

Characterisation of starch from malting barley grown in South Africa

by

Sandra Balet

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Supervisor: Prof Marena Manley

Co-supervisors: Dr Glen Fox

Dr James Lloyd

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Declaration

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Abstract

South African barley is not always suitable for malting purposes due to unpredictable weather conditions. Malted barley is imported from other barley producing countries such as Australia, the United States and Europe. It would be of great importance to characterise the local cultivars in order to lay a strategy for the local plant improvement.

Different analyses were conducted to examine the functional properties of local cultivars for malting purposes. Pasting properties were determined using the Rapid ViscoAnalyser (RVA), amylose contents using size exclusion chromatography (SEC), phosphate contents using an iodine binding assay, particle size distribution using laser diffraction, granule morphology using scanning electron microscopy (SEM) and amylose and amylopectin fine molecular structure using SEC. The SSII gene was amplified using polymerase chain reaction (PCR) allowing allele specific SNP detection. Barley flour was used to examine pasting properties and starch molecular structure. Isolated starch was used for phosphate determination, granule size distribution and starch granule morphology. The DNA was extracted from barley leaves.

PCA biplots were used to relate pasting properties with different cultivars. Multiple factors analysis (MFA) was done to relate different blocks of variables with one another, correlation circle graphs were used to graphically show relationship between the blocks of variables and an individual factor map was used to relate groupings of cultivars. The PCR amplicons were sequenced and analysed using Geneious software. From the pasting properties results, Metcalfe seems to be different from the rest of the cultivars, Baudin and Henrike shows similarity, Cristalia, Marthe and Cocktail showed similarities, as well as Erica, Nemesia, Disa and Houwink. Metcalfe, Baudin and Henrike seemed to be of good malting quality.

There were no differences observed in phosphate contents, granule particle size distribution and amylose contents among the samples. The phosphate contents were between 0.21 to 0.9%, Houwink had the highest phosphate content and Henrike the lowest. Granule particle size was between 15.9 and 18.6 μm and amylose contents were between 23.8 and 25.5%. Baudin had a smaller granule diameter and Metcalfe the largest. Houwink had the lowest amylose content and Nemesia the highest. The phosphate content was low as expected in cereals. Granules were classified as large which is a good indication for malting purposes. Amylose content indicated that these cultivars had normal starch.

There were no differences in the average degree of polymerisation (DPX), the samples showed average DPX between 3,420 and 4,330, Metcalfe had the highest DPX and Henrike the lowest. The amylose and amylopectin fractions in the three regions Amylopectin1, Amylopectin2 and Amylopectin 3 were analysed. There were no differences in AP1 and AP2 regions, however, a difference was observed in Am3 region among the ten cultivars. There was a clear variation in the chain length distribution (CLD)

among the samples and also disruption of linearity in the chains length was observed especially in Marthe, Henrike and Cristalia.

Eight set of primers were used to amplify portions of the SSII gene from the ten cultivars. Four amplified the expected fragment sizes 1055, 1059, 1096, and 1112 bp. The fragments were sequenced and analysed using NCBI blast program query ID 149533 and it was 99% identical to *Hordeum vulgare* SSII gene. From the sequenced results, all cultivars showed the same result and one SNP was identified on exon 5 which change the amino acid from leucine to proline.

Opsomming

Weens onvoorspelbare weersomstandighede is Suid-Afrikaanse gars nie altyd geskik vir moutdoeleindes nie, en word moutgars ingevoer van ander lande wat gars verbou. Daarom is dit uiters belangrik om die plaaslike kultivars te tipeer ten einde 'n strategie vir plaaslike gewasverbetering te ontwerp.

Plakeienskappe is met behulp van die snelle visko-ontleder (RVA) ondersoek; amilose-inhoud is met grootte-uitsluitingschromatografie (SEC) bepaal; fosfaatinhoud met behulp van 'n jodiumbindingstoets; styselkorrelgrootteverspreiding met behulp van laserdiffraksie; korrelmorfologie met behulp van skanderingselektronmikroskopie (SEM); en amilose- en amilopektien- fyn molekulêre struktuur is met behulp van SEC bestudeer. Die SSII-geen is deur middel van die polimerasekettingreaksie ("PCR") versterk om alleelspesifieke SNP-opsporing moontlik te maak.

PCA-bistippings is gebruik om plakeienskappe met verskillende kultivars te verbind. Meerfaktorontleding is onderneem om verskillende blokke veranderlikes met mekaar te verbind; korrelasiesirkelgrafieke is gebruik om die verwantskap tussen die blokke veranderlikes grafies voor te stel, en 'n kaart van individuele faktore is gebruik om kultivargroeperinge te verbind. Die PCR-amplikons is met behulp van die sagteware Geneious aan reeksbeplanning en ontleding onderwerp. Uit die plakeienskapresultate blyk Metcalfe van die res van die kultivars te verskil, terwyl Baudin en Henrike ooreenkomste toon, Cristalia, Marthe en Cocktail gelyksoortig voorkom, en so ook Erica, Nemesia, Disa en Houwink. Die voorspelling kan gemaak word dat Metcalfe, Baudin en Henrike van 'n goeie moutgehalte is.

Geen verskille in fosfaatinhoud, korrelgrootteverspreiding en amilose-inhoud word tussen die kultivars waargeneem nie. Die fosfaatinhoud was tussen 0,21% en 0,9%; Houwink toon die hoogste en Henrike die laagste fosfaatinhoud. Korrelgrootte was tussen 15,9 μm en 18,6 μm , en amilose-inhoud was tussen 23,8% en 25,5%. Baudin toon die kleinste korreledeursnee, en Metcalfe die grootste. Houwink het die laagste amilose-inhoud, en Nemesia die hoogste. Lae fosfaat is te verwagte by graan; die korrels is groot, wat 'n goeie aanwyser van moutgehalte is. Amilose-inhoud dui daarop dat hierdie kultivars oor normale stysel beskik.

Die DPX was tussen 3,420 en 4,330; Metcalfe toon die hoogste en Henrike die laagste DP. Daar is geen verskille tussen die AP1- en AP2-streke nie; tog is verskille in die AM3-streek tussen die tien kultivars opgemerk. Daar is variasie in die kettinglengteverspreiding ("CLD") tussen die monsters, en ontwinging in lineariteit is veral by Marthe, Henrike en Cristalia opgemerk.

Agt stelle aanvoorders is gebruik om gedeeltes van die SSII-geen van die tien kultivars te versterk. Vier daarvan het die verwagte fragmentgroottes 1055, 1059, 1096 en 1112 kbp versterk. Die fragmente is met behulp van 'n navraag (ID 149533) op die BLAST-program van die NCBI bevestig, en dit was

99% identies aan die SSII-geen van *Hordeum vulgare*. Alle kultivars toon dieselfde reeksresultate, en een SNP is op ekson 5 geïdentifiseer wat die aminosuur van leusien na prolien verander.

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List of Abbreviations

| | |
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| AACC | American association for cereal chemist |
| AGpase | ADP-glucose pyrophosphorylase |
| AGP-L | AGpase large subunit |
| AGP-S | AGpase small subunit |
| API | Application programming interface |
| ATP | Adenosine triphosphate |
| BD | Break down |
| CLD | Chain length distribution |
| D | Disproportionation |
| DNA | Deoxy ribonucleic acid |
| dNTPs | Dinucleotide triphosphate |
| DP | Degree of polymerisation |
| DRI | Differential refractive index |
| DMSO | Dimethyl sulfoxide |
| FFF | Field flow fractionation |
| FV | Final viscosity |
| GXE | Genetic by environment interaction |
| Glc-1-P | Glucose -1-phosphate |
| GOPOD | Glucose oxidase peroxidase |
| GBSS | Granule bound starch synthase |
| GWD | α glucan water dikinase |
| ISA | Isoamylase |
| MOS | Malto- oligosaccharides |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NCBI | National centre for biotechnology information |
| PDT | Pedigree equilibrium test |

| | |
|-------|--|
| PPi | Inorganic phosphate |
| Phol | Phosphorylase enzyme |
| PUL | Pullulanase |
| PV | Peak viscosity |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| RVA | Rapid ViscoAnalyser |
| SAB | South Africa breweries |
| SABBI | South Africa barley breeding institute |
| SDS | Sodium dodecyl sulphate |
| SEC | Size exclusion chromatography |
| SEM | Scanning electron microscopy |
| SNP | Single nucleotide polymorphism |
| SS | Starch synthase |
| SSI | Starch synthase I |
| SSII | Starch synthase II |
| SSIII | Starch synthase III |
| SBEs | Starch branching enzyme |
| SDBE | Starch debranching Enzyme |
| TTPV | Time to peak viscosity |

Chapter 1 Introduction

1.1 Introduction

Barley (*Hordeum vulgare*) is the second most important grain used in cereal related industries and the main cereal grain used for malting and beer brewing (Suh *et al.*, 2004; Ma *et al.*, 2014). It is also used for the production of animal feeds. Barley is an excellent source of carbohydrates with starch being the largest component; making up about 65% of the endosperm dry weight (Song & Jane, 2000; Li *et al.*, 2001a; Pycia *et al.*, 2015; Matsuki *et al.*, 2008).

In South Africa, barley is the second most important grain after wheat, where it is grown for malting purposes and animal feed production. The annual production of barley is around 225 000 to 250 000 tons of the produced which 80% is classified as malting barley (Vyver, 2013). As starch is the main component of barley endosperm which converts to malt during brewing it is a major determinant of malt quality. Starch comprises of two polymers amylose and amylopectin. In addition, the endosperm has minor components namely, proteins, non-starch polysaccharides, lipids and minerals (Fox, 2010).

There are several factors that determine starch suitability for specific end uses. These are linked to chemical and physical properties of starch which are affected by the genetic by environment interaction (GxE) (Gous *et al.*, 2013). They include size and branching patterns of starch, amylose to amylopectin ratio and starch granule size distribution. All of these factors were found to have an effect on the physico-chemical properties such as gelatinisation temperature, viscosity and starch swelling (Singh *et al.*, 2003b).

Variation in starch composition occurs within cereal species and is attributed to genetic and environmental factors (Ullrich, 2010). It has been reported that growing conditions, locations and environmental conditions have an effect on the functional properties of cereal starch (Beckles & Thitisaksakul, 2014). In addition, genetic variation of starch biosynthetic genes might affect starch composition and structure and consequently, starch functionality. For example, a starch synthase II (SSIIa) mutation led to an increase in amylose contents and decrease in gelatinisation temperatures in some cereals (Umemoto *et al.*, 2002; Nakamura *et al.*, 2005; Morell *et al.*, 2003).

Different analysis might be performed to identify industrial suitability and improvement of barley cultivars for better quality. For example, identification of single nucleotide polymorphism (SNPs), SNP is the most abundant form of DNA polymorphism which could be used as a genetic markers for breeding applications (Chiapparino *et al.*, 2004) Examination of granule particle size can also be important as large granules were found to be more useful in brewing and in processes such as starch-gluten separation (Morell *et al.*, 1995). Studies had been conducted to examine and relate starch to its relevant

industrial use (Vilaplana *et al.*, 2014; Hoang *et al.*, 2008), pasting properties had been examined to relate barley flour quality to malt extract potential (Zhou & Mendham, 2005).

1.2 Rationale

South African (SA) barley cultivars are not always suitable for malting purposes. Thus malted barley is imported from other barley producing countries. This results in high production costs for the South African Breweries (SAB's) (Visser, 2011). In 2013 almost 75 000 tons of barley were imported to SA as malt or malted barley (Vyver, 2013). Therefore, it is important to study the functionality of the local cultivars, in order to provide local breeders with information to produce cultivars with grain quality attributes suited for malting purposes. It has been suggested that environmental stresses such as changes in temperature and moisture available in the soil have significant effects on barley functionality (Fox *et al.*, 2006; Beckles & Thitisaksakul, 2014). A combination of these factors affects cereals during grain filling (Fox *et al.*, 2003b).

Under high temperature conditions, starch synthesis is affected resulting in reduced starch content (Fox *et al.*, 2003a), which in turn is associated with lower malt extract potential. On the other hand, barley quality has been found to be controlled by multiple genes (G) with a strong interaction with the environment (E). This GxE interaction contributes to the often undesirable variation in barley quality that in turn affects malt quality (Fox *et al.*, 2003b; Ullrich, 2010).

Currently the undesirable combined contribution of GxE factors are of major concern to the barley industry (Fox *et al.*, 2003b) being aware of the demand for malting barley cultivars with specified grain quality characteristics (Pycia *et al.*, 2015). Therefore, determination of variation among barley samples of different malt quality would be essential because it helps breeders to determine future strategies for plant improvement (Gong *et al.*, 2013; Blazek & Copeland, 2008).

1.3 Thesis outline

This thesis comprises of eight chapters namely, an introduction, literature review, research hypothesis, aim and objectives, materials and methods, results, discussion and conclusions and appendices.

Chapter one which is an introduction provides a general idea about the study with emphasis on the research question, gap in knowledge, research rationale and possible solutions to be considered. Chapter two is the literature review and it focused on what has been reported by other researchers concerning the research gap, as well as on the methods that will be used to conduct the study. Chapter three is research hypotheses, aim, and objectives. Chapter four covers materials and methods used to achieve the aim. Chapter five reports the results obtained from the study. Chapter six is the discussion which includes a critical review of the methodology used, followed by Chapter seven and eight which is the conclusions and appendices respectively.

Chapter 2 Literature review

2.1 Introduction

Barley (*Hordeum vulgare*) is a member of the grass family Poaceae, the tribe triticaceae and genus *Hordeum* (Ullrich, 2010; Sologubik *et al.*, 2013; Li *et al.*, 2001a; Li *et al.*, 2001b; BeMiller & Whistler, 2009). It is one of the oldest cultivated crops with archeological evidence supporting the idea that it was originally grown in the Middle East 10 000 years ago (Bringham, 2015). Barley belongs to one of the most economically important plant groups and is known as the fourth most important cereal produced worldwide after wheat, maize and rice (Baik & Ullrich, 2008; Gong *et al.*, 2013; Bertoft *et al.*, 2011).

From a genetic point of view, barley has seven chromosomes ($x=7$), with both diploid ($2n=14$) and polyploid ($2n=4x=28$ & $2n=42$) varieties existing (Slafer *et al.*, 2001) making it one of the most genetically diverse cereal grains. It is classified as spring or winter types, two-row or six-row, hulled or hull-less, and as malting or feed barley (Baik & Ullrich, 2008).

A barley kernel is a single seeded fruit, referred to as a caryopsis (the seed is tightly adhered to the pericarp), and is characterised by the presence of a crease. The crease is the re-entrant region on the ventral side extending along the grain's entire length and is deepest in the middle (Evers & Millar, 2002). It was suggested that during ripening the outer layer, seed coat and pericarp fused together to give the barley seed the caryopsis characteristic (Gubatz & Shewry, 2010).

Barley is a highly adaptable cereal with the ability of growing in different climatic conditions and as a consequence, it has been produced in different parts of the world. It has good malting characteristics that raised its importance and use in malted beverages. Until recently, 90% of the produced barley was used in malting industry (Baik & Ullrich, 2008; Sologubik *et al.*, 2013).

Historically, South Africa has produced barley mainly for malting purposes. Despite this, South Africa still imports it owing to the fact that the varieties grown do not meet the quality specification required by the South African Breweries (SAB) (Vyver, 2013). Growing conditions associated with location, season and planting dates affect the composition and quality of cereal grains (BeMiller *et al.*, 2009).

In South Africa, the dry land cultivation of malting barley is restricted to the Southern Cape, from Botrivier in the west to Heidelberg in the east. The unpredictable weather conditions in the Southern Cape resulted in introduction of barley production to the cooler central irrigation areas of the Northern Cape Province (Anon, 2007).

To meet production requirements, barley is imported from Canada, the United States (US), Australia and Argentina. Malted barley is imported from Canada, the United State, Sweden and France (Anon, 2007). Less dependency on foreign imports would, however, be preferred (Visser, 2011). This will only be possible if malting barley cultivars, suitable for local growing conditions and with the required quality

specifications, could be bred and produced locally. To complement breeding programmes with such efforts, biochemical and/or molecular tests able to rapidly predict barley malting and brewing quality, from small samples, are required (Fox *et al.*, 2003b).

With respect to the composition, cereals vary from one species to another due to genotype by environment (GxE) interaction (Evers & Millar, 2002). Starch is given much attention in different research areas because of the role it plays during food processing and its potential use in a multitude of other industries (Singh *et al.*, 2007; Schirmer *et al.*, 2013). Amylose and amylopectin are the main starch components with great importance and their biosynthesis is catalysed by enzymes encoded by multiple genes that are required for normal starch granule synthesis (Regina *et al.*, 2010).

The end-products of starch biosynthesis reflect the genetic diversity among enzymes involved, as well as environmental factors acting on their expression and activities. Understanding the basic reactions of starch biosynthesis could be of great benefit to agricultural applications by providing the means to manipulate the quality and quantity in cereal grains as well as its suitability for industrial uses (Copeland *et al.*, 2009).

In this literature review, barley grain structure and composition will be discussed focusing on starch as the main component of the grain with specific emphasis on starch composition and structure. In addition, the starch biosynthesis pathway, the enzymes involved, as well as the environmental factors influencing expression and activities of these enzymes will be reviewed in detail.

The review will also include the use of the Rapid ViscoAnalyser (RVA) to determine physical properties, which may or may not be related to environmental influences. Other methods to be reviewed include the enzyme linked assay to determine phosphate contents, laser diffraction to measure granule particle size distribution, scanning electron microscopy (SEM) to examine starch granule morphology, polymerase chain reaction (PCR) to examine genetic variation on barley starch biosynthesis genes (specifically one encoding starch synthase II) and size exclusion chromatography (SEC) to examine starch molecular structure and amylose content.

2.2 Structure and composition of barley grain

Barley grains comprise of three main components (1) an embryo (germ), (2) the endosperm and (3) the outer layer (aleurone layer, husk, and nucellar layer, seed coat and pericarp) (Figure 2.1) (Fox, 2010). (1) The embryo is a very important component because it has ability to grow into a new plant and that is important for species survival. (2) The endosperm is a solid mass of starch located in the center of the grain (Evers & Millar, 2002), which is surrounded by the aleurone layer and its chemical composition is related to malt quality (Fox, 2010).

Endosperm is made of cells filled with starch granules embedded in a protein matrix (Jääskeläinen *et al.*, 2013). (3) The outer layer comprises firstly the aleurone which is a thick layer. The husk surrounds

the grain which is also called the material layers of the grain. It comprises the nucellar layer, which is composed of a thin cuticle layer surrounding the embryo. The seed coat which is also known as the testa or true seed coat is made of a very thick cuticle layer and, the pericarp which is a cuticle layer overlaying the seed coat (Evers & Millar, 2002).

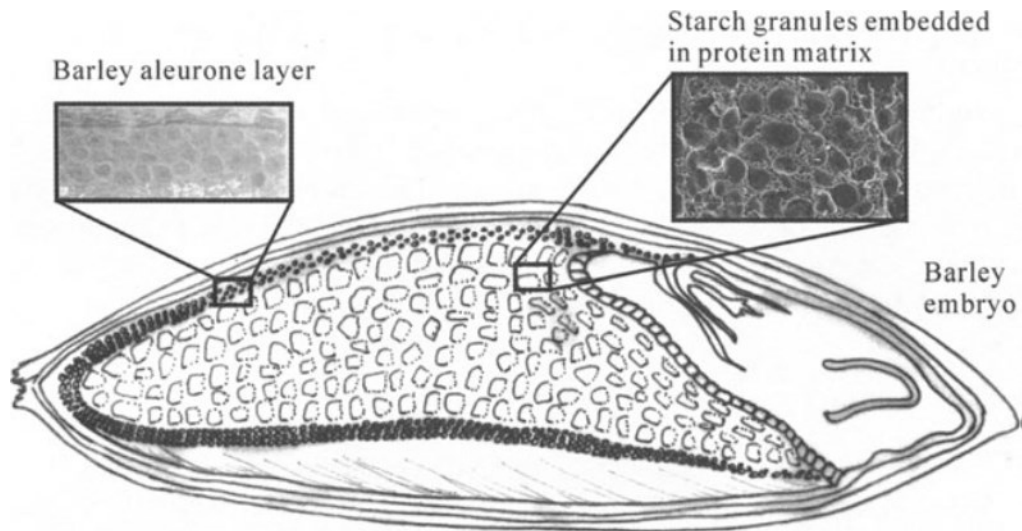


Figure 2.1 Barley structure, comprising the embryo, aleurone layer and endosperm showing starch granules embedded in a protein matrix (Fox, 2010).

Carbohydrates, fiber and protein are the major components of barley seeds. The grain contains up to 80% carbohydrates of which 53 to 67% is starch, 14 to 25% dietary fiber and 9 to 14% crude protein. Barley grain also typically contains ash (3-4%), fat (2-3%), low molecular weight carbohydrates (1-7%), arabinoxylans (4-11%), β -glucans (3-7%) and a small amount of cellulose and lignin. Genotype by environment (GxE) interaction was raised earlier as being the main factor that could cause variation in the chemical composition of barley grain (Oscarsson *et al.*, 1996).

The endosperm also contains minor components such as arabinoxylans and β -glucans, in addition to other substances like phenolic acids, cellulose and proteins. The aleurone layer is composed of arabinoxylans, β -glucan, phenolic acids, proteins and cellulose while the husk is composed of xylan, lignin, and cellulose (Jääskeläinen *et al.*, 2013). Starch composition is central to the functionality of barley grain (Regina *et al.*, 2010) which directly or indirectly impacts on the end use quality (Gous *et al.*, 2013).

2.3 Starch and its composition

Starch is a common component of many plants and the major storage carbohydrate with many important functions. It is the main form in which carbon is stored in plants (Cuesta-Seijo *et al.*, 2013; Carciofi *et al.*, 2012) as well as being the most abundant and renewable polysaccharide in plants after cellulose (Jeon *et al.*, 2010).

