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Critical aspects of starch in brewing

This comprehensive review explores the structure of starch and of starch granules, the enzymology of starch synthesis and of starch degradation and the gelatinization properties of starch. It details the different types of barley starch in terms of their levels of amylose and amylopectin. The impact of starch and its hydrolysing enzymes on wort fermentability is reviewed.

Descriptors: amylases, amylopectin, amylose, analysis, fermentability, gelatinisation, interactions, limit dextrinase, starch, structure, synthesis

1 Introduction

Starch remains the primary source of carbohydrate from which sugars are derived during the brewing of beer, barley also has an inherent high level of beta-amylase and is one of the few cereals that retains a husk after threshing. Barley became the standard grain for beer production in most regions of the world as it could be grown or easily obtained. In regions where barley can not be so readily sourced, alcohol production can be achieved using other grains starch based grains such as wheat, sorghum, millet, rye, rice and maize. Regardless of the grain type, the key common attributes for alcohol production, starch and protein, are present. The complexity and variety of proteins varies between grain types, but starch composition is more consistent.

Starch is comprised of glucose monomers linked together to form chains. However, the basic composition of starch grows into a more complex structure which influences grain quality, the malting process and finally brewing efficiency. The paradox of starch is a molecule with a simple composition but a complex and varied structure (Fig. 1, see page 127).

MacGregor, in a review of future opportunities in malting and brewing, identified barley starch as a topic that required further research in several areas, not least a fuller understanding starch synthesis [1]. Today this is much better understood. MacGregor also identified gelatinization as a critical factor, as well as understanding the effect of starch granule size. Again, there is a wealth of research both in barley and other grains that has furthered our understanding here.

In 2003, *Bamforth* [2] reviewed starch and starch-degrading enzymes and concluded that starch properties and factors affecting gelatinization should also be considered along with other barley grain properties. In addition, Bamforth emphasized that the availability of starch-degrading enzymes in mashing was also crucial [2]. And again, in 2018, Bamforth further expanded his concerns about understanding starch when he stated "… that there is an urgent need to understand the interaction between barley variety, extent of modification and temperature in relation to starch gelatinization" [3].

In recent years, several publications have provided data on the relationship between starch structure and the downstream effects on malting and mashing efficiency and fermentable sugar production. These areas will be reviewed in detail below. However, some studies have specifically explored the effect of gelatinization on starch conversion and it was "recommended" that gelatinization become a routine test to better understand how malt starch will solubilize in a mash [4]. Since then, others have further elucidated the fine structural differences in amylopectin and amylose and discussed how these differences impact on gelatinization and the efficiency of enzyme activity to produce fermentable sugars. But a standard test for gelatinization has not yet been adopted by the industry. This perhaps presents an opportunity for a broad industry study of malt quality, gelatinization properties and the impacts on commercial brewing.

Over the last two decades, new research on starch structure and properties has been published in several reviews and research papers, as reviewed here. These findings challenge us to rethink the paradigm about the role of starch in brewing. More specifically, the focus needs to be on the role that the structure of starch and its gelatinization properties play in the brewing process. In this review we will consider the most recent information on starch structure and its influence on grain, malt and wort quality and variation therein, in conjunction with the starch-degrading enzymes. We will use these recent research findings to make recommendations that will help industry strive to be even more efficient.

2 Barley grain and starchy endosperm

Barley is the grain of focus for this review but in many ways the characteristics of barley starch barley is paralleled by the other grains used in brewing. The structure of all cereal grains is very

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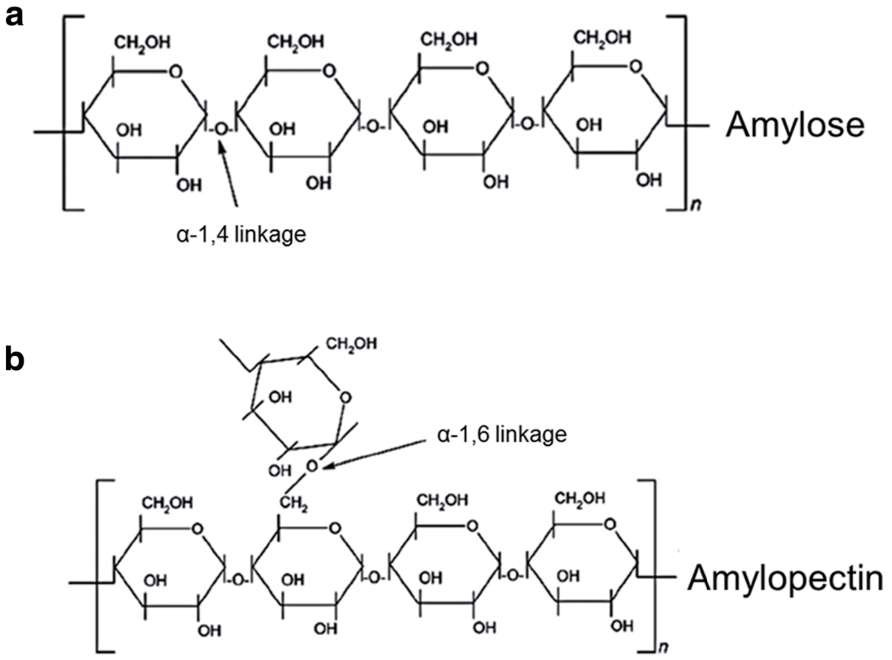

Fig. 1 Structure of (a) amylose and (b) amylopectin with α-(1 → 6) branches [13]

similar with an embryo produced in the first stages of seed development. The embryo becomes surrounded by a layer of cells (the scutellum) and then a storage compartment grows adjacent to the embryo, separated by the scutellum. Additional layers also form, grow, and expand as the endosperm fills until the grain is ready to be harvest. Thus, the three major parts of the grain important for malting and brewing (and by default, distilling), the embryo, endosperm and surrounding aleurone and scutellum cell layers. While the endosperm is the important part for a review on starch, both the endosperm and aleurone layers are key for the discussion on starch-degrading enzymes where the aleurone layers are responsible for synthesis of starch-degrading enzymes [5].

