage. The mucilages also contain amino acids that are potential substrates for laccase, transglutaminase and tyrosinase crosslinking. Both mucilages are thermally stable and can be applied in foods that undergo processing at high temperatures. Amadumbe mucilage exhibits a Newtonian flow

behaviour at low concentrations whilst cactus mucilage displays a pseudoplastic behaviour. Both mucilages show a shear thinning behaviour with an increase in concentration. Amadumbe mucilage may be a potential emulsifier in low viscosity applications such as in beverages and jelly candy, as a substitute for gum arabic. Cactus mucilage can potentially be used as a thickening and emulsifying agent in food applications such as in gluten-free dough and mayonnaise formulations.

T. versicolor laccase crosslinked phenolics and thiols, producing a wide range of homo- and hetero-conjugates, as demonstrated by model reactions. Laccase-mediated oxidation of amadumbe dough resulted in a decrease in dough phenolic and thiol groups, confirming crosslinking reactions. The reduction in Tan δ indicated an improvement in the elastic properties of the laccase-treated dough. Similarly tyrosinase mediated the crosslinking of tyrosine and thiols producing homo- and hetero-conjugates. On the other hand, transglutaminase crosslinking resulted in the formation of glutamyl-lysine isopeptide. Tyrosinase oxidation of amadumbe dough resulted in a decrease in dough thiol content and dough free amino groups, confirming the crosslinking predicted by model reactions. Transglutaminase crosslinking also led to a reduction in dough amino groups. The enzymatic modification of amadumbe dough resulted in an increase in dough G" and G', which confirmed structural modification of dough.

An optimised combination of enzymes resulted in improved dough rheological, physical and sensory properties. Laccase had a positive additive effect on dough G", with either transglutaminase or tyrosinase. Laccase and tyrosinase also had a positive additive effect on G'. The G' and G" of amadumbe dough with a combination of enzymes was higher than that of dough without enzymes or dough modified with a single enzyme system. A combination of enzymes resulted in bread with a higher specific volume and lower crumb hardness compared to bread from dough modified with a single enzyme. The combined effect of cactus mucilage (2% w/w) and optimised combination of crosslinking enzymes resulted in an increase in G' and G" and consequently the best bread which had a high specific volume, low moisture loss and better sensory acceptability.

Overall this study has demonstrated that a combination of enzymes and hydrocolloids is superior to currently used single enzyme systems and could be a viable alternative approach for improving the quality of gluten-free amadumbe bread.

8.2 Recommendations

Future work may look at further characterisation of amadumbe and cactus mucilage molecular weight by SEC-HPLC and structure by NMR. Furthermore, structural properties of these mucilages, with or without exogenous proteins, may be modified in order to design a hydrocolloid suitable for a particular application. For example, enzymatic crosslinking of a hydrophobic protein to the mucilage may result in a potent emulsifier which will further increase gluten-free bread properties.

The polymerization of amadumbe proteins, soy proteins and mucilages due to enzymatic crosslinking were suggested to be the main contributors to improved volume and softness of gluten-free amadumbe bread. Further studies may possibly characterise the formed conjugates and biopolymers for other different uses such as encapsulation or for improving the texture of different bakery products. The combination of enzymes may also be used for other gluten-free products. However, process optimization is necessary to tailor the enzyme combination for each application

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Appendices

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Laccase-mediated crosslinking of gluten-free amadumbe flour improves rheological properties

Vimbainashe E. Manhivi, Eric O. Amonsou, Tukayi Kudanga*

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

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ABSTRACT

The absence of gluten in gluten-free flours presents a challenge to their application in baking. Enzymatic modification of the protein and polysaccharides may result in a network that mimics gluten. In the current study, the effects of laccase on the rheological properties of amadumbe dough were investigated. Thiol and total phenolic contents of dough decreased by up to 28% and 93%, respectively, as laccase activity was increased (0–3 U/g flour). Both G′ and G″ of laccase-treated dough increased significantly due to laccase-catalysed cross-linking of proteins and polysaccharides esterified with phenolics, as demonstrated by relevant model reactions. Tan δ decreased with increase in laccase activity indicating an increase in the elastic character of the dough. The improvement in dough viscoelasticity may enable the retention of adequate carbon dioxide during proofing and production of more acceptable gluten-free bread.

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Composition, thermal and rheological properties of polysaccharides from amadumbe (*Colocasia esculenta*) and cactus (*Opuntia* spp.)

Vimbainashe E. Manhivi[a], Sonja Venter[b], Eric O. Amonsou[a], Tukayi Kudanga[a,*]

[a] Department of Biotechnology and Food Technology, Durban University of Technology, P.O. BOX 1334, Durban, 4000, South Africa
[b] Agricultural Research Council, Roodeplaat-Vegetable and Ornamental Plant Institute, Pretoria, South Africa

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ABSTRACT

The extensive application of hydrocolloids in the food industry, coupled with their short supply and shortcomings, has led to the ongoing search for alternative sources. In this study, the compositional, rheological and thermal properties of amadumbe and cactus mucilages were investigated. The mucilages had a similar qualitative composition of monosaccharides and amino acids, except for the absence of rhamnose in amadumbe mucilage. Fractionation of amadumbe and cactus mucilages on an anion-exchange column yielded four and three fractions, respectively. The fractions eluting with protein showed no β-elimination, suggesting stronger glycosylation bonds such as those in arabinogalactan proteins (AGPs). There was no evidence of thermal depolymerisation of the mucilages up to 195 °C. Cactus mucilage showed a pseudoplastic flow behaviour whilst amadumbe mucilage showed a Newtonian flow behaviour at up to 5% (w/v) concentrations. Amadumbe mucilage may be a potential emulsifier, whilst cactus mucilage can potentially be used as a thickening or emulsifying agent.