Starch is synthesised in many organs within plants and stored in two forms namely, storage and transient starch. Storage starch is used for long term storage in amyloplasts of sink organs such as endosperm and tubers while transient starch is a short term storage in chloroplasts of vegetative organs such as leaves (Mutisya *et al.*, 2003; Tetlow *et al.*, 2004a; Sonnewald & Kossmann, 2013; Regina *et al.*, 2010; Cuesta-Seijo *et al.*, 2013). Native starch is stored in inactive form, hence it becomes suitable for long term storage component in plants (Thitisaksakul *et al.*, 2012). It is insoluble in cold water unless heated to a temperature of approximately 65°C and above (Kossmann & Lloyd, 2000; Tester *et al.*, 2004b). Granules are classified based on diffractometric spectra and not morphological classification, however, this enable to classify starch according to their physical properties (BeMiller & Whistler, 2009).

In general, there are three types of granules pattern, A types and that is found in cereal starches, B type which is found in tuber starches and high amylose starches, type C is found in legumes. In addition, V type which is referred to amylose complexes (BeMiller & Whistler, 2009). Starch granules are found in different sizes, shapes, crystallinity and composition based on their botanical origin (Cuesta-Seijo *et al.*, 2013; He *et al.*, 2013). Granule size ranges from 1 to 100 µm in diameter depending on the starch type. In general, barley and wheat have two types of starch granules namely A and B. A granules have a diameter greater than 10 µm ($\geq 10 \mu\text{m}$), while B type granules are less than 10 µm ($\leq 10 \mu\text{m}$) in diameter (He *et al.*, 2012).

Cereals contain starch granules with various shapes such as polygonal, spherical and lenticular. They can be classified into three morphological groups known as (1) simple (e.g. maize and sorghum), (2) bi- or trimodal. This group varies in size and morphology: (a) 10-35 µm, (b) <5-10 µm) and (c) < 5µm (e.g. barley and wheat) and (3) compound granules (e.g. rice and oat) (Thitisaksakul *et al.*, 2012). In particular, barley has lenticular (A type) and spherical (B type) shaped starch granules with bimodal distribution (Tester *et al.*, 2004b; Eskin & Shahidi, 2012). Both A & B types of starch granules have significant variation in their composition and physico-chemical properties, which contributes to their suitability in food and non-food applications.

As mentioned above, starch is comprised of a number of components. The major component are two distinct polyglucan polymers, namely, amylose and amylopectin (Schirmer *et al.*, 2013). The ratio of the two polymers within the granule is not constant and varies between 0 and 80% depending on the botanical origin of the starch as well as the genotype of the plant (Copeland *et al.*, 2009).

Starch is composed of glucose polymers in the form of semi-crystalline granules with an internal lamellar structure made up of amylose and amylopectin (Jobling, 2004). These fractions are the same with regards to the primary structure, in that they are polyglucans where the glucose moieties linked by α -1 \leftarrow 4 glycosidic linkages with branch point composed of α -1 \leftarrow 6 linkages. The contrast between each other is based on differences in chain length and the degree of branching, both of which influence the physicochemical properties of the starch (Slattery *et al.*, 2000). Amylose is manufactured within the granule, while amylopectin is at the surface of the granule and that could be an underlying reason for their structural differences (Denyer *et al.*, 2001). Almost all barley starches is similar to the other cereal grains, it contains both amylose and amylopectin (MacGregor & Bhatta, 1993; BeMiller & Whistler, 2009). In this section amylose and amylopectin will be discussed in general.

2.3.1 Amylose

Amylose is essentially linear chains of glucose (D-glucan chain) molecules joined by α -1 \leftarrow 4 glycosidic linkages Figure 2.2 with less than 0.1% of α -1 \leftarrow 6 branched points (Kossmann & Lloyd, 2000; Regina *et al.*, 2010). With respect to the molecular weight, amylose is approximately 10^5 to 10^6 Dalton (Tester *et al.*, 2004a; Copeland *et al.*, 2009; Thitisaksakul *et al.*, 2012) and it contains about 9 to 20 branch points leading to approximately 3 to 11 chains per molecule. Each chain contains 200-700 glucose residues resulting in an approximate molecular weight of between 30 000 and 110 000 Dalton (Tester *et al.*, 2004b; Simsek *et al.*, 2013).

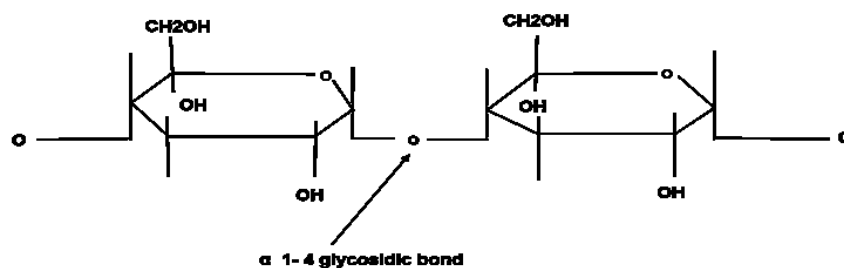


Figure 2.2 Amylose structure, it comprises of linear chains of glucose linked by α -1 \leftarrow glycosidic bonds.

2.3.2 Amylopectin

Amylopectin is also composed of linear chains of glucose (D-glucan chain) linked by α -1 \leftarrow 4 glycosidic bonds, but also contains a high degree of branched points, approximately 5% of α -1 \leftarrow 6 linkages Figure 2.3 (Kossmann & Lloyd, 2000; Tester *et al.*, 2004b; Regina *et al.*, 2010).

The molecular weight of amylopectin is 10^7 to 10^9 Dalton (Tester *et al.*, 2004b; Tester *et al.*, 2004a). Amylopectin is, therefore, larger than amylose in terms of molecular weight and is also more highly organised as the distribution of the linear chains within it is non-random. They aggregate into clusters

where the chains within the cluster form double helices that leads to crystallisation of the molecule (James *et al.*, 2003; Sonnewald & Kossmann, 2013). However, amylose has a longer average chain length than amylopectin (Tester *et al.*, 2004b; Tester *et al.*, 2004a) due to the lower frequency of branch points.

Depending on the botanical origin, amylopectin can contain significant proportions of covalently attached phosphates (Sonnewald & Kossmann, 2013; Singh *et al.*, 2007). This phosphorylation strongly influences the physical properties of the starch (Jobling, 2004). The basic organisation of amylopectin chains is divided into three groups termed A, B and C chains (Figure 2.4). A chains are linked by α -1 \leftarrow 4 glycosidic linkages at the reducing group through C6 of a glucose residue to an inner chain, B chains are those which bearing other chains as branches while, C chains also carry other chains as branches and also contains the sole reducing residue (Buléon *et al.*, 1998).

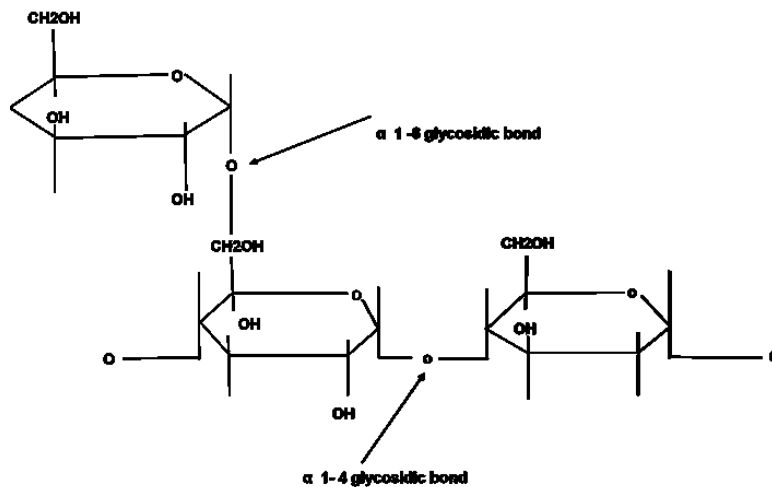


Figure 2.3 Amylopectin structure, it comprises of linear chains of glucose linked by α 1 \leftarrow 4 glycosidic bonds and branches link by α 1 \leftarrow 6 glycosidic bonds.

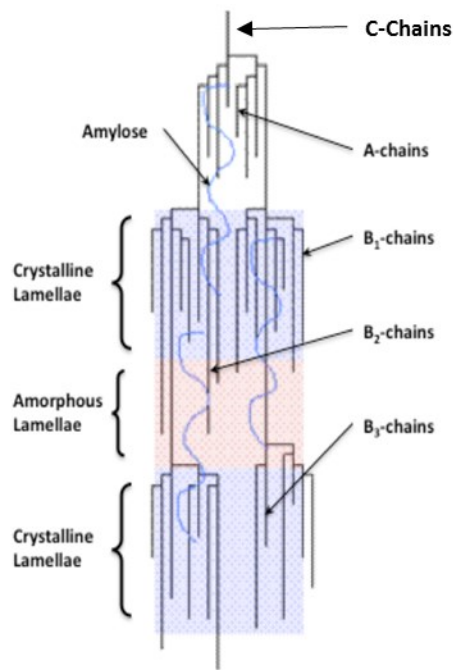


Figure 2.4 Amylopectin cluster structure (Saunders *et al.*, 2011).

2.4 Starch granule structure

Efforts have been made to investigate starch granule structure. In 1937 Hanson and Katz hypothesised that starch granules are composed of crystalline units embedded in amorphous material. Based on this hypothetical model, Badenhuizen demonstrated the presence of naturally resistant units of material under a light microscope (Gallant *et al.*, 1997). Starch granules are roughly spherical in shape and are crystalline, where double helical chains within amylopectin, which form crystalline lamellae, generate the crystallinity. These are interspersed with amorphous lamellae comprising α -1 \leftrightarrow 6 branched regions of amylopectin and amylose (Tester *et al.*, 2004a).

Different methods such as microscopy and enzyme digestion have been employed to study the complex structure of starch granules which was shown to have a hierarchical order of amylose and amylopectin being identified (Tester *et al.*, 2004b). Atomic force microscopy was used to investigate the starch granules at different levels. At the lowest level Figure 2.5 A of organisation, alternating crystalline (hard) shells and the semi-crystalline (soft) shells were identified which are several hundred nanometers in thickness (Gallant *et al.*, 1997).

The hard shells consist of blocklets which are approximately 50 to 500nm in size while, the soft shells are smaller in ranging between 20 to 50 μ m Figure 2.5 B. At the higher level of structure organisation, the blocklet structure shows amorphous radial channels connected by a central cavity with the exterior of starch granule (Kossmann & Lloyd, 2000; Gallant *et al.*, 1997).

At the highest level of structure Figure 2.5 C, one blocklet is observed containing numerous amorphous crystalline lamellae besides the crystalline structure of the starch polymers (Gallant *et al.*, 1997). The crystalline structure is exclusively associated with the amylopectin component while the amorphous regions mainly represent amylose (Singh *et al.*, 2007).

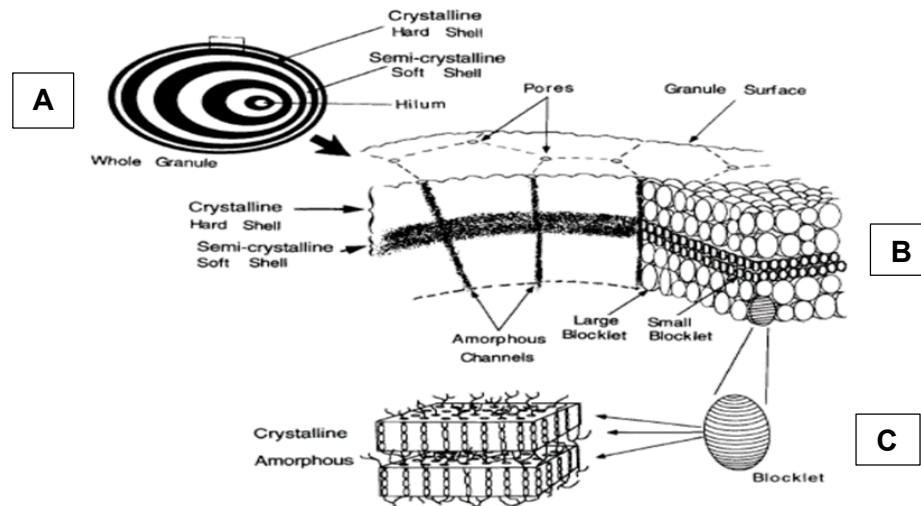


Figure 2.5 Starch granule structure at different microscopic levels (Gallant *et al.*, 1997).

2.5 Starch biosynthesis enzymes and their respective roles

At least four classes of enzymes are required for the successful production of starch. These are, adenosine diphosphate glucose pyrophosphorylase (AGPase; EC 2.7.7.27), starch synthase (SSs; (EC 2.4.1.21), starch branching enzymes (SBE; α -1 \rightarrow 4 glucan-6-glucosyl-transferase, EC 2.4.1.18) and starch debranching enzymes (DBE; EC 3.2.1.41 and EC 3.2.1.68) (Morell *et al.*, 2003; Li *et al.*, 2003). However, other enzymes such as disproportionation enzyme (D) and phosphorylase (Phol 1) are also proposed to be involved in the process, even though their roles are not well understood (Higgins *et al.*, 2013). In the following section each enzyme will be discussed in more detail, with particular emphasis on the pathway in cereal endosperm tissue.

2.5.1 ADP-glucose pyrophosphorylase (AGPase)

The first committed step of starch metabolism is the production of ADP-glucose by ADP-glucose pyrophosphorylase (AGPase). AGPase catalyses the conversion of glucose-1-phosphate (Glc-1-P) and adenosine tri-phosphate (ATP) to inorganic pyrophosphate (PPi) and adenosine di-phosphate glucose (ADPglucose). The ADP-glucose formed is the substrate for starch synthase in the production of amylose and amylopectin (Tetlow, 2011). AGPase is thus a key regulatory enzyme for starch biosynthesis (Faix *et al.*, 2012). In most plants AGPase is present only in the plastid, however, in cereal endosperm tissue it is found in both the cytosol and the plastid (Smith *et al.*, 1997), meaning that ADP-

glucose is synthesized extra-plastidially and later transported into amyloplasts (James *et al.*, 2003; Tuncel & Okita, 2013).

AGPase is composed of two subunits, large (AGP-L) and small (AGP-S) encoded by separate genes. Each of these, play different roles in catalysis and regulation of enzyme by allosteric effectors and redox. The small subunits are responsible for catalytic activity whereas, the large subunits modulate the enzymatic regulatory properties that affect allosteric response (Jeon *et al.*, 2010; Tuncel & Okita, 2013). Mutations in AGPase normally reduce starch contents (Faix *et al.*, 2012).

2.5.2 Starch synthases (SSs)

Starch synthases are also involved in manufacturing the starch polymers. They are comprised of two classes; the soluble starch synthases (SSs) which are found in the plastid stroma and the insoluble, or granule bound starch synthase (GBSS) found within or bound to the granule. These enzymes catalyse starch synthesis by transferring the glucosyl moiety from ADP-glucose to the non-reducing end of the α -1 \leftarrow 4 linkage to form amylose and amylopectin (Ball & Morell, 2003; Li *et al.*, 2003; Morell *et al.*, 2003).

It has been suggested that soluble starch synthase has up to five isoforms depending on plant's species while, the GBSS is present as one isoform (Tetlow, 2011). In particular, cereals have three forms of soluble starch synthase namely, starch synthase I (SSI), starch synthase II (SSII) and starch synthase III (SSIII) (Morell *et al.*, 2003).

2.5.2.1 Starch synthase I (SSI)

Among starch synthase isoforms, SSI is the only one that appears to occur as a single isoform (Jeon *et al.*, 2010). It is required for the synthesis of short glucan chains and its deficiency results in lack of short chains with degree of polymerisation (DP) between 6 and 12 (Wu *et al.*, 2013). It has been proposed that the role of SSI is not yet known (Morell *et al.*, 2003). On the other hand, it has been reported that the activities of some enzymes such as SSI and SSII vary between plant species (Delvallé *et al.*, 2005).

It has been reviewed that the absence or reduction of SSI in potato neither caused starch structural changes nor changes in amylose to amylopectin ratio or chain length distribution (CLD) (Kossmann & Lloyd, 2000; Jeon *et al.*, 2010). That was attributed to low expression of mRNA in tubers compared to other plants (Delvallé *et al.*, 2005).

Compared to other starch synthesising enzymes, SSI has high level of expression in the developing cereal endosperm (Ball & Morell, 2003). In addition it was reported that absence of SSI changes amylopectin structure in rice endosperm (Delvallé *et al.*, 2005).

2.5.2.2 Starch synthase II (SSII)

Starch synthase II (SSII) is suggested to be involved in amylopectin synthesis and it has two isoforms in cereals known as *SSIIa* and *SSIIb*. A recent study suggested that SSII has a specific role in the synthesis of the medium chains with the DP greater than 12 and less than 30 of amylopectin clusters (Wu *et al.*, 2013). It was clear that the deficiency of SSII caused reduction in starch contents and alteration in the amylopectin structure (Kossmann & Lloyd, 2000; Jeon *et al.*, 2010; Tetlow, 2011) as well as reduction in the medium chain with $DP \geq 12$ and ≤ 30 (Wu *et al.*, 2013).

2.5.2.3 Starch synthase III (SSIII)

Starch synthase III (SSIII) is also involved in amylopectin biosynthesis, and its role was observed in the synthesis of long chains with degree of polymerisation greater than 30 which are mainly the chains that extend between amylopectin clusters. Two isoforms were identified in cereals, known as *SSIIIa* and *SSIIIb*. *SSIIIa* is expressed in the endosperm, while *SSIIIb* is in leaves (Ball & Morell, 2003).

Different effects were observed in plant species lacking *SSIII*, in potato it altered chain length distribution and granule shape and in *Arabidopsis* it affected the rate of starch accumulation in leaves (Tetlow, 2006).

2.5.2.4 Granule bound starch synthase (GBSS)

GBSS is essential for amylose synthesis (Kossmann & Lloyd, 2000; Jobling, 2004). In cereals, GBSS has two isoforms GBSSI and GBSSII with GBSSI being responsible for amylose biosynthesis in the endosperm, whereas GBSSII is responsible for amylose synthesis in leaves (Morell *et al.*, 2003). It has been shown that GBSS is also involved in amylopectin synthesis, mainly for the forming of extra-long glucan chains (Fulton *et al.*, 2002). Deficiency of GBSS leads to production of starch with low or eliminated amylose known as waxy starch (Wu *et al.*, 2013).

2.5.4 Starch branching enzymes (SBEs)

Branching enzymes catalyse the hydrolysis of α -1 \leftarrow 4 glycosidic linkages within the polymer and transfers the hydrolysed chain to form α -1 \leftarrow 6 glycosidic linkages. There are two isoforms known as starch branching enzymes I (*SBEI*) and starch branching enzymes II (*SBEII*). These can be differentiated based on their preference of glucan chain transfer (Slattery *et al.*, 2000; Thitisaksakul *et al.*, 2012).

In general, branches introduced by SBE influences the chemical and physical properties of starch (Slattery *et al.*, 2000). *SBE I* produces longer glucan chains with a DP greater than 16, whereas *SBE II* produces shorter chains with DP less than 12 (Jeon *et al.*, 2010). In cereals, *SBEII* is further divided into two isoforms namely, *SBEIIa* and *SBEIIb* which are both having the same expression time and pattern

and are present in stroma and granule. Mutation of *SBEIIb* has a greater impact on grain phenotype compared to when the activity of *SBEIIa* was repressed, although that led to alteration in leaf starch (Ball & Morell, 2003). Generally mutations in SBE's decrease the amount of branch points in amylopectin and increase the amylose content of the starch (Kossmann & Lloyd, 2000).

2.5.5 Starch debranching enzymes (SDBs)

An earlier study supports the role of starch debranching enzyme in determination of amylopectin structure by regulating the branching and maintenance of amylopectin crystallinity (Morell *et al.*, 2003). Two isoforms of SDEs are found namely, isoamylase (*ISA*) and, pullulanase (*PUL*), which is also known as limit dextrinase. These both hydrolyse α -1 \leftarrow 6 glycosidic linkages and differ in substrate specificity. The *ISA*'s are further divided into three isoforms (*ISA1*, *ISA2* and *ISA3*), whereas, pullulanase type of debranching enzyme has only one isoform (Thitisaksakul *et al.*, 2012).

Although it appears counterintuitive, three of these enzymes (*ISA1*, *ISA2* and *PUL*) have been shown to be involved in amylopectin synthesis. Mutations in either *ISA1* or *ISA2* lead to plants accumulating a highly branched polymer (known as phytoglycogen) either instead of, or in addition to, starch. Similarly a mutation in *PUL* can increase phytoglycogen contents when it is combined with either an *ISA1* or *ISA2* mutation (Zeeman *et al.*, 2010).

ISA1 is mostly active on glucan substrates with long external chains, such as solubilised amylopectin, while *ISA2* modulates the stability of *ISA1* rather than contributing to amylopectin debranching (Zeeman *et al.*, 2010). These isoforms are called direct debranching enzymes because their action is directly on hydrolysing without prior transfer of the α -1 \leftarrow 4 chain (Ball & Morell, 2003). Generally, *ISAs* debranch phytoglycogen and amylopectin, while *PUL* debranches pullulan and, amylopectin (Jeon *et al.*, 2010). In many plant species mutation of SDEs genes in barley correlate with accumulation of water soluble polysaccharides such as phytoglycogen a glycogen like structure which is associated with reduction of starch content (James *et al.*, 2003). *ISA3* on the other hand is involved in the starch degradation pathway rather than the starch biosynthetic pathway.

2.6 Starch biosynthesis pathway

An understanding of the pathway of starch biosynthesis, the main genes involved, the way they catalyse the reaction, and the factors influencing their activities are necessary in providing an idea on how to improve and produce plants with high quality and suitable end uses (Morell *et al.*, 1995).

As discussed earlier, several specific enzymes are required for a successful starch synthesis, namely AGPase, starch synthase, starch branching enzymes, and starch debranching enzyme (Preiss, 2009).

This process occurs in a special organelles known as plastids and the biosynthesis can occur either in amyloplasts or chloroplasts (Carciofi *et al.*, 2012; Smith *et al.*, 1995).