The endosperm is the storage compartment with the major components being starch and protein and lesser components (by amount) including cell wall components, phenolic acids and lipids. The endosperm is constructed with cells crammed with starch, proteins, and lipids while the cell walls are composed of mainly of β-glucan (70 %) and arabinoxylan (20 %) [5] . Research conducted many decades ago explored the physiological development of endosperm cells (amyloplasts) and showed the rate of endosperm cell growth, the number of endosperm cells, number of starch granules in a cell and amount of starch were more influence by growing environment than by genetics [6, 7]. There are between 20 x 10^4 to 40 x 10^4 endosperm cells in a barley grain but external stress such as heat influences the final volume and as such the physical size and weight of the grain [8, 9]. These early studies highlighted the effect of high temperature on starch development which are rather poignant today considering the possible future climate and the uncertainty in how grain quality will be affected.

Starch is approximately 80 % of the starchy endosperm with the endosperm storage protein, hordein, being around 70 % of the protein found in the endosperm. While this review is focused on starch there is a strong affiliation between starch and protein [7]. So, some discussion on the interaction between starch and protein will be included.

During the grain filling period, i.e. from fertilization to before the crop is ripe, the synthesis of starch commences from 3 to 5 days after fertilization [10]. Variation in starch properties can be detected as early as 9 days after fertilization and the synthesis of starch increasing until at least 35 days after fertilization with a concomitant increase in weight of thee grain, based on temperature-controlled experiments [11]. However, environmental conditions influence the rate of starch synthesis as well as the final composition [6, 12, 13]. Heat and moisture stress will reduce the rate of starch production as they influence the total number of granules and how the starch granules pack [7]. These abiotic stresses will also influence the bonding between the endosperm protein matrix and the starch granules where higher temperatures produce a more tightly bound hordein matrix, smaller granules, and more tightly packed granules. This increases endosperm texture (also known as hardness) and has negative impacts on malting potential and malting quality [14]. A softer or floury endosperm is more easily modified during malting [15].

Measuring the total starch content is a useful tool when trying to understand the full composition of the grain. Comparing total starch content to total protein content or total lipid content gives a picture of the overall grain composition. But measuring the total starch content of grain to understand malting or brewing potential does not really provide the most useful information. Total starch is positively correlated to malt extract and fermentability, but that relationship can vary and is strongly influenced by starch structure, ratio of large and small (A and B) granules, starch-degrading enzymes, malt modification and mashing conditions. While the interest is in starch hydrolysis during mashing, there is some loss of starch during malting where B granules seem to be preferentially hydrolyzed during malting.

Furthermore, measuring total starch content does not provide details about the amount of amylopectin and amylose or the ratios of these molecules within the starch component. It also provides no information on their three-dimensional structure and hence, no information on how these molecules form the starch granules. Total starch content cannot provide information on the numbers of starch granules or the ratio of A-type to B-type granules.

We now understand that all these details are more important to future malt quality and efficiency in mashing understanding than the total starch content. So, why is the specific composition of starch more important than the total amount? To answer this question, we need to discuss the components of starch in grain (amylopectin and amylose) as well as the development and structure of starch granules. This information provides insight into how the relationship between amylopectin and amylose impacts and influences grain malting quality.

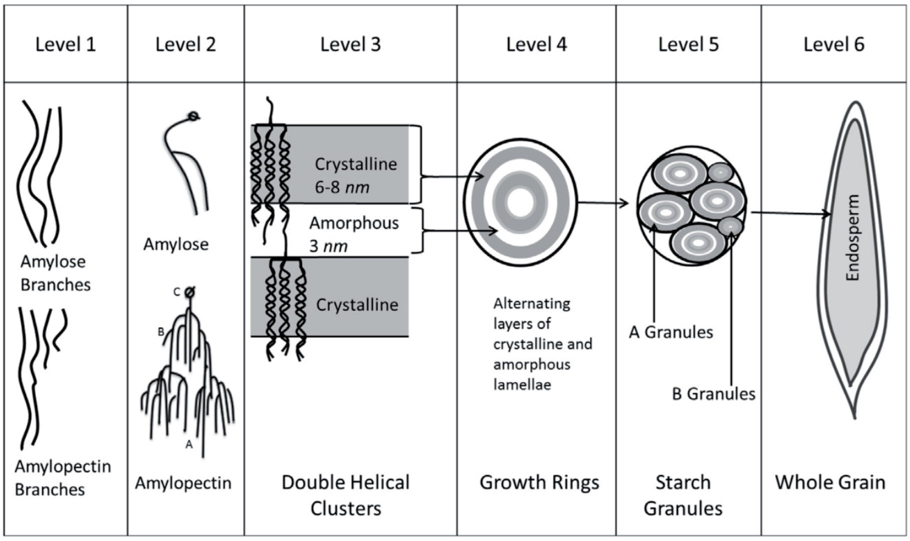

Fig. 2 **The hierarchical structure of starch [31]**

2.1 Amylopectin

Amylopectin is a highly branched (tree-like) polymer made up of all glucose monomers (Fig. 1) [2]. The number of branches, or degree of branching, varies between 4 to 20 %. The structure of amylopectin is composed of three types of chains. The C chain is the base molecule with between 6 to 10 glucose (referred to as degree of polymerization [DP]) forming this shortest chain. B chains begin at the first branching off the C chain and can vary between 12 to 36 DP in length [16, 17]. The final chain branching from the B chains are the A chains, which can elongate to greater than 100 DP. Because amylopectin branch chains are responsible for the formation of crystalline structure in starch granules, the branch chain length of amylopectin molecules significantly affects the gelatinization properties of starch [18].