The starch biosynthetic pathway can be completed in two phases, namely, the initiation and the extension phase (Figure 2.6). The first phase involves initiation of the glucosyl primer whereas the latter phase involves extension of α -glucan primer to produce amylose and amylopectin (Preiss, 2009). During the biosynthetic reaction, amylose and amylopectin synthesis occurs in the same place and at the same time due to the asynchronous action of the starch biosynthetic enzymes (Denyer *et al.*, 2001). However, the modes of enzymatic actions that affect the rate in which these enzymes function are not yet defined (Wu *et al.*, 2013).

2.6.1 Initiation phase

Through photosynthesis, plants harvest light energy to fix reduced carbon dioxide into a simple carbohydrate backbone. The result of this reaction is a number of triose and hexose phosphates including glucose-1-phosphate. AGPase converts glucose-6-phosphate (Glc-6-P) and adenosine triphosphate (ATP) into adenosine diphosphate glucose (ADPglucose), which initiates starch biosynthesis in leaves. During the day chloroplasts synthesise transient starch, which is degraded at night to form glucose, maltose and sugar phosphates. Some of these products are converted to the major transport sugar, sucrose, which is translocated to storage tissues such as seeds and tubers (Asare, 2011).

Sucrose is believed to be the basic source of carbon for starch biosynthesis in cereal endosperm and it is composed of two molecules, glucose and fructose. Catabolism of sucrose in the cytosol produces the substrates for ADP-glucose production, i.e. G-1-P and ATP (Eskin & Shahidi, 2012; Copeland *et al.*, 2009).

As a result of sucrose synthase activity, the cytosolic sucrose is converted to uridine diphosphate glucose (UDP-glucose) and fructose. This reaction is followed by conversion of UDP-glucose into G-1-P by the action of the enzyme UDP-glucose phosphoglucomutase in the presence of pyrophosphate (PPi) (Kossmann & Lloyd, 2000; Tester *et al.*, 2004b).

A transporter in the plastidial envelope is required to import the G-6-P from cytosol to the plastids stroma. It has been proposed that Hv.NST1 is the transporter required for G-6-P in barley. However, the nature of these transporters are not yet clear (Patron *et al.*, 2004). Once G-6-P has entered the amyloplast, it is converted into G-1-P by the enzyme phosphoglucomutase. (Kossmann & Lloyd, 2000; Tester *et al.*, 2004b; Tetlow, 2011) and can then be used to produce ADP-glucose. In cereal endosperm, G-1-P can be directly transported into the amyloplast as G-1-P, or can be converted into ADP-glucose as a result of a cytosolic AGPase. A specific ADP-glucose transporter, known as Brittle-1, is present in the amyloplast membrane in cereal endosperm (Shannon *et al.*, 1998).

2.6.2 Extension phase

Extension is performed by starch synthases and involves the elongation of the linear glucan chain by catalysing the transfer of glucosyl unit of ADPglucose at the non-reducing end of glucan. (Jobling, 2004; Regina *et al.*, 2010; Radchuk *et al.*, 2009). GBSS differs from the other SSs in its exclusive localisation and mode of action. It is this protein that has the ability to extend the linear glucan chains in the absence of branching enzymes (Denyer *et al.*, 2001; Jobling, 2004).

Moreover, GBSS has much higher affinity for malto-oligosaccharides (MOS) and is more active on elongation of MOS than the other starch biosynthesis genes and researchers have hypothesised that amylose is synthesised using MOS as a primer for amylose biosynthesis. (Denyer *et al.*, 2001; Tetlow *et al.*, 2004a; He *et al.*, 2012; Tetlow, 2006).

On the other hand, amylopectin biosynthesis is achieved by SS isoforms in addition to branching and debranching enzymes. The glucosyl moiety is transferred onto the existing glucan chain by starch synthase isoforms. These isoforms have different properties and roles in amylopectin biosynthesis (Jobling, 2004; He *et al.*, 2012). The distribution analysis of amylopectin chains length provided an idea of the role of each gene involved in this process (Zeeman *et al.*, 2010).

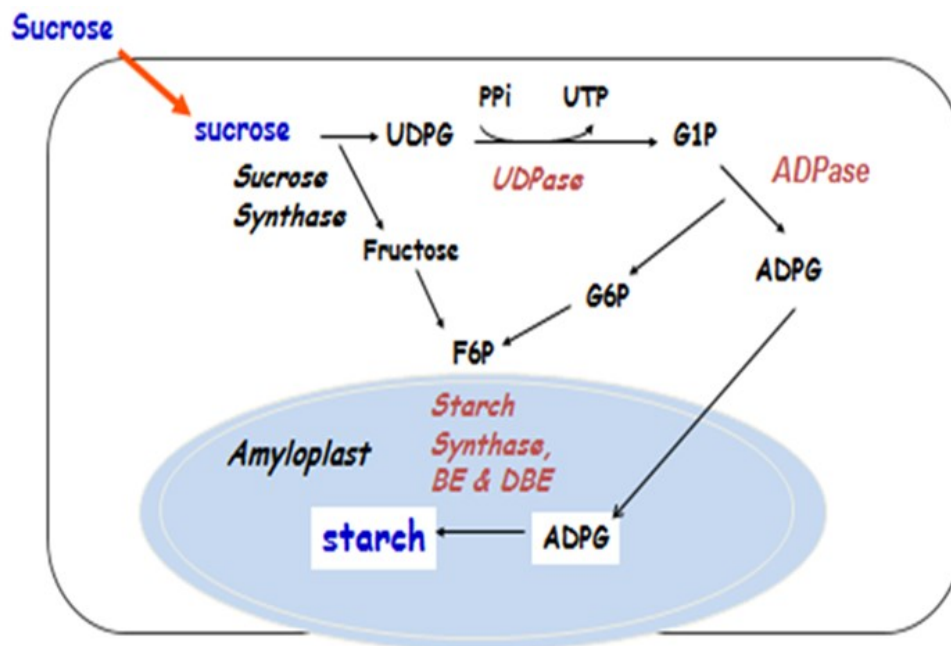


Figure 2.6 The starch biosynthesis pathway in cereal endosperm (Lloyd, 2014).

2.7 Effect of environmental conditions on starch biosynthetic enzymes

Barley is a short season plant that has high potential of adaptability and growth in a wide range of environments including extreme latitudes where other crops fail to grow and survive. It is more resistant to salt, drought and it tolerates high and low temperatures (Slafer *et al.*, 2001). Environmental stresses have been reported to affect the starch biosynthetic pathway. These factors influence the activity of starch biosynthetic enzymes and, as a consequence, alter starch structure and the amylopectin to amylose ratio (Kossmann & Lloyd, 2000).

Starch biosynthetic enzymes determine the structure of starch molecules; hence alteration in their activities could alter starch functional properties. They form physical complexes with one another and their substrates. Thus, this interaction is likely to be important for the proper architecture of the starch granule. Different environmental stresses, previously reviewed, include heat, drought, low temperatures, salinity, nitrogen, carbon dioxide and acidic stress (Thitisaksakul *et al.*, 2012). These will be discussed in general for cereals with some examples of barley, wheat and rice.

Heat stress is defined as an increase in temperature for a specific period of time which results in plant damage (Lipiec *et al.*, 2013). An earlier study in wheat has shown that starch biosynthetic enzymes seemed to be sensitive to high temperatures (Keeling *et al.*, 1993). On the other hand, it has been reported that some of these enzymes such as AGPase showed an increase in activity with increased temperature (Wallwork *et al.*, 1998). A recent study revealed that all starch biosynthesis enzymes are sensitive to heat and their functions are reduced at temperatures above 35°C which was attributed to the denaturation of protein. Barley showed reduction in total starch when grown at temperatures greater than 40°C (Thitisaksakul *et al.*, 2012). In general, the severity of heat stress on cereals determines amylose and amylopectin content (Wallwork *et al.*, 1998; Thitisaksakul *et al.*, 2012).

Drought is also called water stress which is defined as a period of dry weather that causes plant dehydration (Lipiec *et al.*, 2013). It is considered as one of the most important environmental factors that affect crop productivity (He *et al.*, 2012). At high temperatures, plants tend to reduce water losses and this leads to stress. Mostly, this phenomena occurs in dry land farming where the plant may exceed ambient temperature by 5% due to reduction in transpiration rate as it minimizes moisture loss (Fox *et al.*, 2003b).

Soluble starch synthase are more sensitive to water stress than insoluble starch synthase with their activities become quickly reduced. AGPase also demonstrates a reduction in its activity (Thitisaksakul *et al.*, 2012). In addition, it has been reported that water stress affects the expression of starch synthetic genes which consequently affect starch composition and grain weight (He *et al.*, 2012).

Carbon dioxide and nitrogen also have shown an effect in plants biosynthetic pathway, they disrupt the pathway which results in a shift in partitioning between starch and nitrogen containing compounds

such as amino acids and protein (Asthir *et al.*, 2012). It has been reported that high nitrogen is associated with low starch content, while high carbon is associated with high starch content. This result was observed in rice (Uprety *et al.*, 2010). Both nitrogen and carbon are needed by plants for normal growth and carbon dioxide respectively (Thitisaksakul *et al.*, 2012).

Drought and low temperatures have also been found to have an effect on crop development and growth. Drought is considered one of the major problems especially in sub-tropical climates (Hossain *et al.*, 2012). It stimulates *GBSSI* activity which gives rise to production of starch with high amylose. Moreover, it has been proposed that cold temperature also causes reduction in AGPase and starch branching enzymes activity (Thitisaksakul *et al.*, 2012).

High salt concentration in the soil led to plant toxicity as a result of osmotic stress. Generally, high salt causes reductions in amylose content, although this effect is greatly dependent on the genotype of the cereal crops (He *et al.*, 2012). The effect of acid stress also been reported in rice and the available data indicated that there was diminishing of debranching enzymes, α -amylase and β -amylase (Thitisaksakul *et al.*, 2012).

2.8 Malt quality

Malting process is refers to changes that occur in grain's endosperm. Starch is the major contributor for the success of this process. Nevertheless, other compounds such as organic substances and protein are also important in the process (Cook, 2013). A study was conducted on malted barley using the RVA to examine its functionality. The study reported that good quality malt form low and board peak compared to the poor quality malt (Visser, 2011).

It has been reported that barley has a good malting potential and that was attributed to the three celled aleurone layer that ensure extensive and uniform modification of the starch endosperm. However, variation in the pattern and extent of modification is related to the variation in the amount , composition, properties and spatial distribution of protein in particular D hordein (Brennan *et al.*, 1997).

2.9 Techniques and equipment used to examine barley starch / flour malting quality

There are several techniques that can be used to determine starch functional properties such as gelatinisation, amylose to amylopectin ratio, amylose contents and molecular fine structure of its components. As has been discussed above (section 2.5) any changes in the activity of starch biosynthetic enzymes might alter starch structure and contents, which would likely, affect the physico-chemical properties of the starch.

The Rapid ViscoAnalyser (RVA) is typically used to study starch pasting properties. An enzyme linked assay can be used to determine starch phosphate content, laser diffraction to determine granule particle

size distribution, scanning electron microscopy to examine granule morphology, the polymerase chain reaction to amplify and characterise the *SSI/a* gene and size exclusion chromatography to examine amylose – amylopectin structure and amylose contents.

2.8.1 Rapid ViscoAnalyser (RVA)

The RVA was developed by the Australian CSIRO wheat research Unit and Bread Research Institute. This equipment was initially invented as a tool to measure the extent of sprout damage in wheat (Deffenbaugh & Walker, 1989; Zhou & Mendham, 2005; Cozzolino *et al.*, 2012).

RVA analysis allows the identification of physical properties of a sample, which is useful in determining its industrial utilisation. For example, examining the microstructure of starch pastes has been essential for gaining understanding of the relationship between chemical composition and pasting properties of starch (Singh *et al.*, 2003b). The RVA has been used in breeding programs to determine the relationship between barley flour pasting properties and its potential malting quality (Cozzolino *et al.*, 2013; Zhou & Mendham, 2005; Cozzolino *et al.*, 2012).

By definition, RVA is a heating and cooling viscometer that measures the viscosity of a system over a given period of time as it is stirred (Gamel *et al.*, 2012). The temperature profile for RVA includes holding to 65°C, heating to 95°C, holding to 95°C, cooling to 50°C and, holding to 50°C. The time profile depends on pasting type, which can be short or long. The RVA viscometer measures system viscosity with changes in temperature and also provides readings and information on peak viscosity (PV; highest viscosity during heating), time to peak viscosity (TTPV), the time taken by a sample to reach peak viscosity, trough (T; lowest viscosity following peak viscosity), break down (BD; the difference between the peak and trough viscosities), final viscosity (FV; viscosity at the end of the pasting cycle) and set back (SB; final viscosity minus peak viscosity) (Figure 2.7) (Zhou *et al.*, 2007; Zhou & Mendham, 2005).

The values obtained from the RVA pasting curve using starch, depends on the botanical origin of the plant as well as environmental effect on plants such as temperature (Tester *et al.*, 2004a). The RVA is commonly used to determine pasting properties because it is easy to operate and has the ability to set a temperature profile. RVA is time - consuming compared to other viscometers such as Brabender (Deffenbaugh & Walker, 1989; Cozzolino *et al.*, 2012). However, the results obtained from the RVA were similar to those obtained using either an amylograph or an Ottawa starch viscometer (Zhou & Mendham, 2005).

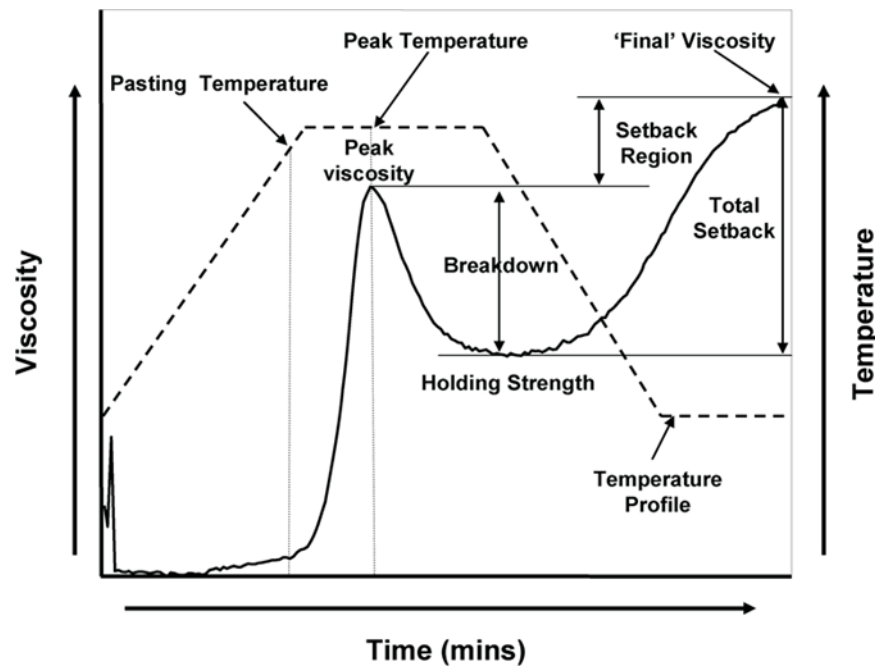


Figure 2.7 A typical RVA pasting profile showing different variables that are measured by the RVA (Saunders *et al.*, 2011).

2.8.2 Enzyme link assay for phosphate determination

Phosphate is the only naturally occurring covalent modification of starch. Its level affects the physical properties of the starch such as paste stability and viscosity (Singh *et al.*, 2003b; Jobling, 2004). It is detected using standard enzymatic assay which is based on acid hydrolysis of starch to its constituent monomers followed by determination of glucose -6- phosphate (Nielsen *et al.*, 1994).

In principle, glycosidic linkages are hydrolysed in hot hydrochloric acid (HCl) and the amount of glucose-6-phosphate residues was analysed enzymatically by the glucose-6-phosphate dehydrogenase catalyzed reduction of nicotinamide adenine dinucleotide phosphate (NADP) (Bertoft, 2004; Carpenter *et al.*, 2012).

2.8.3 Laser diffraction

Laser light scattering technique is used for characterisation of macromolecules (Raeker *et al.*, 1998). It is a powerful technique which is based on scattering electromagnetic waves by particle (Chmelik *et al.*, 2001). This has been used in industries and also in different research areas for particle size measuring (Black *et al.*, 1996).

In principle, light of parallel laser beams is reflected at an angle dependent on the diameter and optical properties of granules. Small granules scatter laser beam of electromagnetic waves at larger angles than large granules (Narváez-González *et al.*, 2007; Chmelik *et al.*, 2001).

A convergent lens focuses the scattered light in a ring form on the detector, where the Fourier spectrum light energy distribution is recorded. The size distribution of particle is calculated according to a complex theory (Chmelik *et al.*, 2001) such as Mie or Fraunhofer theories.

2.8.4 Scanning electron microscopy (SEM)

SEM is commonly used to examine surface materials (Kaláb *et al.*, 1995). It uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimen. The signals that comes from electron sample interactions shows information about samples such as morphology, chemical composition and crystalline structure and arrangement of materials making up the sample (Swapp, 2012).

2.8.5 Size exclusion chromatography (SEC)

Chromatography techniques become one of the most powerful techniques for separating molecules based on a number of different properties including their size. It was invented in 1901 by a Russian scientist called Mikhail Tsvet (Anon, 2015b). SEC is a type of chromatography also known as Gel Permeation Chromatography (GPC). It is the most developed technology which is commonly used for separation of natural and synthetic polymers by hydrodynamic volume (V_h) (Castro *et al.*, 2005; Gaborieau *et al.*, 2008; Cave *et al.*, 2009) or the equivalent hydrodynamic radius (R_h) (Vilaplana & Gilbert, 2010a).

SEC is consist of a pump that push the solvent through the instrument injection port to introduce sample to column which hold the stationary phase. The instrument is equipped with detectors that detect the component as they leave the column. There is a software that monitor the different parts of the instruments and display the results (Anon, 2015a).

SEC control polymers separation is aqueous solution based on their sizes and shapes as they pass through structural pores material column packed with column packed of porous of known size. As the molecular size of the polymer decreases with respect to the pores size, the polymer penetrates into the pore and retarded on the column. High molecular weight polymers such as amylopectin are restarted less than smaller ones such as amylose and so elute first from columns followed by low molecular weight polymers (Mori & Barth, 2013).

The main components of SEC are the mobile and stationary phases. The column holds the stationary phase and the mobile phase carries the samples through where the mobile phase is a solvent and the stationary phase is a packing porous particle (Barth *et al.*, 1994). For starch analysis a water based or polar aprotic solvent such as Dimethyl sulphoxide (DMSO) is recommended as a mobile phase because it dissolves starch with minimal degradation (Vilaplana & Gilbert, 2010a).

Linear polymers such as debranched starch have a unique relation between V_h and molecular weight, however, this is not found with the branched polymers (Gous *et al.*, 2015). The SEC are normally coupled with a detector which provides useful information about different types of distribution of molecules (Castro *et al.*, 2005; Gidley *et al.*, 2010; Vilaplana & Gilbert, 2010a).

Common type of detectors that have been used are, differential refractive index (DRI), multi-angle laser light scattering (MALLS) and viscometry (Vilaplana & Gilbert, 2010a; Gous *et al.*, 2015). Above all, selection of column and mobile phase, column calibration and use of on line detectors are essential for successful separation (Barth *et al.*, 1994).

Amylose content for the ten cultivars was also measured after separation of the debranched starch by SEC. This is a standard method, according to International Standardization Organization, 2011, ISO 6647-2, for amylose content determination (Gilbert *et al.*, 2013). Amylose content is calculated after separation of debranched starch by SEC. This has been calculated as the ratio of area under the amylose branches curve to the ratio of overall amylose and amylopectin branches (Syahariza *et al.*, 2013; Vilaplana *et al.*, 2012).

2.8.6 Polymerase chain reaction (PCR)

PCR is defined as a biochemical technology used to amplify a single DNA sequence by multiple orders of magnitude, thereby generating thousands to millions copies of a specific sequence. In general, the concept behind PCR is amplification the amount of DNA. PCR comprises of three steps. These are usually repeated between 25 to 30 times resulting in exponential increased amounts of a particular sequence of interest (Wilson & Walker, 2010; Schochetman *et al.*, 1988; Bartlett & Stirling, 2003).

The PCR reaction mixture comprises of buffer, nucleotides (dNTP's), primers (forward and reverse), enzymes (DNA polymerase) and template (Erich, 1989; Schochetman *et al.*, 1988) alongside components that aid catalysis such as magnesium (Mg^{+}). The first step is template denaturation, whereby the bond between the two DNA strands is spilt at a high temperature of approximately 95°C. Secondly, the annealing step whereby, oligonucleotide primers which are complementary to the sequence of interest bind to the single stranded DNA. The annealing temperature varies depending on a number of factors such as the length of the primer and the composition of nucleotides within it. However, a typical annealing temperature ranges between 55°C and 65°C. Thirdly, the extension step whereby, the enzyme DNA polymerase adds di-nucleotide triphosphates (dNTP's) to the end of the primer that are complementary to the DNA template, thus creating an exact copy (Erich, 1989; Wilson & Walker, 2010).

The specificity of PCR products are usually analysed and detected by different methods including restriction enzyme digestion and separation by electrophoresis. PCR is considered as a powerful and

precise tool in alternating a particular template sequence. Use of the thermostable *Taq* polymerase simplified the PCR procedure and increased the specificity and the overall yield of the reaction by reducing competition by non-target products for enzyme and primer (Erich, 1989).

However, problems such as temperature might affect the PCR reaction, thus the precise temperature has to be defined and optimised (Wilson & Walker, 2010). In addition, contamination of the sample reaction either with products of a previous reaction or with material from an exogenous source is also a potential problem (Erich, 1989).

2.9 Data analysis

2.9.1 Principal component analysis (PCA)

This is a multivariate technique used for data analysis where observations are described by several inter-correlated quantitative dependent variables (Abdi & Williams, 2010). The main idea of PCA is to minimize the dimension of data of a large number of interrelated variable, while retaining as much as possible the variation present in the data set (Iezzoni & Pritts, 1991). PCA can be applied to any data matrix and it is also recommended as an initial step of any multivariate analysis to obtain a first look at the structure of the data (Wold *et al.*, 1987).

2.9.2 Geneious

The Geneious program is a bioinformatics tool designed to be an easy and flexible desktop software application framework for the organisation and analysis of biological data with focus on molecular sequence and related data types. It provides modules to enable the visualisation, manipulation and transfer of DNA sequences (Kearse *et al.*, 2012). Geneious has a modular design so that additional features can be added as plugins. Also the software had been written in Java which allows it to be used on all major computers (Masters *et al.*, 2011).

2.10 Conclusions

Studies in genetics and biochemistry support the hypothesis that starch biosynthesis is achieved by coordinated action of multiple genes and each gene has a unique role in the starch biosynthetic pathway (Tetlow *et al.*, 2004b). Starch biosynthetic enzymes are affected by several environmental factors such as heat, cold, drought, salinity, acidity, carbon dioxide and nitrogen. These factors might affect their activities which could lead to change in physico-chemical properties of starch (Thitisaksakul *et al.*, 2012).

Genetic variations of starch biosynthetic genes may occur and these might have positive impact on brew house performance. For example mutation of starch synthase IIa gene is responsible for starch with high amylose content in barley and low gelatinisation temperature (Morell *et al.*, 2003) which is good for brew house performance (Fox *et al.*, 2003b). The physico-chemical changes and genetic variations (SNPs) could possibly be an indicator of malt and barley quality, therefore understanding the

genetic variation in relation to the physical properties within barley cultivars which could help plant breeders to determine strategies to improve barley plants. Improvement of the barley plant will bring about an improvement in the quality of the end uses (Tester, 1997).

Chapter 3 Hypotheses, aim and objectives

3.1 Research hypotheses

- Ho: Single Nucleotide Polymorphisms (SNPs) of starch synthase II gene (*SSIIa*) affect barley quality
- Ha: Single Nucleotide Polymorphisms (SNPs) of starch synthase II gene (*SSIIa*) gene does not affect barley quality

3.2 Research aim

- To characterise starch / flour from South African barley cultivars for malting quality

3.3 Research objectives

1. To study barley flour pasting properties using the Rapid ViscoAnalyser (RVA)
2. To determine amylose content using size exclusion chromatography (SEC)
3. To determine phosphate contents spectrophotometrically using an enzyme linked assay
4. To examine starch morphology using scanning electron microscopy (SEM)
5. To determine starch granule particle size distribution using laser light diffraction
6. To examine amylose and amylopectin molecular structure using (SEC)
7. To identify SNPs in SSII gene using allele specific amplification assays

Chapter 4 Materials and Methods

4.1 Experimental design

Ten barley samples were obtained and analysed. Moisture content and the RVA analysis were carried out in triplicate. Phosphate content was carried in four replicates. Eight PCR reactions were performed for each sample, starch morphology was examined using scanning electron microscopy. Particle size distribution was carried out in duplicate and starch molecular structural analysis and amylopectin were carried out in duplicate.

4.2 Materials

Barley seeds and leaves of 10 barley samples were provided by the South Africa Barley Breeding Institute (SABBI) including the following cultivars: Metcalfe, Cocktail, Marthe, Cristalia, Erica, Nemesia, Disa, Houwink, Baudin and Henrike. Metcalfe is imported from Canada, Cocktail, Marthe and Cristalia are introduction from Europe but are grown in Northern Cape under irrigation, Erica, Nemesia, Disa and Houwink are grown in Southern Cape. These cultivars were received in September 2013.

4.2.1 *Sample preparation*

4.2.1.1 *Milling*

The ten barley cultivars were ground in a centrifugal mill (Retsch Model Zml, Haan, Germany) fitted with 0.5 mm size sieve.

4.2.1.2 *Storage of biological material*

Leaves from each cultivar were put in a plastic bag and stored at -20°C.

4.3 Starch isolation

Twenty five grams of mature grain from each cultivar was weighed, followed by grinding in a coffee grinder which were lightly damage to remove the outer layer. The grinds were steeped in a small volume of water on ice for two hours.

The steeped grinds were ground using the manual grinder to release a starch suspension, which was passed through a 75 µm sieve. The step was repeated and more water was added to the sieve to wash through the remaining starch granules. The suspension was centrifuged at 10 000 g for 15 min at 4⁰ C. Supernatant and sediment (protein) on top of starch was removed with a spatula and discarded.

The pellet was suspended in 2% (w/v) sodium dodecyl sulphate (SDS) at room temperature. The starch suspension was centrifuged at 10 000 g for 10 min at 20⁰ C before the supernatant and the protein were discarded. This step was repeated twice after which the pellet was transferred to 50 mL tubes and re-suspended in small volume of acetone pre-chilled at 20⁰ C and was again centrifuged at

1000 g for 10 min. The acetone was carefully aspirated and the starch recovered. The step was repeated twice followed by rapidly air-drying of the starch and storage at -20°C (Tester & Morrison, 1990).

4.4 Moisture content determination

Moisture contents for the ten cultivars were determined as described earlier (Emvula, 2012). The moisture dishes with the lids were washed and dried in a vacuum oven (Heraeus Model RVT 360, Henau, Germany) at 130°C for 30 min followed by cooling in a desiccator for 40min.

After cooling, the containers and the lids were weighed and recorded as (W1). About 2 grams of barley flour was weighed and transferred to the moisture dishes, which were weighed as (W2) before heating. The moisture dishes with the samples covered with lids were placed in the vacuum at 130°C for 60 min. Moisture dish, lid and sample were weighed as (W3) after heating.

The heated moisture dishes were placed into the desiccator to cool for 45 min. After cooling, the moisture dishes with the dried samples were weighed and recorded as W3. The moisture contents were determined by calculating the differences in weight lost which is expressed as percentages of the initial sample weight, the experiment was carried in triplicate.

Moisture content equation

$$\text{Equation 1 : \% Moisture} = (W2 - W3 / W2 - W3) \times 100$$

Where by:

W1 = weight of moisture dish and lids

W2 = weight of moisture dish, lid, and sample before drying

W3 = weight of moisture dish, lid, and sample after drying

4.5 Barley flour pasting properties

The flour pasting properties were determined by the Rapid ViscoAnalyser (RVA) model 4500 (Perten Instruments, Australia) according to American Association for cereal Chemist (AACC) international Method ISO 76-21.01. The Thermocline software was used to record data from the temperature standard profile. Barley flour (3.5 g) was used and the final weight was adjusted to 28 g by addition of water that was calculated based on the moisture contents for each cultivar based on a 14% moisture basis. The standard profile used was as follows; stirring initially at 960 rpm at 50°C with the stirring frequency being reduced to 160 rpm for the rest of period. The temperature was increased for 50°C to 95°C for 4 min 30 sec and was then held at the temperature for 7 min 30 sec, followed by cooling to 50°C for 11min. The test end after 13 min and the viscosity recorded every 4 sec.

4.6 Covalent phosphate determination

One hundred and twenty-five milligrams of the isolated starch was weighed, followed by addition of 0.5 mL of 0.7 M HCl. The mixture was heated at 100°C for 4 h after which 100 µL was transferred to a microcentrifuge tube and 100 µL of M KOH was added.

30 µL of the neutralized solution was added into a microtitre well, followed by addition of 230 µL assay buffer. The composition of the assay buffer was as follows, 100 mM MOPS pH 7.5, 10 mM MgCl₂, 2 mM EDTA, and 2 mM NAD. The reaction started by adding Glc-6-P dehydrogenase from *Leuconostoc* (Nielsen *et al.*, 1994). The samples were measured at 340 nm absorbance using spectrophotometry.

$$\text{The equation used for G-6-phosphate calculation} = \frac{\Delta \text{OD} \times 0.28}{6.22}$$

Where by ΔOD = Changes in the absorbance

Volume= volume of the microtitre well

6.22 = Molar absorption coefficient of NADH (mol cm⁻¹)

4.3.5 Starch granule particle size determination

Granule size distributions were determined according to the International Standardization Method ISO 13320 for particle size analysis. Starch from ten barley cultivars were measured in aqueous (water) suspension using Laser diffraction, Saturn Digsizer 5200 v1.10. The samples were passed through a beam of a monochromatic light source (Laser). The light scattered by the particle at various angles which is measured by detectors. The numerical values relating the scattering pattern were recorded and analysed using Mie theory. The conditions of measurement were; flow rate 12.01/m, circulation 60sec, ultrasonic intensity 50%, ultrasonic time 30 sec

4.3.6 Starch granule morphology

Small amounts of starch from each cultivar were sprinkled on different double stick tape fixed on aluminum and coated with gold palladium. After 3 minutes the samples were removed from coating machine and (Sodhi & Singh, 2003) were examined using a Scanning Electron Microscopy (SEM) - Leo 1430 VP (Zeiss, Jena, Germany).

4.3.7 Starch molecular structure

Ground barley seeds were mixed by inverting with 0.5 mL protease in tricine buffer (pH 7.5, 250 mM) and incubated at 37°C for 30 min in a 2 mL microcentrifuge tube. The mixture was centrifuged at 4000

g for 10 min. The supernatant was discarded and starch was suspended in 1.5 mL DMSO containing 0.5% (w/w) LiBr-DMSO-LiBr in a shaking thermomixer running at 80°C and centrifuged at 350 rpm for 20 h.

The suspension was mixed by inverting to ensure no clumps of the precipitate adhere to tube wall in the first 2 h. The solubilised starch was precipitated by washing with 10 μ L absolute ethanol and centrifuge at 4000 x g for 10 min to remove non-starch component. The precipitated starch was dispersed in 0.9 mL hot water and heated in a boiling water bath until all precipitated was dispersed roughly 10 min.

One hundred microliters acetate buffer (pH 3.5, 0.1 mM) and 2.5 μ L isoamylase from *Pseudomonas* species from (Megazyme, Wicklow, Ireland) were added and the starch dispersion was cooled to room temperature. This mixture was vortexed and incubated at 37°C for 3 h. The resulting was debranched starch dispersion which was adjusted to pH~7 with 0.1mL NaOH. The dispersion was heated at 80°C for 1 h (Wu *et al.*, 2014).

The debranched fractions were performed using combined GRAM pre-column 100 and 1000 analytical columns (PSS) with a flow rate of 0.6 mL min⁻¹ DMSO/ LiBr 0.5% w/w at 80°C (Vilaplana & Gilbert, 2010b). SEC was calibrated using Mark-Houwink parameters for linear starch and pullulan standard in DMSO/LiBr at 80°C. It has been reported that Universal calibration assign a true hydrodynamic volume to a given sample eluting at a given elution volume. Mark-Houwink parameters related intrinsic viscosity or hydrodynamic volume and degree of polymerization (DP) of a linear standard such as Pullulan to intrinsic viscosity or hydrodynamic volume and DP of a second linear polymer such as starch (Vilaplana & Gilbert, 2010b).

The calibration was performed by injection of pullulan standards with molecular weight ranging from 342 to 1.66 $\times 10^6$ gmol⁻¹. The Mark-Houwink parameters for pullulan in DMSO/LiBr (0.5 weight %) at 80°C $K = 2.427 \times 10^{-4} \text{ dl g}^{-1}$ and $\alpha = 0.6804$ and the results were analysed using dn/dc of 0.0853 mL/g⁻¹ (Vilaplana & Gilbert, 2010b).

Differential Refractive Index (DRI) detector gave SEC weight distribution of the linear glucans as a function as $(\log v_h)$, $W_{de}(\log v_h)$. It was converted to a function of $\log x$, $N_{de}(\log x)$ using Mark-Houwink parameters $K = 0.015 \text{ dl g}^{-1}$ and $\alpha = 0.74$ for de-branched starch. $N_{de}(x)$ and $W_{de}(\log X)$ were related by $W_{de}(\log x) = X^{2N_{de}(X)}$ (Wu *et al.*, 2014).

All SEC molecular size distributions were normalized and the idea for normalisation was that the area under each de-branched SEC weight distribution for each elution volume slice is proportional to relative amount of starch in that slice (Vilaplana & Gilbert, 2011). Also it has been reported that the normalisation is done in order to yield the height of the highest peak for comparison among different samples (Syahariza *et al.*, 2013)

The data recorded after the SEC separation and detection was processed using WinGPC software (PSS) and further analysis was performed to obtain the SEC eight distribution $w(\log v_h)$ and the size dependence of the weight average molecular weight $m_w(v_h)$ (Vilaplana & Gilbert, 2011).

4.3.8 DNA extraction

DNA was extracted using Gene JET plasmid Miniprep kit (Thermo Scientific). One gram of barley leaf was weighed whilst keeping the sample frozen in liquid nitrogen. The leaf was ground using a manual grinder in 350 μL of lysis buffer. The ground sample was transferred into 2 mL microcentrifuge tube, 50 μL of binding buffer and 20 μL of RNase were added followed by vortex for 1 min. The sample was incubated at 65°C for 10 min in shaking water bath.

Thirty microliters of precipitation solution was added and the solution was mixed by inverting for 2-3 min, followed by incubation in ice for 5 min. Then sample was centrifuged for 5 min at 20 000Xg. The supernatant of approximately 450-550 μL was transferred through a silica column into clean microcentrifuge tube. The bound DNA was then washed by buffer and lastly the purified DNA was eluted using 400 μL elution buffer. The DNA concentrations were measured.

4.3.9 SSII gene amplification

PCR reactions for DNA samples were performed using PCR, allele specific amplification for SNP detection. Eight primers sets were synthesised and used to amplify part of SSII gene from barley. From eight primers sets, four amplified DNA with the expected fragment size, which is shown in (Table 4.1) The PCR was run on 1% (w/v) agarose TAE gel, 5 μL of sample + 1 μL of loading dye, 6 μL of 1kb molecular ladder and was run at 80 volts for 90 min.

Table 4.1 Four pair of primers used to amplify different parts of the SSII gene

| Forward primer (sequence) | Reverse primer | Product size |
|-----------------------------------|----------------------------------|--------------|
| 5'GAGTAAGAACGGCACGCTGATC3' | 5'GGATCCAAATGAGTGGATCTACTCTC3' | 1055 |
| 5'CTGCTATATAATAGTACTGCCTCCATTCC3' | 5'GTTTCTACAGAATAATTGGGATGACTGC3' | 1059 |
| 5'GGGGTTTGGTTAGAGCGACC3' | 5'GAGTGCCGTGTGCCAATC3' | 1096 |
| 5'CATGTGGTGACTGCACGC3' | 5'GCTGCAGCATGCTCTCCAG3' | 1112 |

Table 4.2 PCR mixture preparation

| Reagents | Volume, uL | Concentration | Master Mix, uL |
|---------------------|------------|----------------|----------------|
| Water | 21.75 | - | 935.25 |
| 2.5mM dNTPs | 2 | 2.5 mM | 86 |
| 10x Buffer | 3 | 1x | 129 |
| ITAQ DNA polymerase | 0.25 | - | 10.75 |
| Forward primers | 1 | 10 mM | - |
| Reverse primers | 1 | 10 mM | - |
| Template DNA | 1 | 10 ng/ μ l | - |

Table 4.3 PCR reaction Condition

| PCR variables | Temperature setting | time |
|-----------------------|---------------------|----------|
| Initial | 94°C | 2:00 min |
| Denaturation | 94°C | 0:30 sec |
| Annealing temperature | 64°C | 0:30 sec |
| Extension | 72°C | 1:00 min |
| Final extension | 72°C | 10 min |
| 34 cycle | | |

4.5 Data analysis

4.5.1 Principle component analysis (PCA)

PCA biplot were used to relate pasting properties with different cultivars. A biplot is a combination of the PCA scores plot and loadings. Multiple factors analysis (MFA) was performed to relate different blocks of variables with one another. Correlation circle graphs were used to graphically show relationships between the blocks of variables, and individual factors maps (similar to scores plots in a PCA) was used to relate groupings of cultivars.

4.5.2 Geneious

PCR products were sent to Australian Genome Research Facility Ltd for sequencing. All the sequencing results were analysed using Geneious software, version 6.1.8 (Drummond *et al.*, 2011).

Chapter 5 Results

Table 5.1 Shows Quality traits measured to examine malting quality of barley flour / starch. These Traits are amylose contents, phosphate contents, granule particle size and degree of polymerisation

| Cultivar | Amylose content, % | Phosphate content, % | Granule size, μm | DP |
|-----------|--------------------|----------------------|-----------------------------|-------|
| Metcalfe | 24.4 | 0.4 | 18.62 | 4,330 |
| Cocktail | 25.0 | 0.3 | 17.8 | 3,440 |
| Marthe | 25.3 | 0.6 | 16.7 | 3,420 |
| Cristalia | 24.9 | 0.3 | 16.5 | 3,450 |
| Erica | 25.3 | 0.3 | 16.7 | 3,770 |
| Nemesia | 25.5 | 0.5 | 16.0 | 3,540 |
| Disa | 24.4 | 0.3 | 17.4 | 3,500 |
| Houwink | 23.8 | 0.9 | 16.4 | 3,500 |
| Baudin | 23.9 | 0.4 | 15.9 | 3,500 |
| Henrike | 24.7 | 0.2 | 16.1 | 3,680 |

Table 5.2 Amylopectin and amylose in three fractions regions AP1, AP2 and AP3 of the debranched barley starch measured by SEC

| Cultivar | Zone AP1 (Xde 5-30) Xde,w | Zone AP2 (Xe 30-100) Xde, w | Zone AM 3 (Xde \geq 100) Xde,w |
|-----------|---------------------------|-----------------------------|----------------------------------|
| Metcalfe | 18 | 37 | 1.64×10^3 |
| Cocktail | 18 | 37 | 1.28×10^3 |
| Marthe | 18 | 37 | 1.26×10^3 |
| Cristalia | 18 | 37 | 1.29×10^3 |
| Erica | 18 | 37 | 1.39×10^3 |
| Nemesia | 18 | 37 | 1.30×10^3 |
| Disa | 18 | 37 | 1.32×10^3 |
| Houwink | 18 | 37 | 1.27×10^3 |
| Baudin | 18 | 37 | 1.34×10^3 |
| Henrike | 18 | 37 | 1.38×10^3 |

5.1 Barley flour pasting properties

Figure 5.1. Shows pasting properties viscogram for the ten cultivars. Cocktail, Marthe, Cristalia, Erica, Nemesia, Disa and Houwink demonstrated very high in peak viscosity, setback, final viscosity, peak time and trough and that is shown by close association with these variables compared to Metcalfe, Baudin and Henrike.

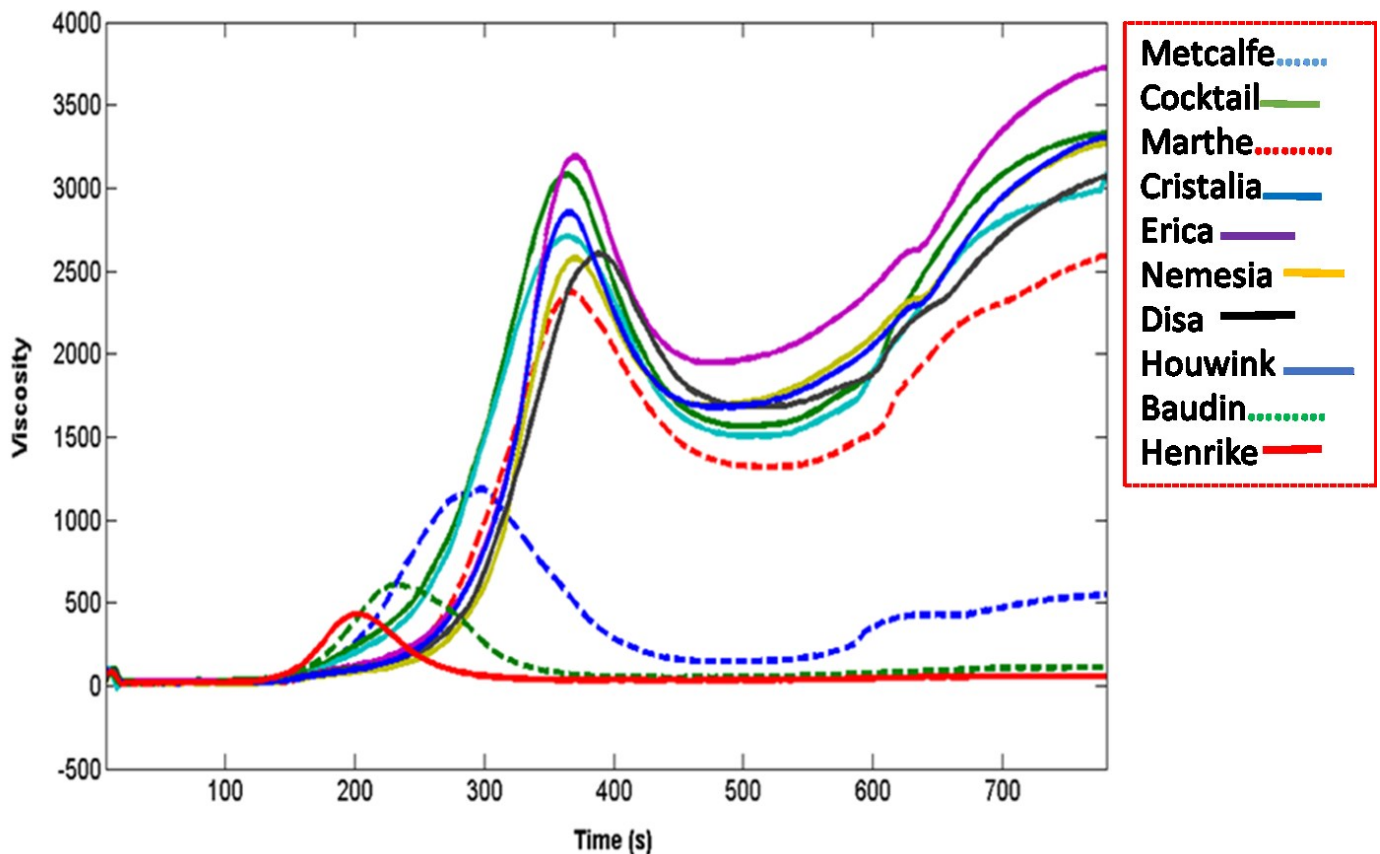


Figure 5.1 Pasting properties viscogram of the ten barley cultivars measured using a Rapid ViscoAnalyser (RVA).

5.2 Analysis on functionality of barley starch / flour

Figure 5.2 illustrates the correlation circle graphs which graphically show relation between the blocks of variables, pasting properties, amylose content, covalent phosphate, granule size distribution and degree of polymerisation. The RVA parameters cluster to one end of dimension while the DP and granule sizes are in the opposite direction.