A single amylopectin molecule has a molecular weight of approximately 5 x 10^8 [19] and is the primary component responsible for the swelling power and viscosity development of a starch paste after the starch granules are hydrated and the rate of swelling increases with higher temperatures [20]. Amylopectin branch chains can be very long, reaching up to 700 – 1000 DP [21]. These extra-long branches extend between the crystalline and amorphous layers of the starch granule.

2.2 Amylose

Amylose is a more straight chained polymer (Fig. 1), and has a lower molecular weight than amylopectin, and is in the range of 10^5 to 10^6 [22]. The proportion of amylose is between 20 % to 30 % of

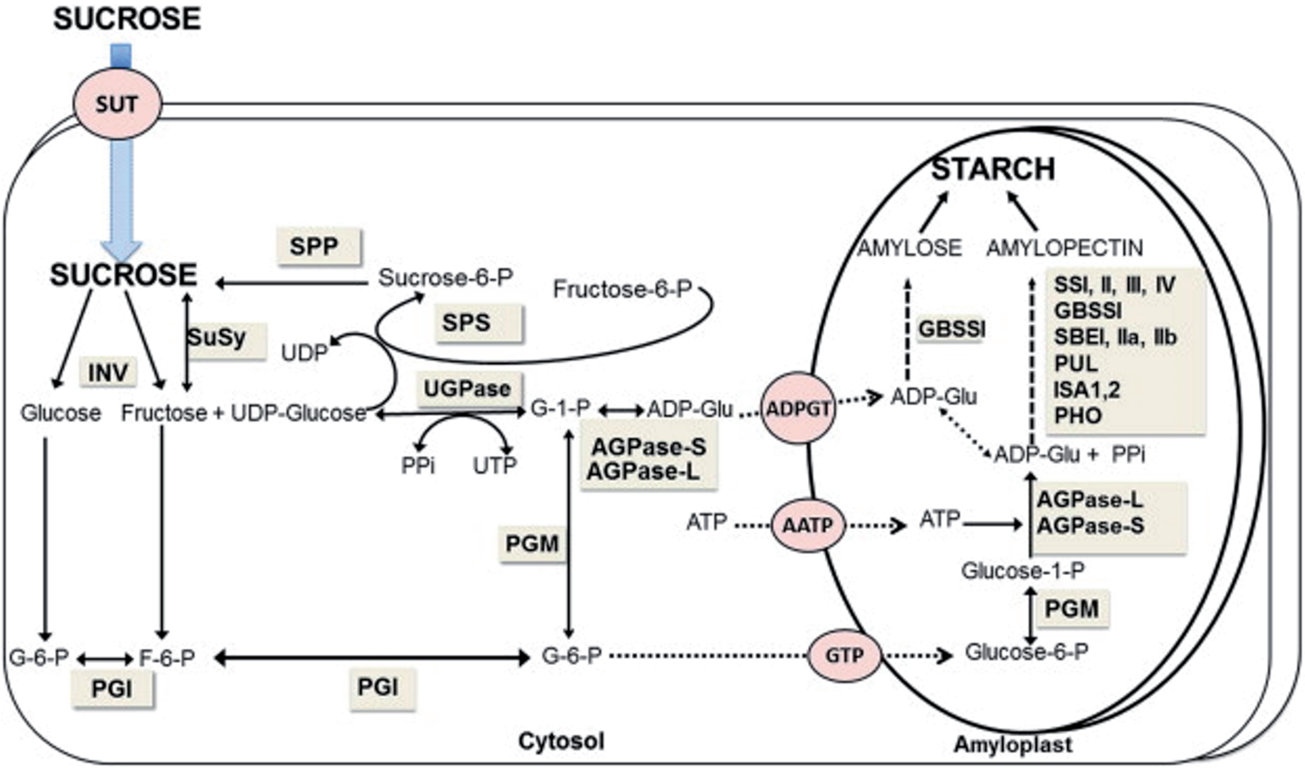

Fig. 3 **A simplified model of the starch biosynthetic pathway in a cereal endosperm cell. Enzymes are highlighted in boxes, and membrane transporters are circles. The legend is as follows. INV – invertase; SuSy – Sucrose synthase; PGI – Phosphoglucoisomerase; PGM – Phosphoglucomutase; UGPase – UDP-glucose pyrophosphorylase; SPS – Sucrose Phosphate Synthase; AGPase-S – Small subunit of ADPglucose pyrophosphorylase; AGPase-L – Large subunit of AGPase; ADPGT – ADPglucose transporter (Brittle-1 or Bt1); AATP – plastidic ATP transporter; GTP – Glucose-6-Phosphate transporter; SS – Starch synthase; GBSSI – Granule bound starch synthase; SBE – Starch branching enzyme; Pul – Pullulanase; ISA – Isoamylase; PHO – Starch phosphorylase. Effects of environmental factors on cereal starch biosynthesis and composition [28]**

the starch in normal starch barley, hence there is 80 % to 70 % of amylopectin [12]. There are some high amylose varieties with up to or greater than 50 %, produced by mutations in the amylopectin synthesis pathway [16].

The general structure of an amylose molecule is a long, sparsely-branched chain composed of thousands of glucose molecules . The small degree of branching on amylose consists of long-chain side branches (Fig. 2). Amylose chains form a helical structure that surrounds a lipid molecule, forming an amylose-lipid complex. Amylose is found in the amorphous layers of starch granules (Fig. 2) and is freely leached when granules are broken. The rapid release of amylose adds to the rate that an increase in viscosity will occur.

3 Starchy off-types and quality

When amylopectin is over expressed or in some cases, amylose is under-expressed, we have very high amylopectin (waxy). Conversely, when amylose is over-expressed, we have a high amylose type. There have been numerous studies exploring the genetic variation in the starch synthesis genes (Section 4). Several studies have highlighted variations in structure of very high amylopectin barleys [23], based on genetic changes in starch synthesis proteins. However, at best, these only measured test weight on the grain. However, it has been shown that there are variations between waxy and high amylose barley and both forms exhibited unacceptable malting quality when compared to a normal starch barley [24]. However, this study only looked at the total amounts of amylopectin or amylose, and not the structural impacts of varying their ratios. Other studies have also investigated the fine structure of amylopectin and amylose and malting quality in normal starch barleys [25-27].

4 The synthesis of starch

Starch is an overall term for the complex interaction between amylose and amylopectin and their deeper, three-dimensional structure. To understand these molecules and their interactions, it is necessary to know how they form in the endosperm. Two synthesis pathways occur simultaneously; (i) a simple pathway where the granule-bound enzyme starch synthase (GBSS) adds glucose molecules to the non-reducing end of glucose chains to produce amylose; and (ii) the more complicated multiple enzyme pathway where starch synthase (SS) I, SSII, starch branching enzyme (SBE) and starch debranching enzyme (SDE), add on glucose molecules to produce amylopectin (Fig. 3) [28].

The SBEs and SDEs add and remove branches (composed of glucose chains) via an α-(1 → 6) linkage, respectively (Fig. 3). The internal conditions dictating the relationship between SBE and SDE, and what governs the timing of branching or de-branching have yet to be understood. While SBE is important for adding branches to chains, rather less studied is SDE. Several SDEs exist in barley, but isoamylase is the most abundant. However, another SDE, more familiar to brewers, is limit dextrinase. Limit dextrinase, is one of the key starch degrading enzymes developed during malting (Section 9), and as such, is of particular interest to the brewing community.

Table 1 Variation in amounts of amylopectin and amylose

Type	Amylopectin (%)	Amylose (%)
Normal	70 – 80	20 – 30
High amylopectin (waxy)	> 90	< 10
Hi amylose	< 40 – 50	> 50 – 60

Limit dextrinase has been identified in the developing barley seed [29]. During seed development, the expression of limit dextranase decreases toward the end of grain fill and any limit dextrinase present at that point is bound to an inhibitor protein. During germination, additional limit dextrinase is synthesized and the bound limit dextrinase is released. However, there is no correlation between limit dextrinase activity during grain fill and limit dextrinase activity during germination suggesting independent regulation of the debranching function. During grain fill, limit dextrinase is controlling the degree of branching for amylopectin and granule structure while during germination, limit dextrinase hydrolyses α-(1 → 6) branches to enable other enzymes to produce sugars for the growing embryo.

The starch branching enzymes are not limited to adding branches to amylopectin. They will also add long-branch chains of glucose molecules to existing amylose molecules. However, the key enzyme for amylose synthesis is GBSS which is located within the granules. Granule-bound starch synthase remains within the granules after seed ripening and, like most of the starch synthesis enzymes, can survive intact through the malting and brewing processes. This is also the case with proteins external to, but associated with, the granule like hordeins, the hordoindolines, and transporter proteins, which are also found in beer [30].

The starch synthesis process is a highly evolved and efficient metabolic system. If genetic mutations occur that modify the function of any the starch synthesis enzymes the branching structure of amylose or amylopectin as well as the ratio of these molecules can be altered. For example, a mutation in GBSS will result in a decrease in amylose, and in some cases almost no amylose production. This form of high amylopectin grain is termed waxy. Conversely, if any of the amylopectin synthesis enzymes are modified then an increase in amylose will be the result, although a starch with approximately 40 % amylose composition appears to be the maximum possible deviation. Severe mutations in the amylopectin pathway is usually lethal to grain development. High amylopectin or amylose forms of barley have been studied but often show little potential with limitations in grain yield and grain quality [23]. Table 1 shows some of the variation between amylopectin and amylose that can result from a disruption in the normal synthesis pathways.

The focus is often on the synthesis of starch in the developing grain, but starch is produced in the growing plant as well. Starch is produced during the night portion of the diurnal cycle. The starch is broken down by the same enzymes used by germinating grain into glucose and used to make sucrose. Very little research investigating starch structural variation in plant systems other than grain has been undertaken. However, in both the growing plant and the developing grain, the production of starch is as a carbohydrate source and energy reserve.