5.3 Analysis on functionality of barley starch/ flour

Figure 5.3 shows correlation between the ten cultivars and the quality traits measured. Cultivar (8) Houwink, Nemesia (6), Marthe (3), Disa (7), Erica (5) cocktail (2) and Cristalia (4) are towards one dimension whereas, Metcalfe (1), Baudin (9) and Henrike (10). PC1 is 59% and PC 2 17%.

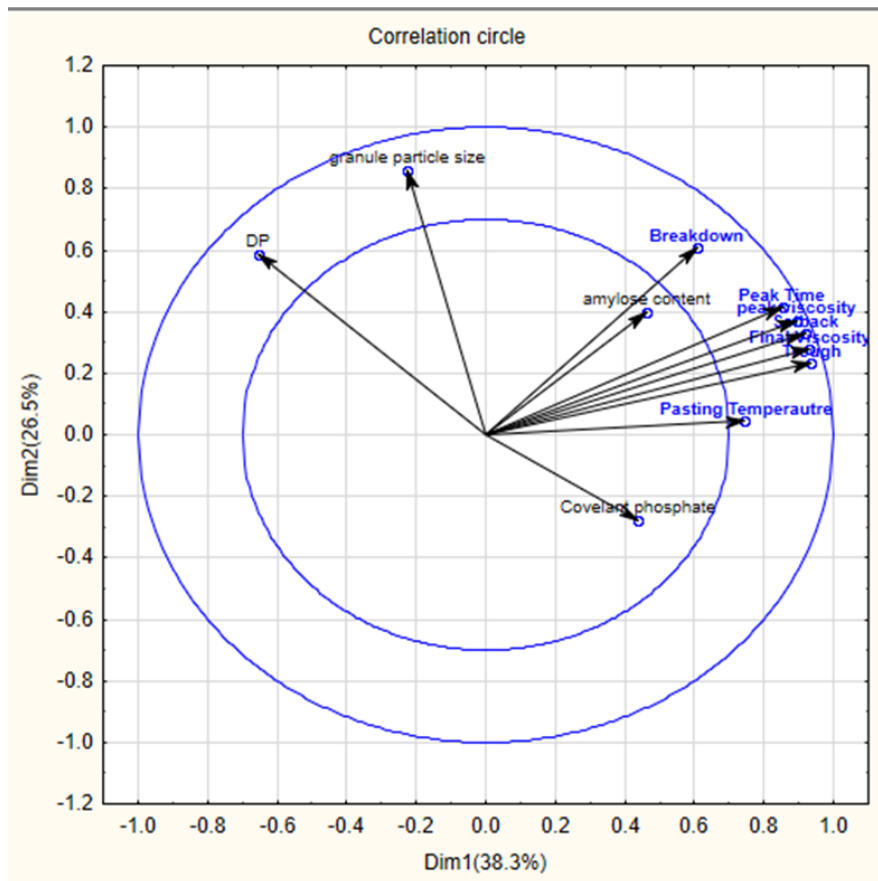


Figure 5.2 Correlation circle graphs indicating correlation between blocks of variables.

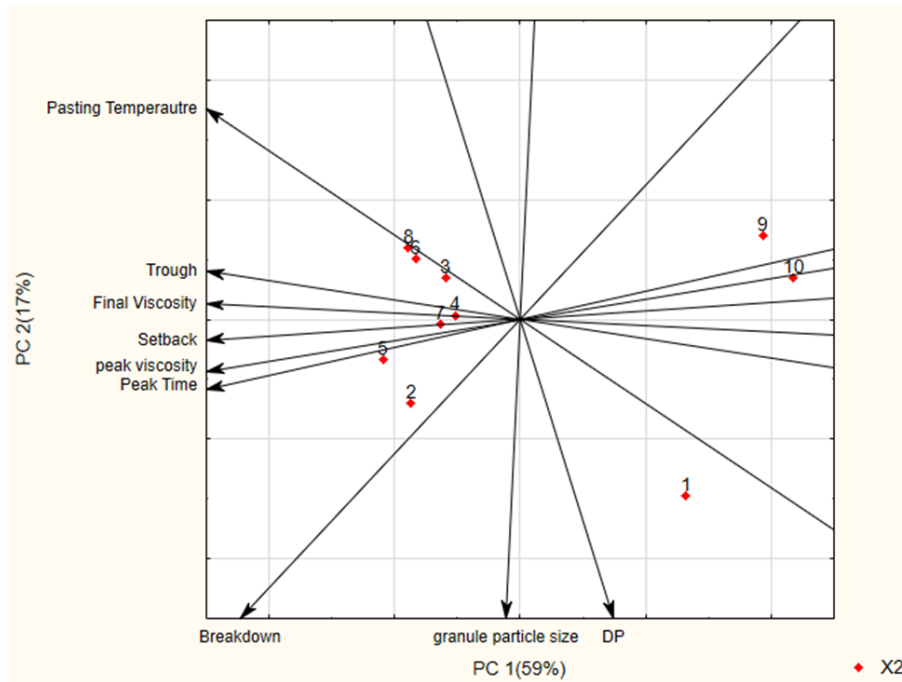


Figure 5.3 PCA biplot indicating correlation between blocks of variables and the cultivars.

5.4 Individual factor map for the ten cultivars

Figure 5.4 shows grouping of cultivars with regard to all the parameters measured (Pasting properties, amylose and phosphate content, granule size and degree of polymerisation). This demonstrates that some of the cultivars share common characteristics. Specifically (1) Metcalfe seems to be different from the rest of the cultivars. (2) Cocktail and (5) Erica correlate well, (3) Marthe and (6) Nemesia also showed positive correlation. However, it seems there is a trait in each cultivar that makes it different from the rest of the cultivars.

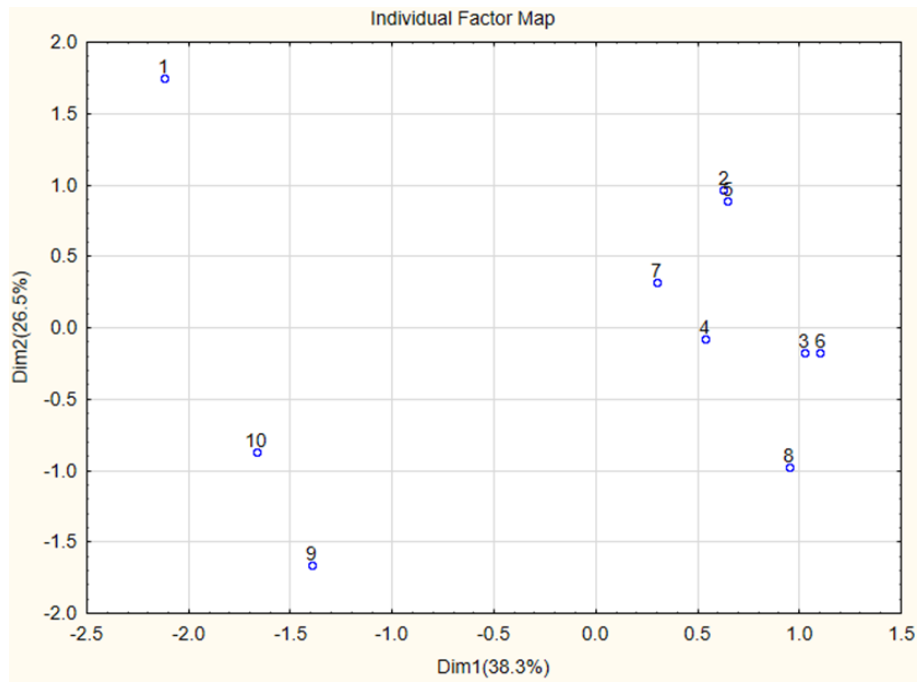
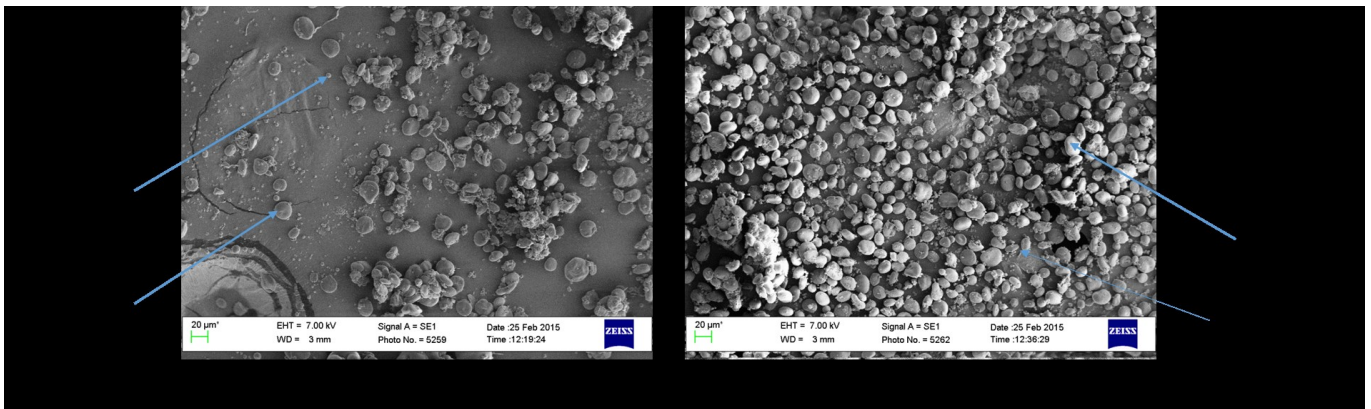


Figure 5.4 Individual factor map for the ten cultivars with regard to the traits previously measured.

5.5 Starch granule morphology

Figure 5.5 shows granule morphology for the ten barley cultivars. All cultivars showed two types of granules large A type granules and small B type granules. Lenticular and spherical shapes were also observed.



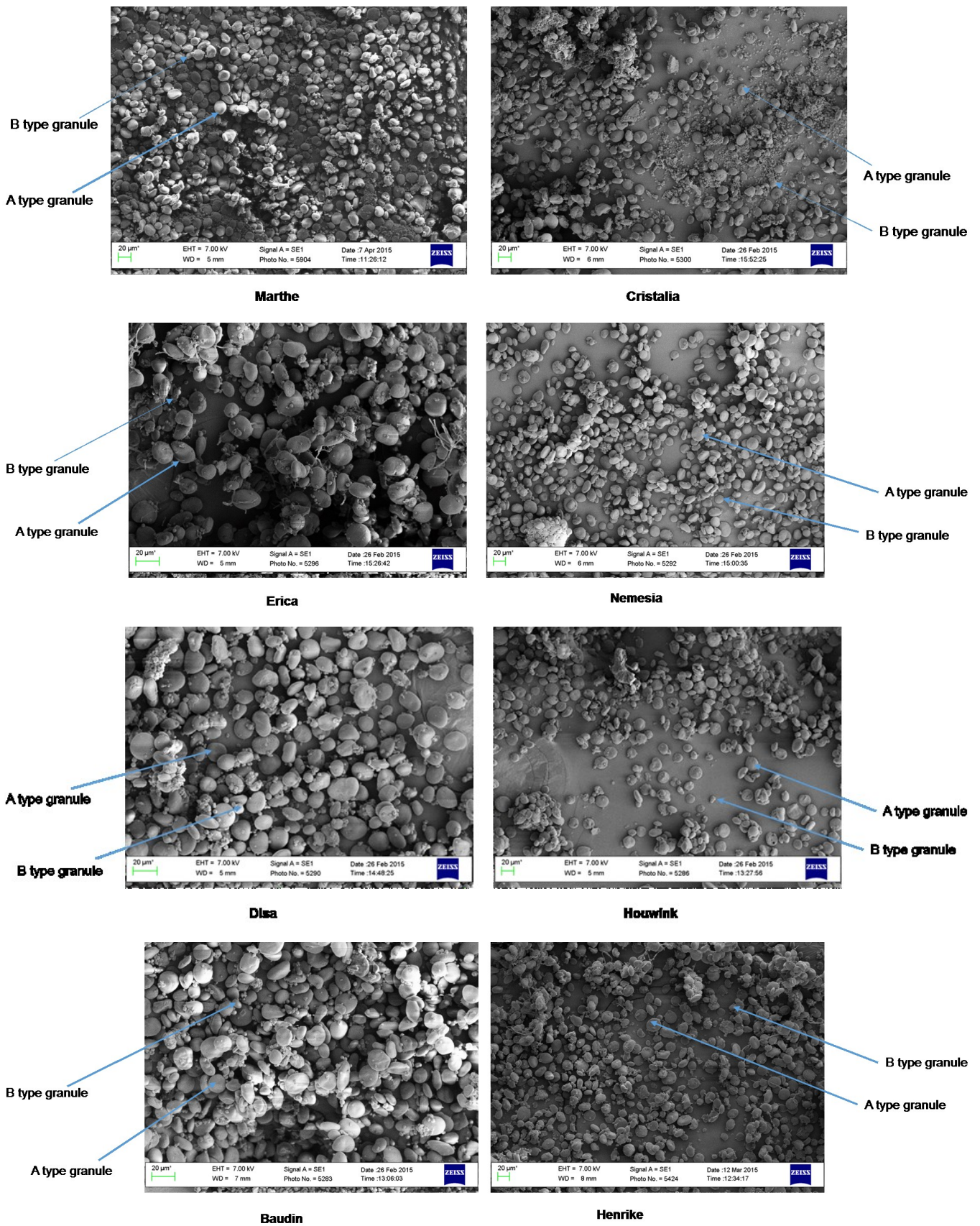


Figure 5.5 Granule morphology examination for starches from the ten barley starches of the ten barley cultivars examined under scanning electron microscopy (SEM).

5.6 Analysis of chain length distribution of debranched barley starch separated by SEC

Figure 5.6 shows debranched barley starch fine molecular analysis. Structural variation was observed in the chains among the ten cultivars. Most cultivars showed chain length distribution (CLD) for amylopectin between ~ 5 and 100 and amylose between 100 and 10 000. Long chains of amylose and amylopectin were observed at the ends of the chains.

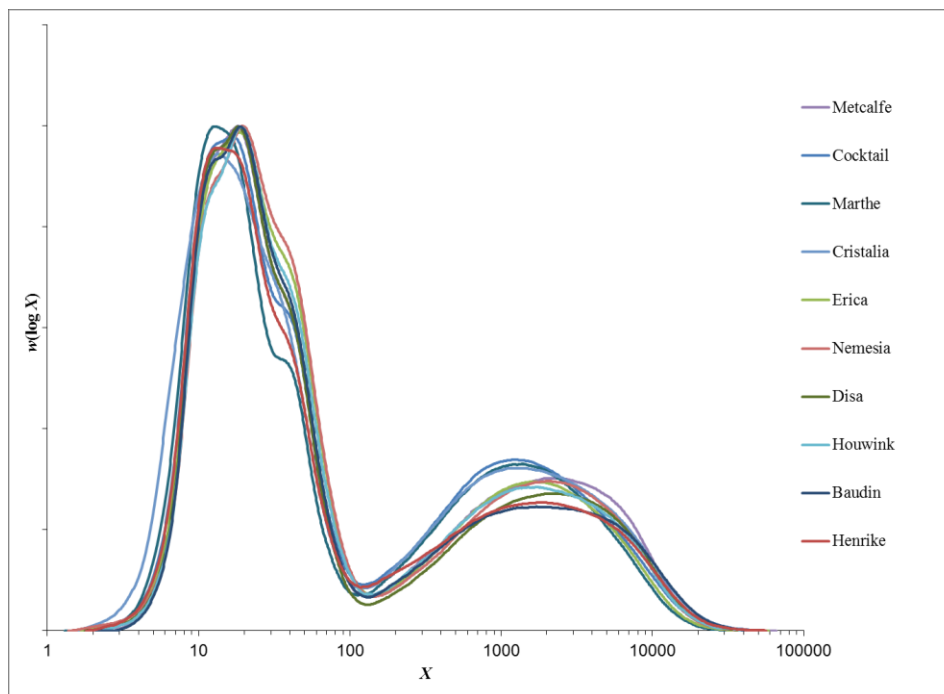


Figure 5.6 Debranched barley starch samples were separated by SEC. The weight chain length distribution (CLD) was plotted as logarithm of the number distribution $N(X)$, as a function of degree of polymerisation for the ten barley cultivars.

5.7 SSII gene identification

The *SSII* gene was amplified in segments using the polymerase chain reaction. Eight sets of primers were tested, but only four gave the expected fragments with most of the cultivars. Bands 2, 3, 4 and 7 are well amplified. Bands 1, 5 and 8 did not amplify well with the ten cultivars. Band 6 showed multiple bands with all cultivars. The expected size for the fragments for band 2, 3, 4 and 7 are **1055 bp**, **1059bp**, **1096bp** and **1112bp** respectively.

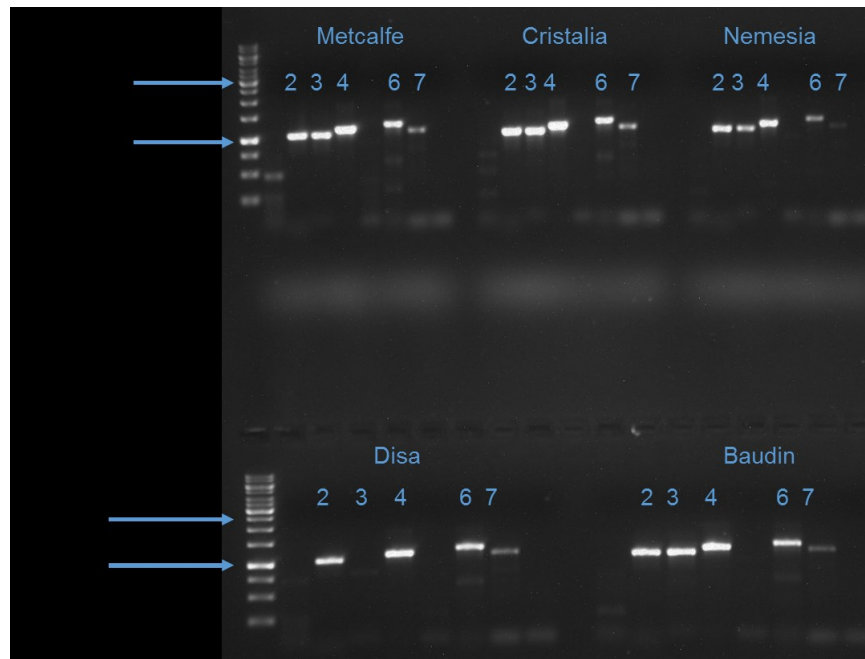


Figure 5.7 eight primers tested on DNA from Metcalfe, Cristalia, Nemesia, Disa and Baudin to identify the SSII gene.

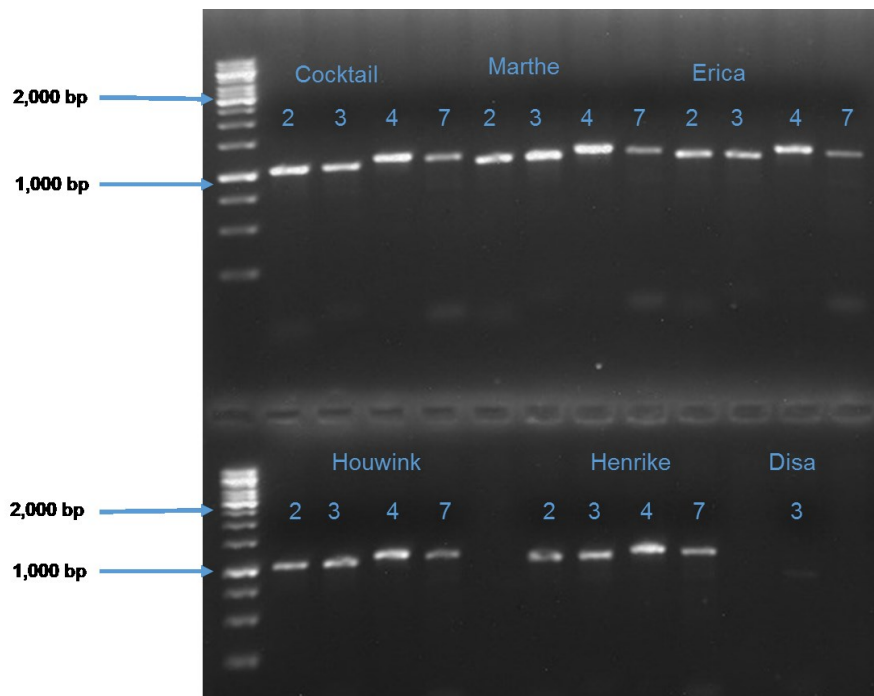


Figure 5.8 Primers 2, 3, 4 and 7 tested on Cocktail, Marthe, Erica, Houwink and Henrike. PCR conditions and primer 3 combination were changed for Disa.

5.8 Sequencing of SSII

Bands 2, 3, 4 and 7 from the PCR amplification Figure 5.5 and 5.6 for the ten cultivars were submitted for PDT (pedigree equilibrium test) sequencing. PDT is a program that allows test of linkage and association in data. Four fragments were mapped to SSII gene for *Hordeum vulgare L.* The sequencing results were the same in the ten cultivars. Figure 5.7 shows part of the sequencing results (Exon 5) to indicate the SNPs found. There is a change in the fourth amino acid in exon 5 from Leucine in reference to proline in the ten cultivars.