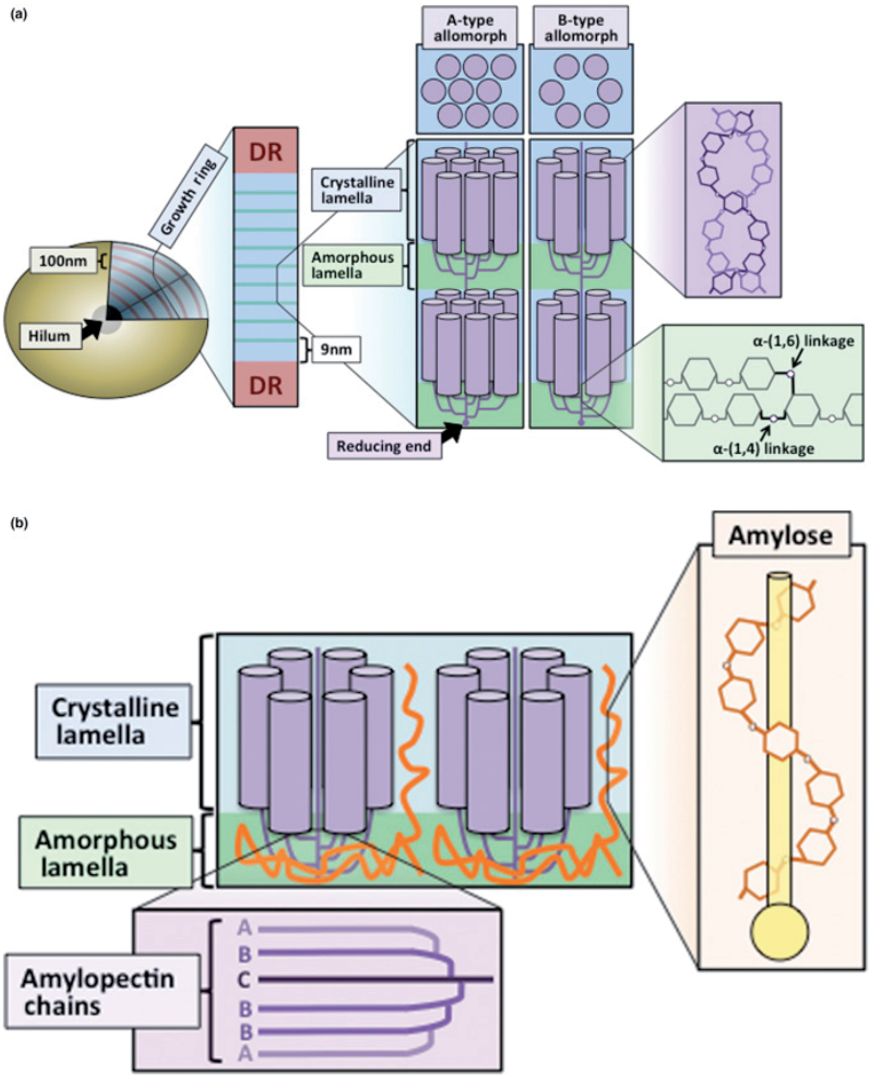

Fig. 4 **Amylopectin and amylose within the granule. (a) Diagrammatic representation of the molecular organization of the starch granule and amylopectin, with macro- to microstructure illustrated from left to right, respectively. (b) Diagrammatic representation of amylopectin glucan chain classification and the intercalation of amylose within the crystalline region of a starch granule growth ring [32]**

5 Fine structure

The components of starch can be divided into six structural levels ranging in size (from nm to mm) and complexity and corresponding to their location within the starch granule structure, as shown in figure 2 [31]. Individual linear starch molecules comprised of α-(1 → 4) linkages are the first structural level (Fig. 2). The second structural level is classified by branched starch polymers composed of individual linear molecules interspersed with α-(1 → 6) linkages. Structural level three refers to the clusters of double helices formed by the vast number of short branches of amylopectin, which further develop into the alternating layers of crystalline and amorphous lamellae- the fourth level of starch structure (Fig. 2). Many these alternating layers of crystalline and amorphous lamellae finally form the ‘growth rings’ in the granule as the fifth level of starch granule structure. Figure 4 shows a more graphical illustration of the interactions and placement of the molecules where amylopectin forms the crystalline layer and amylose locates itself in the amorphous layers [32]. But amylopectin can extend itself across an amorphous layer, increasing the crystallinity of the structures.

Figure 3 divides the development of starch during the grain fill period into a linear sequence. If the availability of glucose is not a limiting factor, amylose chains can grow to more than 50,000 DP and the A chains of amylopectin could grow to over 100 DP. Glucose production pathways in a living plant system often have reduced enzyme activity due to influences such as heat and moisture stress during the time constraints of grain fill. This impacts on the availability of glucose for chain elongation and results in much shorter chains [33]. We can see the impacts these stresses have during grain fill through physical characteristics such as a higher level of screenings, reduced grain size and increased protein content (specifically hordein content) [34]. These in turn influence gelatinization, starch hydrolysis and fermentable sugar production [35].

6 Measuring starch quality, content, and structure

Standard malt and beer analysis laboratories are not geared towards undertaking complex analyses of starch quality. However, this type of specialized research has given us a better understanding of the fine structure of starch and how that affects starch quality regarding downstream brewing performance.

The basic structure of amylose and amylopectin (Levels 1 and 2 in Fig. 3) may be characterized using Nuclear Magnetic Resonance (NMR), size exclusion chromatography (SEC), fluorophore-assisted carbohydrate electrophoresis (FACE) and multiple-angle laser scattering (MALLS). The lamellae of starch, with each lamella being about 9 nm in width, can be characterized using X-ray diffraction (XRD) and small angle X-ray scattering (SAXS). The growth rings vary from 120 nm to 500 nm and can be analyzed by scanning electron microscopy (SEM) and sometimes by confocal laser scanning microscopy (CLSM). Granules can be characterized using light microscopy or CLSM.

Size-exclusion chromatography

Size exclusion chromatography (SEC – a type of gel permeation chromatography) has been widely used to determine the molecular size distributions of (bio)polymers by sorting molecules according to their size in solution [36, 37]. Large polymers elute early as smaller polymers are caught up in the column matrix. SEC is used in conjunction with different detectors including MALLS and differential refractive index (DRI). Combining such detectors provides meaningful information concerning the molecules under investigation, including information on both linear and branched polymers. MALLS can measure the weight-average molecular weight and z-average radius of gyration of molecules (*R*g) with a given molecular size, while the DRI detector defines the total weight (concentration) of those molecules. A SEC profile for amylopectin (peak 1) and amylose (peak 2) is shown in figure 5.

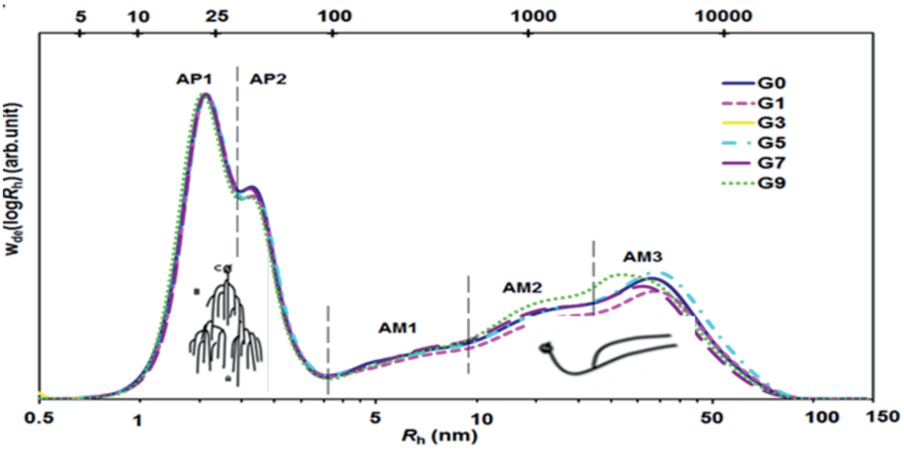

Fig. 5 **Size exclusion chromatograph showing amylopectin in the first peak, with the size of B and A chains as AP1 and AP2, respectively. Amylose is seen as AM1 DP <700, AM2 (DP < 1600 and AM3 (DP < 10,000). The X1 axis shows size of the molecules with the X2 axis showing DP. The Y axis shows the molecular weight (modified from [17])**

These technologies are expensive but necessary for quantifying the fine structure of polymers like starch. With this level of structural analysis and quantification we can then better understand the effect of the starch synthesis enzymes discussed above. While these methods and technologies provide quantitative data on structure, they do not give any indication of how the starch synthesis enzymes interact and importantly how quality the enzyme can start to hydrolyse starch depending upon gelatinization temperature. Understanding these interactions during grain fill would ideally involve running complex real-time PCR or other quantitative enzyme assays simultaneously on all the starch synthesis enzymes in barley. These are costly and unrealistic options at present so current best practice involves using modelling to investigate the activity of starch synthesis enzymes.

Rapid ViscoAnalyzer

The rapid viscoanalyzer (RVA) is a heating and cooling viscosity method that measures the viscosity of a sample over a specified time while it is stirred. During a RVA test, the process of pasting is observed in a starch or wholemeal flour and water slurry subjected to constant stirring and heat, i.e. the formation of a gel as the starch granules swell and totally dislocate following gelatinisation. The test is divided into 5 stages:

- (1) the addition of water to the starch/flour sample;
- (2) heating;
- (3) holding at a maximum temperature;
- (4) cooling; and
- (5) a final holding stage.

Consequently, the observed RVA profile reflects the complex interactions of the starch and water, affected by temperature and time. (Fig. 6). A recent review [38] highlighted many years of study investigating variation in starch properties and linking these to barley and malt quality [39, 40], based on the rate and temperature at which starch becomes gelatinized, then the subsequent changes in viscosity as the paste cools and further changes in viscosity based on starch properties.