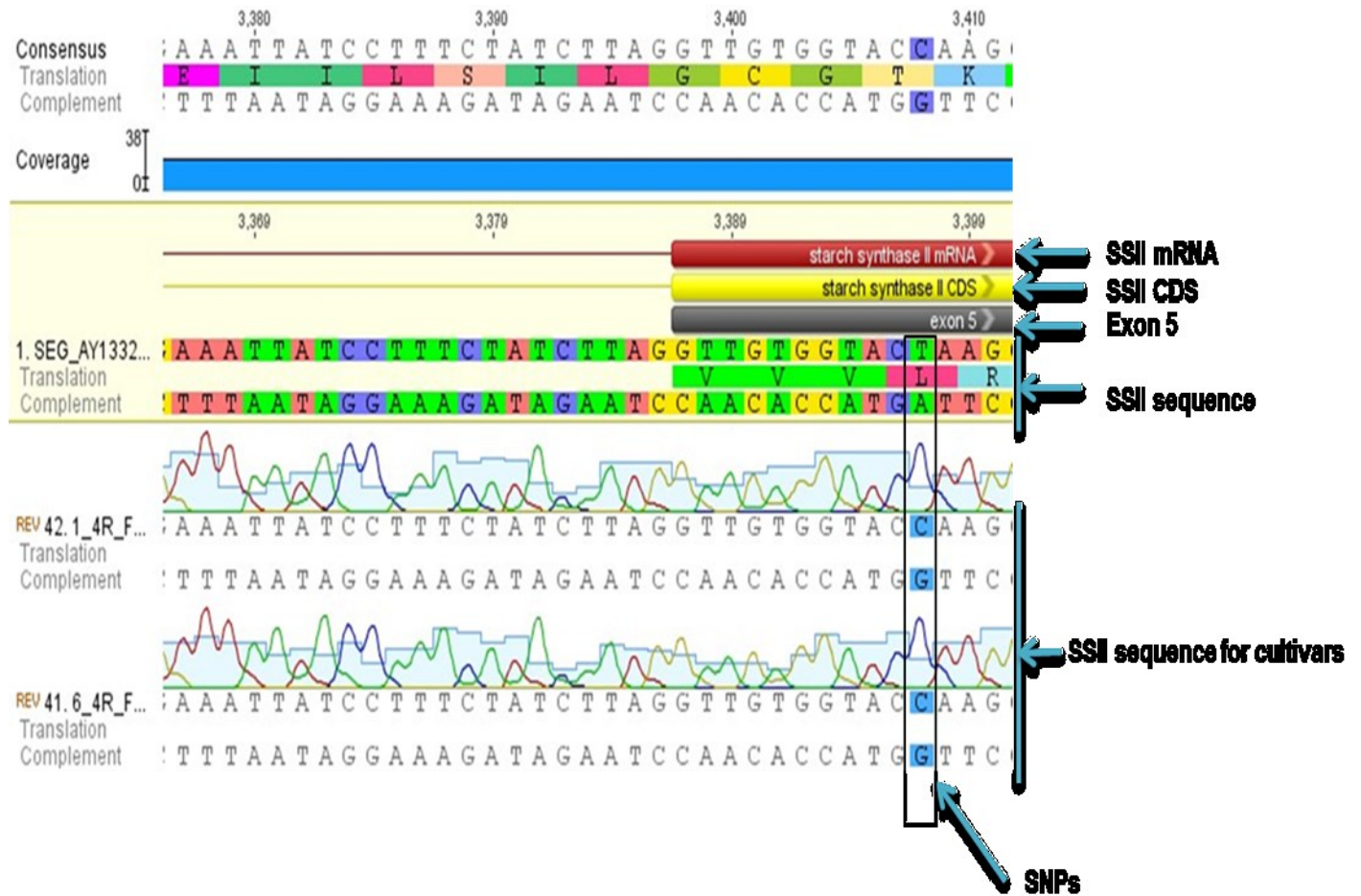


Figure 5.9 Pedigree Disequilibrium Test (PDT) sequence for SSII sequence result.

Chapter 6 Discussion

6.1 Use of barley flour vs starch

Barley flour was used to examine pasting properties, amylose and amylopectin and starch fine molecular structure. Purified starch was used for determination of phosphate contents, granule particle size distribution and starch granule morphology. DNA was extracted from the leaves. The flour used was produced by hammer milling fitted with a 0.5 µm sieve size and the purified starch was extracted as described by Morrison and Tester (1997).

It has been reported that milling techniques that are commonly used to produce flour from grain might cause damage to starch granules, therefore the functionality of the starch might be affected (Donald, 2004; Hasjim *et al.*, 2013). Common millings techniques that have been used are, ball milling, hammer milling and pin milling. It has been observed that in all these techniques, force is applied to break grain into fine particles sizes. However, the degree of damage differs from one technique to another (Lindeboom *et al.*, 2004; Li *et al.*, 2014a).

Recently, cryogenic mills have been preferred over the other types of mills such as hammer mills because it was reported that they produce less damage in the starch fine structure. On the other hand, both techniques cryogenic and hammer mill were reported to have an effect on the starch structure (Hasjim *et al.*, 2013).

Hammer mills are commonly used in cereal research (Visser, 2011; Guelpa *et al.*, 2015) because they are easy to operate and maintain (Al-Rabadi *et al.*, 2012). Because of that cryogenic mills are not widely used in industry mainly in research environments (Li *et al.*, 2014a). For this reason a hammer mill was used in this study instead of a cryogenic mill.

With respect to pasting properties, barley flour has been reported to be closely related to malting quality. In particular, pasting temperature shows significant correlation with malt extract (Zhou & Mendham, 2005; Zhou *et al.*, 2008). It has been reported also that there is a significant correlation between pasting properties of wheat flour and wheat starch (Blazek & Copeland, 2008). Use of flour produced by a hammer mill for starch characterisation is, therefore, still relevant in cereal research as the use of flour to study starch functionality would be more practical in a breeding program (Zhou & Mendham, 2005). As the focus of this study is to provide local breeders with information that would contribute in improvement of local barley plants flour, rather than starch, was used.

Amylose and amylopectin molecular structure was also examined using barley flour. Previously, a study was successfully done to understand the role of starch structure in the solubility and swelling power of rice flour (Hasjim *et al.*, 2012). Notably, the establishment of methods to determine the

amylose and amylopectin ratio from flour using SEC (Simsek *et al.*, 2013). The molecular weights of amylopectin and amylose from flour was also determined using SEC (Ovando-Martínez *et al.*, 2013).

6.2 Critical review of methodology

6.2.1. Pasting properties by the Rapid Visco Analyser (RVA)

Rheological methods play an important role in measuring cereals' physical properties which are related to their functionalities based on composition and structure (Panozzo & McCormick, 1993; Higley *et al.*, 2003). Pasting properties of starch was first measured by Caesar in 1932 using the Consistometer (Deffenbaugh & Walker, 1989).

Currently, the Rapid Visco Analyser (RVA) and Brabender Visco Amylograph are mostly used to measure paste viscosity (Singh *et al.*, 2003a; Singh *et al.*, 2003b). The first RVA did not have a computer that controls temperature, rather the temperature was increased manually using a thermometer (Hazelton & Walker, 1996). RVA developers recognised the problem which led them to improve the equipment by incorporating computer technology which resulted in more reproducible result in better versatility and more rapid measurements. In addition, development of the paddle has ensured homogeneity of the sample and optimal heat transfer (Hazelton & Walker, 1996). The RVA has several advantages over the Amylograph which include small sample size, ability to set a temperature profile and ability to report to a computer (Deffenbaugh & Walker, 1989; Zhou & Mendham, 2005).

Depending on the profile, RVA analysis can be completed within approximately 20 min. The instrument is easy to operate and it is versatile in terms of test procedures using different temperature/time profiles. The parameters measured by the RVA are similar to the other viscometers such as the Brabender-amylograph (Deffenbaugh & Walker, 1989; Ryu *et al.*, 1993). The RVA is economical, fast and a small amount of sample is needed (Zhou *et al.*, 2007). Analysis of the RVA viscograms with multivariate analysis such as PCA allows comparison of different traits between samples of different genotypes (Cozzolino *et al.*, 2012).

The RVA was used to study pasting properties of barley flour. All the necessary steps such as accurate measuring of water and sample using a sensitive balance, mixing of the slurry with paddle before using the equipment, accurate calculation of moisture contents using the software were all considered to make sure that correct results were obtained.

6.2.2 Amylose content determination by size exclusion chromatography (SEC)

Different methods are available for measuring amylose contents. These include colorimetry (iodine binding capacity), potentiometry (Zhu *et al.*, 2008; Gibson *et al.*, 1997; Yun & Matheson, 1990), and near infrared (NIR) spectroscopy (Gibson *et al.*, 1997). However, each of these methods has its limitations.

Colorimetry is not accurate because the iodine binds to the amylopectin long chains that result in formation of amylopectin complex which absorb light at the same wavelength as amylose. A standard curve is also needed and it is generated by measuring starches from different sources in order to select a specific standard curve for amylose and that is always a challenge (Zhu *et al.*, 2008).

The potentiometric method is based on titration techniques and is time-consuming. The DSC (differential scanning calorimetry) requires a standard curve which is also a challenge (Zhu *et al.*, 2008). NIR spectroscopy is rapid but it requires a reference method for calibration (Gibson *et al.*, 1997).

An amylose-amylopectin assay was developed by Megazyme as an alternative approach for measuring amylose contents. It is based on precipitation of amylopectin with the lectin Concanavalin A (Gibson *et al.*, 1997). However, it has been reported that underestimation of amylose contents could be a problem (Vilaplana *et al.*, 2012; Ovando-Martínez *et al.*, 2013).

Amylose contents was determined by SEC according to International Standardization Organisation, 2011, ISO 6647-2 (Vilaplana *et al.*, 2012). It has been explored that determination of amylose contents after separation of a polymers by SEC can be promising (Zhu *et al.*, 2008). This method is based on calculation of the ratio of the area under the curve (AUC) of amylose branches to overall AUC of amylose and amylopectin branches (Vilaplana *et al.*, 2012). Although this analysis is costly and time consuming, it is promising and good results can be obtained (personal communication; Glen Fox, 2015, The University of Queensland, Brisbane, Australia, 20 May 2015).

6.2.3 Covalent phosphate determination by standard enzymatic assay

Glucose-6-phosphate (Glc-6-P) and Glucose-3-phosphate (Glc-3-P) have been separated by high performance anion exchange chromatography (HPAEC) but estimation of phosphate contents from these components was difficult because of degradation (Bertoft, 2004).

Other methods include ^{31}P -nuclear magnetic resonance (P-NMR) spectroscopy for measuring of esterified phosphorous contents and chemical analysis of the total starch bound phosphorous (Bertoft, 2004). P-NMR spectroscopy was also recommended for determination of starch phosphate contents as it can determine the amounts bound at both the C6 and C3 positions (Jane, 2009), however it is relatively insensitive. Phosphate contents were measured by ashing the organic matter followed by measuring the inorganic phosphate using calorimetric assay (Carpenter *et al.*, 2012). G6P was measured enzymatically using Glucose 6 phosphate dehydrogenase in the presence NAD. This method was used because of its simplicity and is straight forward and reliable (Nielsen *et al.*, 1994). All the steps during the experiment were done carefully to avoid errors such as pipetting and setting of the spectrophotometry. The experiment was repeated four times to ensure reproducibility.

6.2.4 Starch granule morphology by scanning electron microscopy (SEM)

A common technique that has been used to study starch granule morphology is microscopy. By introduction of improved analytical and microscopic techniques, different levels of the granule organisation have been visualised. Light microscopy (LM) is commonly used but introduction of high resolution microscopes such as SEM, atomic force microscopy (AFM) and transmission electron microscopy (TEM), more detail about internal and external structure of starch granules can be revealed (Gallant *et al.*, 1997).

LM was the first microscope used to study granule structure, but SEM became popular and commonly used. SEM has two main advantages over LM. Firstly, the magnification of SEM is much greater than LM and secondly, the resolution is better in SEM (Jane *et al.*, 1994).

SEM has played a vital role in barley research. In the 1960's, the three dimensional architecture of barley chromosome was studied by SEM (Iwano *et al.*, 1997). The endosperm and cell wall of barley grain was also visualised by SEM (Brennan *et al.*, 1997) as well as the microstructure of barley after malting was observed by SEM (Wijngaard *et al.*, 2007).

SEM was used to examine morphology of barley starch. Three magnifications were used 250 x, 500 x and 1000 x however; only 1000 x magnification results were included. The most important things that were considered, i.e. sample preparation, proper coating of the starches and adjustment of the focus to ensure good quality images are obtained.

6.2.5 Starch granule particle size distribution by laser diffraction

Techniques that have been used to examine starch granule particle size include microscopy, micro-sieving, electrical resistance and laser light scattering. Common microscopies that have been used are LM and SEM, even so, both have limitations. Microscopy is capable of obtaining information about the sample but more information can be obtain by laser light scattering (Lindeboom *et al.*, 2004).

The sieving technique is based on separation of the granules in a specifically designed sieve, but larger granules seems to be more oval in shape due to granule aggregation (Lindeboom *et al.*, 2004). Electrical resistance is performed by using a coulter counter to separate the particles based on how they affect the electrical resistance as they pass through an electrically conductive liquid. However, this method is not capable of measuring particles of diameter 3 μm (Lindeboom *et al.*, 2004; Raeker *et al.*, 1998).

The laser light scattering technique is fast and objective with the ability of measuring size greater than 100 000 μm , as well as possibility of measuring small granules of 0.1 μm in diameter (Lindeboom *et al.*, 2004).

6.2.6 Debranched starch molecular structure by size exclusion chromatography (SEC)

Different techniques such as SEC, field flow fractionation (FFF), ultracentrifugation and hydrodynamic chromatography have been used to examine molecular structure of starch (Gidley *et al.*, 2010). The iodine test was also used because it has been reported that amylose forms complexes with iodine which gives a blue colour, while amylopectin remains brown (Copeland *et al.*, 2009).

High performance anion exchange chromatography (HPAEC) coupled with an amperometric detector is commonly used but the result obtained is not directly proportional to the carbohydrate contents. Essentially longer chains do not detected as efficiently as short chains. In addition, it has been reported that capillary electrophoresis and DNA sequencer were used to examine amylose and amylopectin chain profile. However, the results presented molar distribution rather than the weight distribution (Bertoft, 2004).

SEC and FFF coupled with detectors are commonly used to provide structural information on the fine molecular structure of polymers (Vilaplana & Gilbert, 2010a). It has been proposed that FFF reduces shear scission which is one of the main limitations of SEC but there is no published literature to support the prediction (Gilbert *et al.*, 2013; Vilaplana & Gilbert, 2010a).

SEC has become a useful technique and more developed for measuring molecular weight and molecular weight distribution of polymers. The use of detectors in SEC has expanded its use, it became possible to determine molecular conformation and long chain branching with the use of the sensitive molecular detectors (Bertoft, 2004).

There are two main limitations in SEC, band broadening, shear scission and low recovery of large molecules (Gous *et al.*, 2015; Syahariza *et al.*, 2013). Band broadening is a common instrumental limitation found in chromatographic techniques and shear scission is degradation of high molecular weight polymers during elution (Vilaplana & Gilbert, 2010a). As it has been emphasised earlier SEC is costly, complex and few laboratories can do this analysis yet it provides useful information on fine molecular structure of natural and synthetic polymers (personal communication; Glen Fox, 2015, The University of Queensland, Brisbane, Australia, 20 May 2015).

Another key thing to remember is that there is no comprehensive technique that provides complete information on the chain length distribution of debranched starch (Vilaplana & Gilbert, 2010a). However, fluorophore–assisted carbohydrate electrophoresis (FACE) has been assessed to be superior to the other techniques such as HPAEC and SEC (Wu *et al.*, 2013).

In fact, FACE is preferred when studying amylopectin chains because it prevents band broadening, but long chains of amylopectin and amylose with DP greater than ~100 is well observed with SEC (Wu *et al.*, 2014). Different researchers have proved that SEC coupled with multiple detectors became most

available and developed technology for size separation of polymers (Vilaplana & Gilbert, 2010a; Gaborieau *et al.*, 2008; Gous *et al.*, 2015; Ward *et al.*, 2006).

6.2.7 Identification of genetic variation in barley starch synthase II (SSII)

The polymerase chain reaction (PCR) is the main method used to amplify DNA from plant and animal materials. This technique has caused a revolution in molecular biology by increasing the capacity of identification and manipulation of genetic materials (Bartlett & Stirling, 2003).

We tried to amplify the entire SSII gene from the ten cultivars but no PCR product was found when they were examined using gel electrophoresis. Different primers were used and the PCR conditions were optimised in terms of temperature using gradient PCR and gel purification, however, no PCR product was obtained.

This led us to amplify specific regions in the SS II gene (Table 4.1). Based on that, eight sets of primers were synthesised which amplified the sequence at specific sizes (Table 5.1). Out of eight primers, four pairs of primers gave the expected fragment sizes on gel electrophoresis. The fragments were sequenced for further confirmation. Much optimization was deformed to try and optimize primer pairs that did not lead to amplification, especially in terms of altered annealing temperatures, however, no amplicons were ever found.

Primer design is one of the challenges needed to be considered before performing PCR. Poorly designed primers might result in no PCR amplifications. Therefore, a number of web-based programs were developed to help scientists with primer design (You *et al.*, 2008).

When designing primers using the normal methods, and it is difficult to find good primers for the particular sequence, the whole source sequence will be discarded whereas, with the primer design application, different options are available with the aim that one might obtain the expected result (Rozen & Skaletsky, 1999).

On the other hand, molecular markers are common technologies used for identification of DNA polymorphisms. These include first generation molecular markers such as random amplified polymorphic DNA (RAPD and RFLPs), the second generation molecular markers such as amplified fragment length polymorphism (AFLPs), while third generation molecular markers include single nucleotide polymorphisms (SNPs) (Gupta *et al.*, 2001).

First and second generation molecular markers are time consuming and expensive because they are gel based assays. Among the mentioned techniques, sequencing becomes an effective method to detect SNPs (Waters *et al.*, 2008).

SNPs are the most common form of DNA polymorphism; they can be used in plants as single genetic markers for breeding applications. Availability of DNA sequence in the databases has made it possible to identify SNPs by database mining (Chiapparino *et al.*, 2004).

6.3 Data analysis

6.3.1 Principal component analysis (PCA)

The idea behind PCA is extraction of important information as a set of new variables called principal component. PCA is a multivariate statistical techniques and almost used by all scientific disciplines (Abdi & Williams, 2010). It has been proven that combination of PCA with instrumental method results can help in generating more information and also it has the ability to determine more than one component at a time that show relationships between physical characteristics of different samples (Cozzolino *et al.*, 2014).

6.3.2 Geneious

It is important for a scientist to select an appropriate bioinformatics tool for data analysis for the success of any research (Kearse *et al.*, 2012). Geneious is a bioinformatics tool used for analysis of biological data (Masters *et al.*, 2011).

The Geneious program was created to overcome the limitations raised from other bioinformatics programs. It takes the advantage of modern computing and the platform includes automated database searching, data backup functionality and an extensible Application Programming interface (API) for the integration of novel bioinformatics analysis tools (Kearse *et al.*, 2012).

6.4 Research findings

6.4.1 Pasting properties

Pasting is referred to as changes in viscosity during the event of gelatinisation (Zeng *et al.*, 1997). Pasting occurs after the gelatinisation process and gelatinisation happens when starch / flour is heated in the presence of water when it expands due to hydration. As the granules take up water they expand resulting in rupture of the granules because of structure disruption and break down of hydrogen bonds (Tester *et al.*, 2006; Lii *et al.*, 1996; Srichuwong *et al.*, 2005).

When the gelatinised granules are continually heated, amylose leaches out of the granule resulting in a paste which consists of a molecular dispersion of dissolved starch molecules and discontinuous phase of swollen granules and granule fragments (BeMiller, 2011). The rate and extent of swelling and breakdown depend on the type and amount of starch, the temperature gradient, shear force and composition of the mixture (Copeland *et al.*, 2009; Schirmer *et al.*, 2013).

As the temperature increases, the starch granules continue to swell as they absorb more water, causing an increase in viscosity over time and a peak viscosity forms when high number of swollen granules are present (Thomas & Atwell, 1999).

After peak viscosity with continued heating and shear is reached, the viscosity of the starch paste reduces quickly due to disintegration of the granules and leaching out of solubilised starch polymers from the swollen granules to the solution. The polymers can also align themselves in the direction of shear which further reduces the viscosity (BeMiller, 2007).

Upon cooling the viscosity again starts to increase, the increase in viscosity during the cooling period indicates the tendency of different components present in the hot paste to re-associate with decrease in temperature (Kaur *et al.*, 2007). Figure 5.1 shows RVA viscogram which for the ten cultivars and the parameters measured by the RVA. Cocktail, Marthe, Cristalia, Erica, Nemesia, Disa, and Houwink were higher in the pasting parameters, whereas Metcalfe, Baudin and Henrike had indicated lower pasting parameters.

Looking at each variable separately Appendix 8.1, peak viscosity is the highest viscosity during heating, high peak viscosity indicates the ability of the granule to swell freely before it breaks down. Starches with high peaks are less resistant to break down and have low viscosity after reaching maximum peak (Singh *et al.*, 2003b).

Low peak viscosity is an indication of granule resistance to the swelling. Amylose plays a role in inhibition of the swelling due to the formation of amylose lipid complexes (Cozzolino *et al.*, 2012). Lowest peak viscosity and breakdown also indicates strong cohesive forces within the starch granules, high thermal stability and lower retrogradation (Li *et al.*, 2014b). Low peak viscosity was characteristic of Henrike, Baudin and Metcalfe followed by the rest of the cultivars.

A study showed that peak viscosity had significant negative relationship with malt extract (Cozzolino *et al.*, 2012). Another study revealed that good quality malted barley formed low and broad peaks, while poor quality malted barley formed high and narrow peaks (Visser, 2011). Based on that, it would be predicted that Metcalfe, Baudin and Henrike would have better malting properties.

In addition, barley contains lipids, the surface lipids inhibit water movement into the granules and the external lipid forms a complex with the amylose which reduce swelling properties and, therefore, amylose leaching from the granules which reduces the peak viscosity (Raeker *et al.*, 1998).

Peak time is the time required by a sample to reach maximum viscosity (Zhou *et al.*, 2007). It has been reported that peak time (PT) and peak viscosity (PV) of barley starch had a significant negative relationship with malt extract (Cozzolino *et al.*, 2012). A shorter time to peak viscosity is favorable for

good malt quality (Wang *et al.*, 2010). Henrike, Baudin and Metcalfe had short time to peak viscosity compared to the other cultivars. Again, an indication that these cultivars are of good malting quality.

Pasting temperature is sometime referred to as gelatinisation temperature because it can be used to estimate gelatinisation temperature, however, the two are different. Pasting temperature is (the temperature which initial swelling of starch granule take place when heated in water), while gelatinization temperature is (the temperature where break down of bonds within starch molecules) (Anon, 2008). For a good malt quality, lower pasting temperature is required (Wang *et al.*, 2010; Fox *et al.*, 2003b). At low temperature, sufficient gelatinisation would occur and that is a good sign during processing (Zhou *et al.*, 1998).

The ten cultivars had showed gelatinisation temperature between 68.3 to 89.2°C. Henrike demonstrated the lowest temperature and Nemesia the highest temperature. It has been revealed that high amylose starch has high gelatinisation temperature (Fox *et al.*, 2003b). On the other hand, It has been observed that mutations in starch branching enzymes (BEIIb) leads to an increase in gelatinisation temperature because of an increase in the proportion of long chains within the amylopectin cluster (Cuevas *et al.*, 2010). In general, gelatinisation temperature is controlled by many factors such as, the molecular structure of amylopectin, starch composition and granule architecture (Singh *et al.*, 2003b; Schirmer *et al.*, 2013).

Breakdown happens as a result of disintegration of gelatinised starch during continuous heating and it is influenced by amylose contents (Gujral *et al.*, 2013). The more amylose that leached from the granule the more it increases breakdown. However, amylose also forms complexes with lipids which inhibits breakdown resulting in reduction of free amylose. Granule rigidity also inhibits breakdown (Singh *et al.*, 2003b). Metcalfe, Henrike and Baudin had showed reduction in breakdown compared to the rest of the cultivars.

Trough ,(the lowest viscosity after cooling) and peak viscosity have a negative relationship with malt extract (Cozzolino *et al.*, 2012). Henrike, Baudin and Metcalfe showed low troughs compared to the other cultivars. Setback viscosity is the difference between the final viscosity and peak viscosity. It indicates the retrogradation tendency of the starch after gelatinisation (Li *et al.*, 2014b). It has been observed that type A granules have greater setback viscosity than type B granules because the type A granule consist of more amylose and long chains of amylopectin than type B granules (Ao & Jane, 2007). It has been reported high amylose and long chain amylopectin chains affect pasting properties by increasing gelatinisation temperature which is not a good indication as it has been mentioned earlier (Zhang *et al.*, 2004; Cuevas *et al.*, 2010).

From the RVA viscogram, peak viscosity, setback, final viscosity, peak time, trough and pasting temperature seems to be the major factors responsible for variation in pasting properties among the samples.

6.4.2 Amylose content

Many applications of starch depend on its amylose and amylopectin ratio (Gibson *et al.*, 1997). Normal starch contains 20-30% amylose and 70-80% amylopectin (Jane *et al.*, 1999; Ma *et al.*, 2014). However, it varies with botanical origin and is affected by genetic and environmental (G×E) factors (Singh *et al.*, 2006; Hu *et al.*, 2010).

On the other hand, it has been recognised that amylose content is one of the most important determinants of starch functionality because it affects gelatinisation, pasting and swelling properties (You & Izydorczyk, 2002). It could be important to look at some mutations in cereals that affected amylose contents; for example the *amo1* and *sex6* mutants resulted in high amylose contents (Matsuki *et al.*, 2008). The barley *shrunk* mutant (*SSII*) has more than 60% amylose compared to normal barley (Yamamori *et al.*, 2006; Morell *et al.*, 2003). In addition, it was reported that environmental factors such as high temperature during grain filling affects granule size distribution which also results in high amylose content (Svihus *et al.*, 2005).

Table 5.1 shows the amylose contents for the ten cultivars were between 23.8 and 25.5%. Nemesia had the highest content (25.5 %) followed by Marthe, Erica (25.3%), Cocktail (25.0%), Cristalia (24.9%), Henrike (24.7 %), Metcalfe (24.4%), Disa (24.4%), Baudin (23.9%) and Houwink (23.8 %).

A study has reported that amylose contents for waxy starch in barley varies between 0 to 9.1%, in normal starch (from wild-type varieties) between 23.0 to 32.7% and high amylose starch between 33.4 to 48.7% (Tang *et al.*, 2002). The SEC results showed that the ten cultivars have normal barley starch.

The results obtained from SEC confirmed the accuracy of the method (International Standardization Organisation, 2011 - ISO 6647-2) for amylose content determination (Vilaplana *et al.*, 2012). It has been explained that this method is accurate due to a clear separation between amylose and amylopectin chains at DP ~ 100 and the amylose contents for normal starch measured by SEC is ~ 25% (Gilbert *et al.*, 2013). This was observed in the ten cultivars.

6.4.3 Covalent phosphate content

It was important to analyse phosphate contents in this study because phosphate groups alter physicochemical properties of starch (Tetlow, 2006). These phosphate groups are covalently attached to the C3 and C6 position of starch and their function is to support granule plasticity and hydration (Carpenter *et al.*, 2012; Carciofi *et al.*, 2011).

Phosphorous in starches is found in three forms namely, phosphate monoester, phospholipids and inorganic phosphate (Tester *et al.*, 2004b). Most of the cereals starches contain phosphate in the form of phospholipids (Lysophospholipids) (Svihus *et al.*, 2005).

Covalently bound phosphate for the ten cultivars was measured and it ranges from 0.2 to 0.9 % (Table 5.1). It was reported that cereals contain little or no starch bound phosphate and the level varies with botanical sources (Glaring *et al.*, 2006). It has been reported that GWD (α -glucan water dikinase) is present in all plants and it catalyses phosphorylation. Suppression of this enzyme in potato led to reduction in starch bound phosphate by 90% and also mutation of this gene in Arabidopsis caused reduction in the phosphate content of leaf starch. Activity of this enzyme could be the reason cereals synthesize starch with low covalent phosphate of approximately $\leq 0.01\%$ (Mikkelsen *et al.*, 2004).

Despite its low level in cereals, phosphate has an effect on starch physical properties. In rice, it has been found that phosphate suppressed viscosity and lower gelatinisation temperature (Wang *et al.*, 2000). In other starches such as those in potato, phosphate contents affect paste viscosity and gel forming capacity. Also it prevents crystallisation and final viscosity (Blennow *et al.*, 2002). The level of phosphate in the ten cultivars could be natural or due to variation in growing locations.

6.4.4 Starch granule particle size distribution

Granule particle size distribution has been expressed in a different ways such as, average length of the major or minor maximum diameter, average granule volume or average surface area. However, it depends on the technique used (Lindeboom *et al.*, 2004). In this study, granule particle size distribution was presented as granule diameter.

Granule size is mostly related to the biological sources from which the starch is isolated. The granules are classified into three classes namely, large granules with $>25 \mu\text{m}$ diameter, medium 10-25 μm and small 5 -10 μm (Lindeboom *et al.*, 2004). (Table 5.1) shows granule size distribution for the ten cultivars showed average granule diameters of between 15.87 and 18.62 μm . The largest particle size was observed in Metcalfe (18.62 μm) followed by Cocktail (17.84 μm), Disa (17.42 μm), Marthe and Erica had the same distribution (16.69 μm), Cristalia (16.45 μm), Houwink (16.43 μm), Henrike (16.03 μm), Nemesia (16.14 μm) and Baudin (15.87 μm).

It has been shown that large barley starch granules range from 15 to 25 μm and the small granules are less than 10 μm . On the other hand, it has been demonstrated that the large granules are about 80 to 90% of the total granule amount (Gubatz & Shewry, 2010) whereas, the small granule make up 10 to 15% of the total granule amount (Tester *et al.*, 2004b).

Granule size would not be the major factors for physicochemical differences among the ten cultivars. However, the ratio of large to small granule would contribute to the variation among the cultivars. It has

been demonstrated that large granules contain higher amylose contents whereas, small granules contain high lipid contents (Svihus *et al.*, 2005) that in turn would affect physicochemical properties. On the other hand, it has been found that granules of the same botanical origin might still have different granule sizes and morphology (Eliasson & Gudmundsson, 1996; Eliasson, 2004).

6.4.5 Degree of polymerisation (DPX)

Structural characteristics of linear chains such as debranched starch which is examined by SEC coupled with detectors can be present as the number distribution $N_{de}(X)$ of chains of degree of polymerisation (DP) or weight distribution $W_{de}(X)$ of chains of degree of polymerisation (Vilaplana & Gilbert, 2010a). Size distribution for debranched samples is presented as an average weight degree of polymerisation because of band broadening (Vilaplana & Gilbert, 2010b).

Table 5.1 shows the DP was calculated as an average weight DP for each cultivar. The ten cultivars had showed average DP between (3,420) and (4,330). Metcalfe had the highest DP (4,330) followed by with Erica (3,770), Henrike at (3,680), Nemesis (3,540), Baudin, Houwink and Disa had the same DP (3,500). Cristalia (3,450), Cocktail (3,440) and Marthe (3,420).

Different researchers have reported on average DP in cereal including barley. Barley starch has an average DP of 1120 and 20 for amylose and amylopectin respectively (Bul on *et al.*, 1998). It has also been shown that the amylose fraction is composed of molecules with weights of between 190 to 260 000, whereas amylopectin comprise of 3.6 to 4.1 million (Tester, 1997). On the other hands, It has been reported that the weight average degree of polymerisation for debranched barley starch is 2.73×10^6 (BeMiller & Whistler, 2009).

The properties of debranched molecules in the collected fractions for the three regions amylopectin at region 1(AP1), region 2 (AP2) and amylose at region 3 (AM3) in the chain length were further analysed (Vilaplana & Gilbert, 2010b; Syahariza *et al.*, 2013).

Table 5.2, AP1 and AP2 zone showed the same values. It has been reported that when the distributions are normalised to AP1, AP1 and AP2 become the same in all collected fractions. This indicates that branch CLD for amylopectin are not influenced by their macromolecular size (Vilaplana & Gilbert, 2010b). The explanation for is that, the calibration curve enable the elution volume (V_{el}) to number of debranched monomer (X_{de}) from different fractions .Practically, amylose rich fractions give sufficient signal with Malls for this process, whereas amylopectin does not because of it small molar mass (Vilaplana & Gilbert, 2010b).

The data were normalised to amylopectin highest peak AP1 that is why the amylose 3 (AM3) was calculated as an average area under the curve (AUC) of amylose branch for each cultivar. AM3 showed significant variations for the different fractions. It has been noticed that amylopectin (AP1 & AP2) is well

observed when the results are presented as weight distribution whereas amylose (AM3) is well observed when the results are presented as number distribution (Syahariza *et al.*, 2013).

It has been reported that chain length distribution is composed of short chains of Amylopectin with average chain length 17 – 25 and amylose between 10^3 – 10^4 however, this depends on the plant species (Vilaplana & Gilbert, 2010b). This report is more similar to what has been obtained in Table 5.2.

In addition, it has been reported that the length of the amylopectin chains in particular B, size of the crystalline lamella increases with increase in barley amylose content (BeMiller & Whistler, 2009).

6.4.6 Starch granule morphology

From the morphological examination (Figure 5.5), the ten cultivars had showed two types of starch granules, large and small granules. It has been reported that barley starch has two types of granules, large disk shaped ones known as A type granules and small spherical type B granules. The granules are formed during different developmental stages which result in variation in their sizes and shapes (Suh *et al.*, 2004; Ao & Jane, 2007). The ten cultivars showed both types of granules lenticular A and spherical B types. Different sizes of granules were also observed.

Barley granules have been described as ones that has an equatorial groove on the large granule with indentations on the surface, which could be attributed to close packing. Also, the activity of enzymes on the granules can result in pore formation. Additionally, it has been reported that cereals contain more pores of different diameter on the surface of the granule (0.1 – 0.3 μ m). This was suggested to be openings to channels that goes inside the granule potentially to the hilum (Eliasson, 2004; Eliasson & Gudmundsson, 1996). Importantly, starches isolated from different botanical sources have different morphological characteristics (Pérez & Bertoft, 2010).

Some granules were described as loosely packed which were referred to as mealy granules, whereas the ones which are densely packed are referred to as steely (Chandra *et al.*, 1999). The same terms were indicated as characteristics of simple and compound granules. Simple granules formed when one granule is initiated in an amyloplast while, compound granules are formed when more than one granule is being initiated at the same time (Suh *et al.*, 2004).

The small holes that were observed on the surface of some cultivars such as Metcalfe, Marthe, Nemesia, Houwink and Henrike could be attributed to initial stages of hydrolysis by α -amylase and gluco-amylase. It has been reported that malt α -amylase attacks large barley granules and this leads to the formation of pinholes on the granule surface (MacGregor & Bhatta, 1993).

On the other hand, it has been suggested that the presence of holes on the surface of starch granules is a result of an unbalanced level of starch synthase and hydrolase and induced the premature autolysis (Li *et al.*, 2013). It was noticed in barley and wheat that inhibition in starch branching enzyme

increases amylose contents. This cause changes of the granule morphology. Large granules appear to be sickle-shaped and their hilum are hallow and aggregate granule with large size, hallow and elongated (Cai *et al.*, 2014). On the other hand, it has been reported that when barley starch was examined under scanning electron microscopy, pin holes were observed on the surface of both normal and high amylose starch (BeMiller & Whistler, 2009).

There was a reduction in the number of small granules in Nemesia and Houwink. The reduction in the number of small granules might be as a result of genetic by environment interaction (GxE). It has been reviewed that down regulation of starch debranching enzymes in particular pullulanase type debranching enzyme in barley leads both to reduction in the number of small granule and also to changes in granule shape (Tetlow, 2006) . On the other hand, this can also depend on how much starch goes onto the SEM plate.

Granule size and shape affect the physicochemical properties of starch. Variation in sizes and shapes of A and B type granule in cereals such as in barley become an important determinant for its industrial use (Ao & Jane, 2007). Large granules are preferred by the brewing industry for good brew house performance (Morell *et al.*, 1995) and large granules were characteristic of the ten cultivars.

It has been reported in barley, that small granules are characterised by high pasting temperature and swelling properties (Lindeboom *et al.*, 2004; Myllrinen *et al.*, 1998). On the other hand, large granules have a greater peak, final viscosity, breakdown, and setback viscosity than smaller granules. B type granules have higher pasting temperature which can reach up to 94.3°C and a lower peak viscosity (Sologubik *et al.*, 2013).

Figure 5.2 shows correlation circle graphs which graphically shows correlation between blocks of variables, pasting properties, amylose contents, phosphate contents, granule particle size distribution and degree of polymerisation (DP). When the percentages of the two dimensions were added, it gives value of 64.8% which shows a positive correlation. The RVA parameters cluster to one end of dimension, while the amylose contents are towards the RVA parameters. This would be expected because amylose content plays a great role in pasting properties. Amylose correlates well with pasting temperature, which would be expected. It has been reported that increase in amylose contents is associated with an increase in gelatinisation temperature (Fox *et al.*, 2003b) and it has been explained that gelatinisation temperature and swelling are controlled by molecular structure, granule architecture and starch composition (Singh *et al.*, 2003b).

The DP and pasting properties are negatively correlated which could be attributed to variation between the DP and pasting properties. DP is also negatively correlated with the amylose and phosphate contents. DP correlates well with the granule size than amylose and phosphate contents.

It has been hypothesised that cultivars might have the same chemical composition but different molecular structure which, in turn affects their functionality. This could be attributed to the polymerisation process in particular phosphorylation reaction that integrated with starch biosynthesis in the plastids. It has been reported that α glucan water dikinase (GWD) preferentially phosphorylated longer chains in amylopectin because it restricts require α -1 \leftarrow 6 bond in its glucan substrate. In addition, it has been observed in potato that GWD shows increase in activity when the DP increases from 27.8 - 29.5. GWD is found in all plants kingdom including barley (Mikkelsen *et al.*, 2004).

The presence of this phosphate groups affect physical and chemical properties of starch granule which are important for starch metabolism in plants (Carciofi *et al.*, 2011). (Figure 5.3) PCA biplot relates pasting properties, DP and granule size. From the plot, amylose and phosphate contents did not appear in the plot because of their low R^2 -values of 0.211 and 0.247 (Appendix Table 8.1), respectively. (Figure 5.2) the correlation are each on the outer circle is (0.7 – 1). The pasting properties were grouped in the one dimension and some cultivars such as Cocktail, Marthe, Cristalia, Erica, Nemesia, Disa and Houwink are toward the pasting parameters and that demonstrate strong correlation. On the other hand, Metcalfe, Baudin and Henrike did not correlate well with the pasting properties. Breakdown and pasting temperature are completely in opposite direction which indicates no correlation with samples and the other pasting parameters.

Figure 5.4 shows an individual factor map for the ten cultivars where cocktail and Erica seems to be similar. Marthe and Nemesia also showed close relation. However, each cultivar seems to have certain trait that makes it different from the rest of the cultivars. This would be attributed to the structural variation among the cultivars because from the analysis of the chain length distribution there is a clear variation in amylose and amylopectin chains of the ten cultivar (Figure 5.6).

6.4.7 Analysis of chain length distribution (CLD) of debranched barley starch

Molecular structure is referred to molecular size distribution of individual branches of the polymers and it is commonly known as the chain length distribution (CLD) for the debranched starch and degree of branching (DB) for the branched molecules (Syahariza *et al.*, 2013).

It is important to understand the relationship between starch structure and functionality in order to improve the quality of it industrial use (Simsek *et al.*, 2013) as starch structure determines starch use in many end applications (Morell *et al.*, 1995). It has been found that changes in starch cluster structure causes variation in physicochemical properties between plants of different genotypes (Umemoto *et al.*, 2004). In this study, the CLD was studied at the second structural level and this is the number distribution of linear glucan chains α -1 \leftarrow 4 glycosidic linkage which comes as a result of debranching by isoamylase

type debranching enzymes. This is presented as a function of degree of polymerization (DP) (Wu *et al.*, 2014).

Figure 5.6 shows CLD for the ten barley cultivars plotted as a logarithm of the number distribution $N(X)$, as a function of degree of polymerisation (DPX) (Cuevas *et al.*, 2010; Castro *et al.*, 2005; Wu *et al.*, 2014). All cultivars exhibit a bimodal pattern of de-branched starch (Copeland *et al.*, 2009; Eliasson, 2004).

CLD of starch by SEC does not provide information on individual DPs for each chain but it gives ranges for the whole chain length distribution of the debranched starch (Vilaplana & Gilbert, 2010a). From the results, DP for amylopectin and amylose for the ten cultivars ranges from ~ 5 to 100 and 100 to 10 0000, respectively.

Structural variations among the cultivars were observed in the chain length. All cultivars did not fall under the same distribution; each cultivar formed a unique bimodal distribution. That can be attributed to differences in amylose and amylopectin chain lengths. There is variation in amylose peak height among the cultivars and that would be attributed to differences in amylose contents (Syahariza *et al.*, 2013).

Cultivars with higher amylose contents such as Cocktail, Marthe and Cristalia showed higher amylose peaks compared to the rest of the cultivars. Elevation of the chains was shown at the end of higher amylopectin fractions. The same finding was also observed by Wu *et al.* (2014) who also noted no clear separation between amylose and amylopectin molecules (Vilaplana *et al.*, 2012).

Some cultivars showed the same values for amylose and phosphate contents, granule particle size distribution and average DP, yet they differ in structure in term of the debranched chain length. Hence, it can be concluded that the starch fine structure could play a vital role in the functionality because starches might have the same chemical composition but different structures, leading to different functionality. Basically, this reflects the activity of the enzymes controlling starch biosynthesis. For example, starch synthases classes are responsible for elongation of chains while debranching enzymes carry out chain termination. Thus, any alteration in activities of these enzymes could affect the biosynthesis and consequently the chain length distribution (Cuevas *et al.*, 2010).

The differences in molecular structure could be attributed to different genotypes which result in variation in chemical composition and properties of starch (Song & Jane, 2000). It has been highlighted that environmental stress alters starch biosynthesis by acting on the starch biosynthetic enzyme and this leads to changes in structure and composition of starch granules (Beckles & Thitisaksakul, 2014).

Correcting of the band broadening effect in SEC through deconvolution is essential because it has been clarified that band broadening alters position of peaks and causing features such as shoulders to

disappear (Castro *et al.*, 2005). The ten cultivars were also plotted as lnN (X) plots. Firstly, this helps comparison of data without effect of normalisation and secondly, it shows features that arise as a result of variation at the highest DPs (Cuevas *et al.*, 2010).

From the lnN(X) plot results (Appendix 8.2) the ten cultivars showed shoulders at DP ~10. Interruption of the linearity was also observed among the cultivars and divergence was shown at DP ~100 which can be attributed to separation of amylose and amylopectin chains. The interruption of linearity was clear in Marthe, Cristalia, Cocktail and Henrike.

6.4.8 Identification of *SSII* gene from barley

SSII is found in both the granule and the soluble fractions during early endosperm development. It becomes available in starch granule from the mid-grain filling (Morell *et al.*, 2003). *SSII* gene is responsible for the manufacturing of medium chains of amylopectin with degree of polymerisation 13 to 25 (Cuesta-Seijo *et al.*, 2013). Its deficiency shows reduction in starch contents, amylopectin chain length distribution and it also alters granule morphology (Ball & Morell, 2003; Ullrich, 2010; Tetlow, 2006).

In barley, different mutants that lead to increase in amylose contents have been identified such as *amo 1* lead to increase in amylose content of 44 to 47%. Further research, has reported that the gene responsible is located at chromosome 5 and a slight change in starch branching activity was observed (Bertoft *et al.*, 2011).

Shrunken grain mutant M292 led to a novel high amylose starch and it has been proven that this mutant is present in the *SSIIa* gene which is located at the *starch excess-6* (*sex 6*) locus on chromosome 7H (Clarke *et al.*, 2008). The same mutant has an effect on amylopectin synthesis because of lack in the *SSIIa* protein (Bertoft *et al.*, 2011).

The same finding has been reported in rice, the *SSII* gene is located at the *alk* locus on chromosome 6H. Mutation of this gene in rice affected the amylopectin structure of two cultivars and caused reduction in the activities of other enzymes such as branching enzyme. It has been confirmed that a reduction in *SSIIa* activity is said to be responsible for the low gelatinisation temperature starch from some rice cultivars (Umemoto *et al.*, 2002; Umemoto & Aoki, 2005).