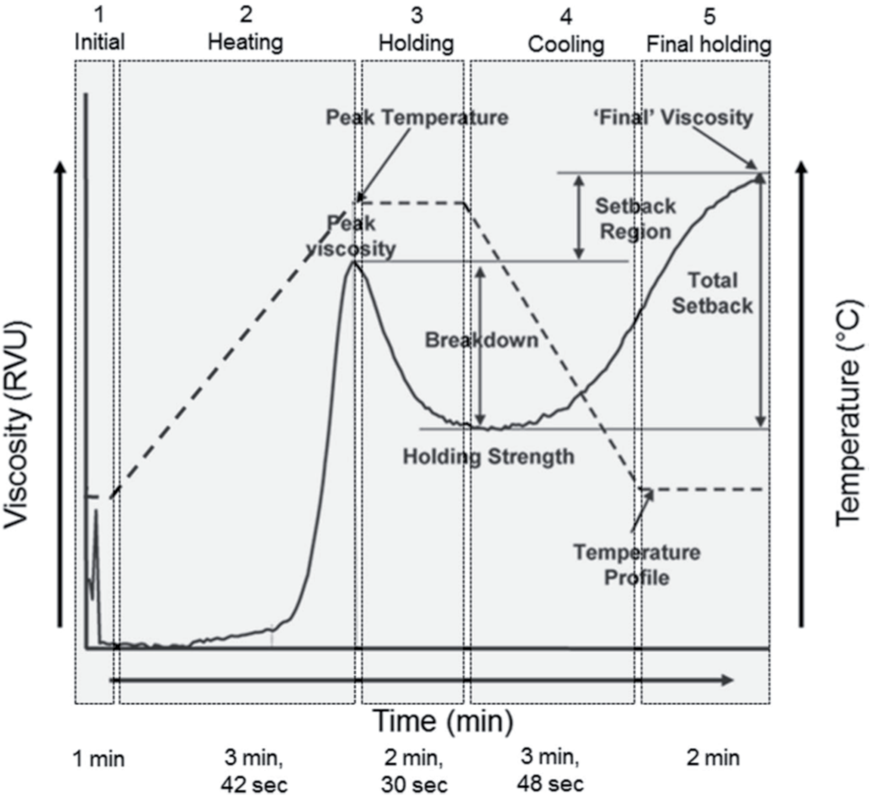

Fig. 6 **Temperature profile and changes in paste viscosity using a Rapid ViscoAnalyzer**

6.1 Mathematical fitting of amylopectin and amylose chain length distribution

While it is possible individually measure starch synthesis enzymes in an *in-vitro* environment, it is not as yet possible to do so *in situ*. To that end, researchers have developed models to predict the chain length sizes and their distribution in amylopectin and amylose. This was initially done for rice, where genomic analysis revealed the gene sequences and amino acid sequences of all starch synthesis enzymes.

Using laboratory-determined fine structure and chain length distribution (CLD) data, the CLDs can be fitted to both amylopectin and amylose models of starch synthesis. The two models reduce these data to a small number of biologically meaningful parameters. These in turn can be used to find statistically valid correlations between molecular structure and the properties of interest.

The amylopectin CLD can be fitted with the Wu-Gilbert model [41]. This model assumes that the CLDs of amylopectin chains are produced by the action of three sets of starch biosynthesis enzymes: 1. starch synthase, 2. starch branching enzyme, and 3. starch debranching enzyme. The amylopectin CLD is controlled by several enzyme sets, with each set comprising one each of an isoform of SS, SBE and SDE. The model has two parameters for each enzyme set: for the i^{th} set, these are $\beta_{\text{Ap},i}$ (the subscript Ap is for amylopectin), the ratio of the activity of SBE in that set to that of SS, and the relative contribution of each enzyme set to the entire amylopectin CLD, $h_{\text{Ap},i}$. For example, a sample with a high value of $\beta_{\text{Amylopectin},\,i}$ has high activity of SBE and/or low activity of SS in enzyme set (i) which results in a low rate of elongation for chains confined to a single lamella ($X \leq 30$), and thus more short amylopectin chains over the whole single lamella range.

The above discussion is important to better understand how the starch synthesis enzymes build the fine structure of starch, making it is easier to explain some of the vagaries around the relationships between starch and malting and brewing performances. But it is not starch alone that is involved, as several other players are present with starch and play a role in starch hydrolysis.

7 Starch has many close friends

The purpose of germination is the development of enzymes to degrade some of the endosperm substrates into smaller molecules that supply energy to the growing embryo. The endosperm is essentially a storage unit, is packed with starch and protein during grain-fill.

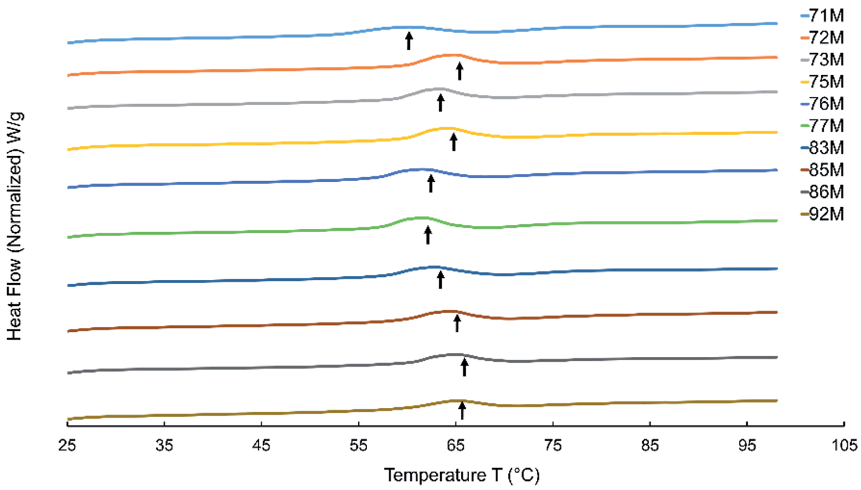

Fig. 7 **Variation in gelatinization temperatures between malt samples [27]. The arrow points to the peak gelatinization temperature**

While we are focusing on starch, there are several proteins that associate with the starch granules, other than the starch synthesis proteins within the granules as well as the ever-present hordein matrix [41]. Surface proteins play a role of restricting the swelling of starch granules during pasting [42]. Some proteins are in the interior part of starch granules, which include a diverse group of proteins having different biological functions, including the starch synthesis enzymes [43, 44].

7.1 Hordeins

An increase in total protein, means an increase in hordein content as hordein is approximately 20 % to 40 % of total protein. The environmental conditions that result in a reduced starch expression and smaller grain size, usually results in higher protein content, and higher hordein, which in turns results in a tighter packing of starch granules, impacting on endosperm texture (hardness) [45, 46]. By default, a higher protein will result in lower starch with negative impacts on texture. A low protein, high starch grain will have a flourier (softer) endosperm which is a result of more amylose, with large (A) granules [19]. Grain hardness is a physical measure and is positively related to traits such as high malt friability, higher wort extract and higher wort fermentability and variation in fermentable sugars where a softer grain is better for these traits [15, 28, 47, 48]. Low protein and low hordein is positively related to higher grain hydration rates, higher friability, and improved malt modification [26]. However, the up-side increase in protein content is an increase in β-amylase as there is a portion bound up in the hordein [35, 49] while some beta-amylase is free. An increase in β-amylase should also result in an increase in wort maltose, but the amount of extract is negatively related protein content, reported as early as 1930 [50], so less starch will be available in the mash. More on β-amylase in section 9.2.