Reduction of *SSIIa* activity in rice and wheat leads to decrease in gelatinisation temperature due to a decrease in the proportion of long chains of the amylopectin cluster (Cuevas *et al.*, 2010). This showed a 80% decrease in amylopectin and the chain length distribution in barley (Clarke *et al.*, 2008). In Maize, a mutant at the *sugary 2* (*su2*) locus, which encodes *SSIIa*, altered amylopectin structure and causes increase in amylose content by 40% which in turn changed physicochemical properties in useful ways. *Sugary* codes for the *SSIIa* gene (Zhang *et al.*, 2004).

As far as good malt quality is concerned, high amylose and low gelatinisation temperature is advantageous (Fox *et al.*, 2003b). A finding by Morell *et al.* (2003) could be important for barley breeders especially those who would like to improve barley for malting purposes. They found that the increases in amylose contents (72%) in *sex6* mutants led to a decrease in gelatinisation temperature (Morell *et al.*, 2003). Therefore, there was a need to characterise this gene in order to find whether it influences quality traits measured such as pasting properties particularly pasting temperature and amylose content. By identifying these, possible strategies might lead to improved quality of the local cultivars.

Table 4.1 shows four pairs of primers that gave the expected fragments (Figures 5.7 and 5.8). The PCR products from *SSII* DNA samples were submitted to the Australian Genome facilities for sequencing. The sequencing was successful and could be compared with sequence of the barley *SSIIa* gene at the gene bank library (Morell *et al.*, 2003).

The fragments were further confirmed at the National Centre for Biotechnology, they are 99% identical to the *Hordeum vulgare SSII* gene under query ID 149533. One single nucleotide polymorphism (SNP) was identified (Figure 5.9) in all cultivars. This was present within exon five of the gene which changed one amino acid from leucine to proline. As proline is known to affect protein secondary structure due to its conformational rigidity, it may be expected that such an alteration could potentially affect the kinetics of the *SSIIa* protein. The mutants that are commonly used for breeding malting types have been found at chromosomes six (6H) and three (3H) (Slafer *et al.*, 2001). However, the SNPs identified could be referred to as breeding SNPs because it was consistently present in all cultivars.

Looking at the pasting temperature, all cultivars showed a pasting temperature between 68.3 and 89.2°C. This is clearly higher than gelatinisation temperature of between 54 and 65°C previously reported in barley flour (MacGregor & Bhatti, 1993). This could be hypothesised to potentially be caused by a loss in starch branching enzyme (*SBEIIb*) activity because it has been reported that loss of *SBEIIb* in rice leads to an increase in gelatinisation temperature due to increase in the proportion of long chain with an amylopectin cluster (Cuevas *et al.*, 2010). However, this would need to be confirmed in the ten cultivars. Moreover, it has been highlighted that heat stress causes an increase in gelatinisation temperature and that was attributed to changes in double helix length within crystalline lamellae (Gous *et al.*, 2015). Therefore, GxE interaction should also be examined more carefully.

Chapter 7 Conclusions

7.1 Summary of findings

Barley is the most important grain used in the brewing industry. It is a plant that can adapt to different environments. Plant grown in different environments might have different physicochemical properties as a result of GxE interaction.

As far as good malt quality is concerned, characterisation of barley starch is required. Knowing the characteristic of each cultivar, plant improvement through a breeding program might be established. Basic analysis such as pasting properties, starch granule morphology, granule size distribution, starch structural analysis, chemical composition and genetic variation test could be good indicators for barley malting potential.

From the study, the ten cultivars grouped into four based on their pasting properties, Henrike and Baudin showed the closest relationship. Marthe, Erica, Nemesia, Disa, Houwink also grouped together. Metcalfe seems to be different. However, it is closer to Henrike and Baudin than the rest of the cultivars. Cocktail and Cristalia are most related to each other, but they also showed similarity with Marthe, Erica, Nemesia, Disa, and Houwink.

The ten cultivars showed amylose contents between 23.8 and 25.5% which demonstrates that is roughly the amount reported in wild-type varieties, however, small variations among them exist. Nemesia was the highest with 25.5%, Marthe and Erica had 25.3%, Cocktail 25.0%, Henrike 24.7%, Cristalia 24.9%, Disa and Metcalfe 24.4%, Baudin 23.9%, Houwink 23.8%.

All cultivars showed low covalent phosphate level between 0.2 and 0.9 %, which is natural in cereal starches but different locations might affect level of phosphate due to variation of phosphate in soil. Houwink had the highest phosphate contents (0.9%), followed by Marthe (0.6%), Nemesia (0.5%) and Baudin had 0.4%. Cocktail, Cristalia, Erica and Disa showed the same phosphate level 0.3%. Henrike showed had the lowest phosphate level (0.2 %).

The degree of polymerisation for the ten cultivars ranges from (3,420) to (4,330). Metcalfe showed the highest DP (4,330), Henrike (3,680), Erica (3,770), Nemesia (3,540), Baudin, Disa and Houwink had the same average DP (3,500), Cristalia (3,450), Cocktail (3,440) and Marthe (3,420). DP seems to be the major factor that causes variation among the samples when the variables were analysed.

The properties of debranched molecules in the collected fractions for the three regions amylopectin 1 (AP1), amylopectin 2 (AP2) and amylose 3 (AM3) in the chain length were further analysed. AP1 and AP2 shows the same values due to normalisation, but, there is variation observed in AM3 in the ten cultivars.

The granule size distribution was reported as an average granule diameter the ten cultivars showed large granules between (15.87 μm) and (18.62 μm). Metcalfe indicated that half of its granules are above size average of (18.62 μm), Cocktail (17.84 μm), Disa (17.42 μm), Marthe and Erica indicated (16.69 μm), Cristalia (16.45 μm), Houwink (16.43 μm), Nemesia (16.03 μm), Henrike (16.14 μm) and Baudin (15.87 μm). The cultivars had both A and B types of granules. Spherical and lenticular shapes were also observed. Some large granules seem to have pores on the surface.

The ten cultivars showed chain length distribution of between ~ 5 and 100 and 100 and 10 000 for amylose and amylopectin, respectively. There is structural variation among the cultivars and the long chains were also well observed.

A SNP was found in exon five of the *SSII* gene which was consistent in all cultivars, which can be referred to as a breeding SNP. Although the SNPs are consistent in all cultivars, there is still compositional and structural variation among the cultivars. These SNPs could not be responsible for differences in properties among the samples; in other word it would be a silent SNP.

There are two main important requirements for good brew house performance, high amylose contents and low gelatinization temperature (GT). From the results, the ten cultivars showed a high GT. According to the literature, loss in starch branching enzymes activity is responsible for increase in GT because of increase in the proportions of long chain with DP12 to 24. In addition, *BE* mutation normally lead to decreased starch which is negatively correlated with the brew house performance.

Mutation of the *SSII* gene led to starch with novel amylose content and low gelatinisation temperature. That is because of increase in proportion of short chains with DP 6 to 10 and decrease in 12 to 24 chains.

7.2 Conclusions

From the study, it can be concluded that cultivars might have the same chemical and physical properties yet they differ in fine molecular structure. These structural variation could determine starch functional properties as well as it reflects the effect of starch synthase classes, starch branching and starch debranching enzyme, the controlling genes of starch biosynthesis. In addition, GxE interaction possibly caused variation in starch fine structure and that leads to differences in composition which could result in different functional properties.

Our recommendations are therefore; further amplification and sequencing of the *SSII* gene is required, with the aim of targeting different regions for confirmation on another SNP. Characterisation of starch branching enzyme might be important to relate with the functional parameters measured.

On the other hand, different techniques are available and can be used to achieve a goal but selection of suitable technique is crucial. The RVA, SEM, PCR, laser light scattering techniques and spectroscopy are useful and commonly used in cereal research because they provide good results when the right procedures are followed.

Notably, SEC is gaining attention in the area of starch molecular structure. Few laboratories are committed in doing this analysis because of its complexity. However, efforts have been made by the SEC specialists and data has been published showing the developmental stages of SEC. SEC is promising and need to be considered in the molecular structure research of both natural and synthetic polymers.

7.3 Achievements

- Data on characteristics of local barley cultivars is available and can be used by other researches whom wish to conduct a study on these cultivars. Erica, Nemesia, Disa and Houwink are grown in Southern Cape (Dry land). Cocktail, Marthe, and Cristalia are grown in the Northern Cape (under Irrigation).
- Sequences for four pairs of primers that can amplify *SSII* gene are available and can be used by other researchers.

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Chapter 8 Appendices

8.1 pasting properties

Table 8.1 Pasting properties for the ten cultivars measured by Rapid ViscoAnalyser (RVA), these are presented as average for each parameter

| Cultivar | Peak viscosity | Trough | Break down | Final viscosity | Setback | Pasting time | Pasting temperature |
|-----------|----------------|---------|------------|-----------------|---------|--------------|---------------------|
| Metcalfe | 1184 | 145 | 1039 | 546.3 | 401.3 | 5.0 | 72.7 |
| Cocktail | 3099 | 1563 | 1536 | 3326.7 | 1763.7 | 6.1 | 72.1 |
| Marthe | 2377.7 | 1319 | 1058.7 | 2583.7 | 1264.7 | 6.1 | 85.5 |
| Cristalia | 2714.7 | 1503.3 | 1211.3 | 2989 | 1485.7 | 6.1 | 75.3 |
| Erica | 3231.7 | 1946.3 | 1285.3 | 3720.7 | 1774.3 | 6.1 | 86.1 |
| Nemesia | 2583 | 1687 | 896 | 3263.3 | 1576.3 | 6.2 | 89.2 |
| Disa | 2610.7 | 16871.3 | 926.3 | 3058.7 | 1377.3 | 6.5 | 88.5 |
| Houwink | 2857.3 | 1677.7 | 1179.7 | 3303.3 | 1625.7 | 6.1 | 90 |
| Baudin | 606.7 | 46.7 | 560 | 109.3 | 62.7 | 3.9 | 71.2 |
| Henrike | 430.7 | 29.3 | 401.3 | 57 | 27.7 | 3.4 | 68.3 |

8.2 Calibration curve

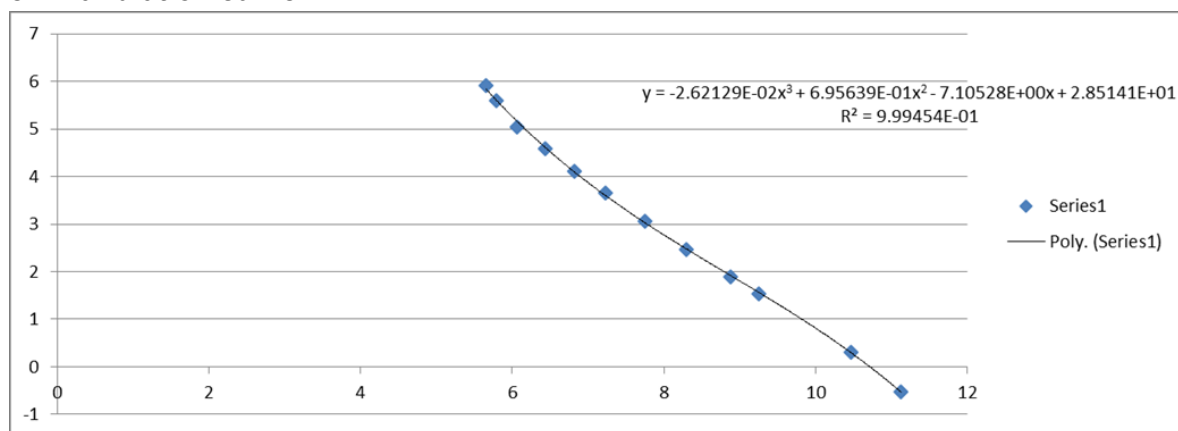


Figure 8.1 Calibration curve used to assign the hydrodynamic volume to the ten cultivars.

8.3 Chain length distribution plotted as $\ln N(X)$ for each cultivar

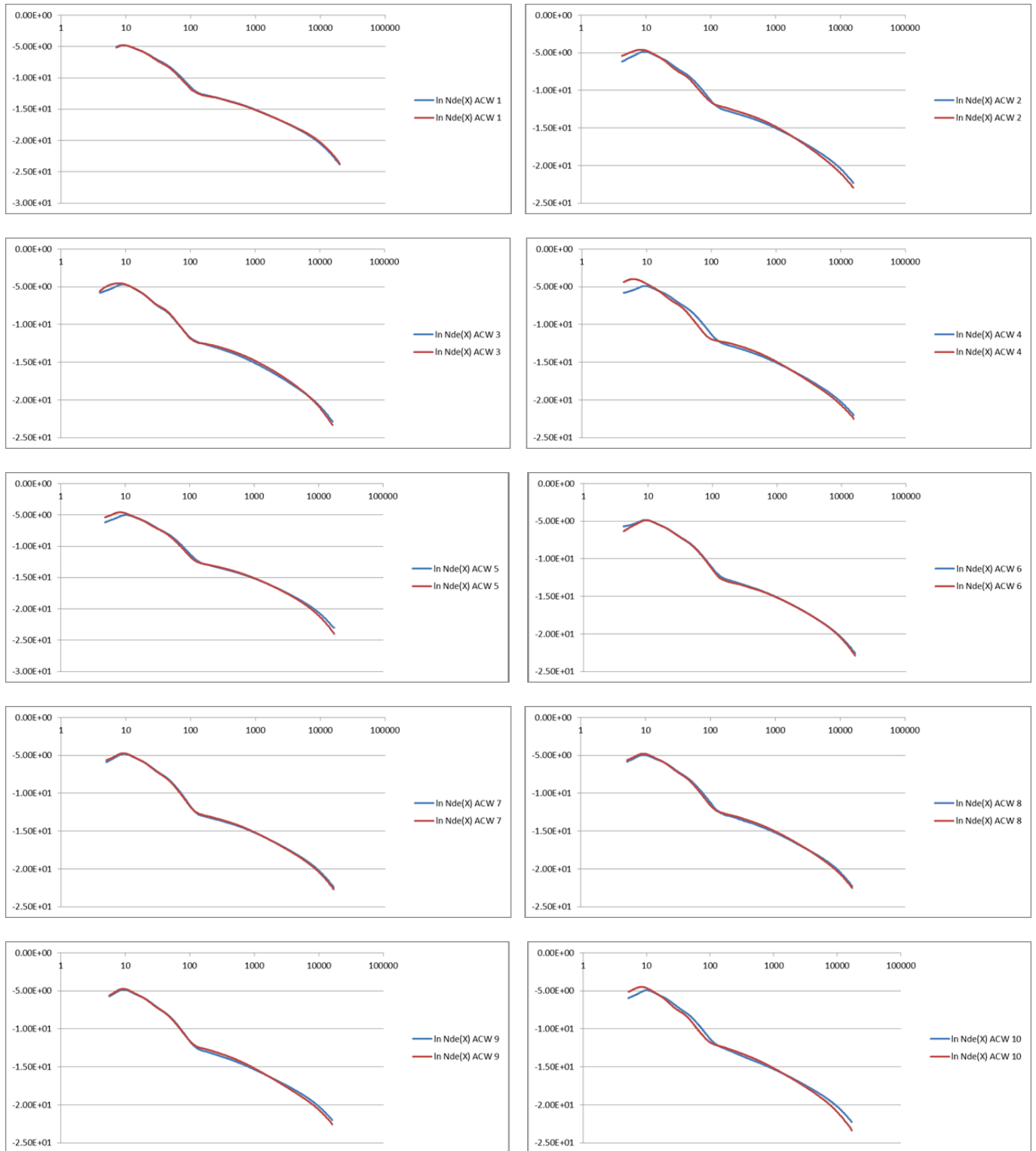


Figure 8.2 $\ln N(X)$ plots for the ten cultivars.

8.4 Average weight chain length distribution for each cultivar

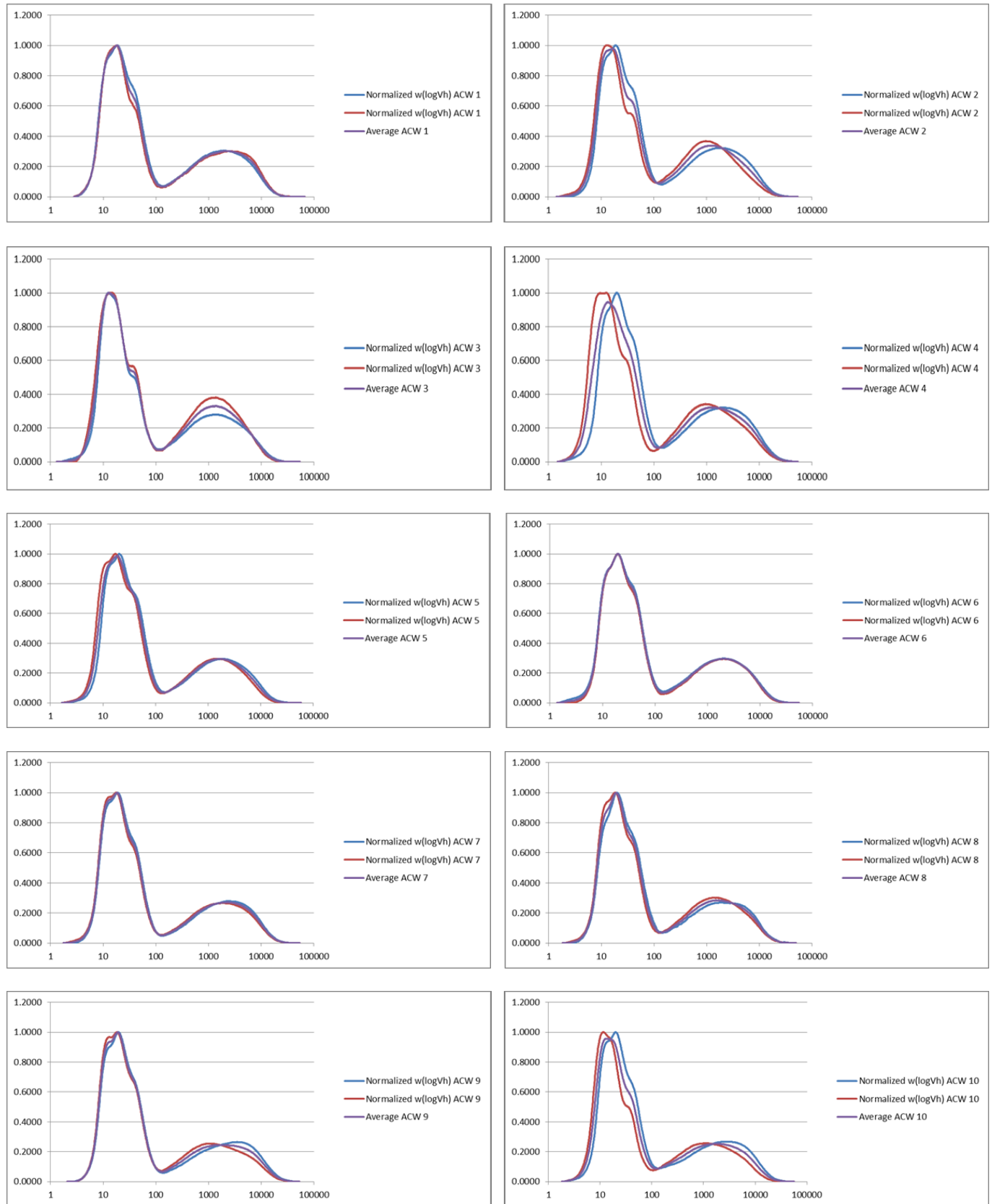


Figure 8.3 Normalised chain length distribution (CLD) for the ten cultivars.

8.5 Quality parameters measured and their R² value

Table 8.2 Quality parameters measured and their R² Values

| Parameters | Var number | R ² |
|---------------------|------------|----------------|
| Final viscosity | 5 | 0.984 |
| Peak viscosity | 2 | 0.982 |
| Setback | 6 | 0.982 |
| Trough | 3 | 0.975 |
| Peak time | 7 | 0.943 |
| Granule size | 10 | 0.857 |
| Break down | 4 | 0.845 |
| DP | 12 | 0.692 |
| Pasting temperature | 8 | 0.645 |
| Covalent phosphate | 9 | 0.247 |
| Amylose content | 11 | 0.211 |

8.6 Line plot of R²

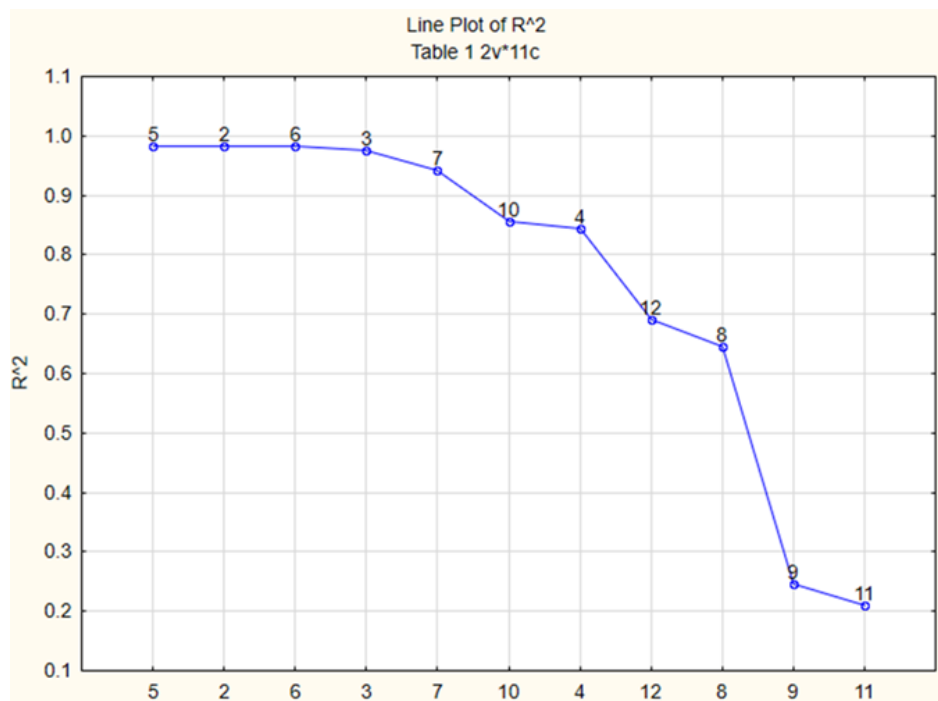


Figure 8.4 Line plot R² for the parameters measured