7.2 Other proteins

There are smaller proteins bound to the granules which are believed to bind to the hordein as well. These are known as friabilins [51] and hordoindolines [15] which have different roles. These, in conjunction with the amount of hordein and the size and packing of the starch granules, give a texture to the endosperm [52]. Lower amounts of these combined with more large granules as well as thin endosperm cell walls give a softer or floury textured endosperm. Thus, a flourier endosperm is more conducive to rapid endosperm hydration as well as positive relationships to malt extract [52]. Another protein bound in the granules is protein Z4 which survives malting, brewing and has been linked to foam properties [53].

7.3 Lipids

Most cereal grains are low in lipid and fatty acid content (less than 6 % w/w) and with barley its around 2 – 3 % [54]. Most lipids are in the embryo but there are also lipids found in the endosperm and specifically with starch granules. These are mostly overlooked in any quality testing but impact several aspects of malting and brewing efficiency. It has been suggested that endosperm lipids associated with starch, influence the gelatinization temperature, leaching of soluble polysaccharides, and contribute to the swelling of starch granules [21, 54]. As seen in figure 4 there is a lipid internally bound within amylose and several studies have shown how the presence of lipid can reduce the hydrolysis of amylose [55, 56]. Other than the possible negative effects of lipids on starch gelatinization and digestion, they can adversely affect beer quality with negative impacts on flavour and foam stability. However, 18 : 2 lipids have been shown as an essential nutrient for yeast [57]. The level and the type of lipids in beer depend on the raw materials (barley and malt) as well as the brewing process [54].

8 Gelatinization and fermentability

Starch gelatinization is the irreversible disruption of the organized molecular structure within the starch granule exposed to water and heat. When starch granules are exposed to hot water, the amorphous regions (composed of mostly amylopectin) begin to swell first. As the starch molecules begin to dissolve and are "opened up," so that water molecules progressively become associated with the hydroxyl groups (-OH) on the outside of the molecule. This swelling disrupts the layered structure of the granules. Smaller granules which typically have higher amylose levels and more protein on their exterior, gelatinize at slightly higher temperatures than the more amylopectin-rich large granules. Gelatinization also depends on starch granule composition (amylose to amylopectin ratio, proteins, and lipids), molecular structure of amylopectin (chain lengths, degree of branching, molecular mass) and the average chain lengths of amylose.

Before starch structure could be quantified, it was possible to investigate the effect of starch granule sizes. Large-granule barley starches gelatinize earlier than small ones, despite their higher enthalpies [56]. The gelatinization range of small granules is wider and is attributed to the higher number of granules per unit weight of starch [10] with B granules being around 80 % of total granules in number. B-granules may negatively affect malting and brewing [57]. While ideally little starch should be degraded during malting, about half of the B-type granules are degraded, representing a decrease in ethanol production [59] but the developing embryo does need sugar so granules in endosperm cells close to the scutellum and aleurone layers would be partially degraded during germination [60]. Furthermore, the small B granules remaining after malting only partially gelatinize during mashing, while the large A-type granules fully gelatinize. These residue partially degraded B granules could pose a problem in beer quality with 'retrograded starch' a risk of causing beer haze.

In relation to fine starch structure, both the length and degree of branching of amylopectin and average chain lengths of amylose impact on gelatinization temperature in different ways [61]. For example, gelatinization temperature is negatively correlated to the amount of short amylopectin chains (B and A). The effect of this is a negative correlation between $h_{\mathrm{Ap},3}$ (the amount of longer amylopectin chains, DP 37 ~ 64) and maltose and maltotriose content [30].

The total amylose content is positively correlated with gelatinization temperatures of malt but the molecular size of amylose can be negatively correlated with gelatinization. The positive correlations between $\beta_{1,\mathrm{Am}}$ and gelatinization temperature supports the current understanding that short amylose chains are important in determining gelatinization temperatures because of the way they affect crystallinity. *Quek* et al also found the effect of amylose and amylopectin chains lengths and fermentable sugars but the latter was also influence by the starch hydrolyzing enzymes [36].

When testing malt quality, measuring a fermentability of wort is based on a standard three-day test with a specified yeast. Such as method is highly used within barley breeding programs [62, 63]. However, this is a time-consuming process. Researchers have with varying degrees of success used malt traits to predict fermentability for convenience [64, 65], but these were focused more on starch-degrading enzymes and malt modification. Patindol et al, however measured the fine structure of amylose and amylopectin and developed a model to predict real degree of fermentation (RDF) [19]. These authors included gelatinization temperature with starch structural sizes and found useful models to predict RDF. The models improved with gelatinization temperatures added.

Before discussing the starch-degrading enzymes, we should summarize. Starch is the source of fermentable sugars for beer fermentation. While measuring starch structure is very complex and detailed and it unlikely there is a single genetic alternation in the starch synthesis genes which would be easily expressed and stable across environments. However, there are some simpler sur-

Table 2 Starch-degrading enzymes

Enzyme	E.C. number	Mode of action	Main products	Notes
α-Amylase	3.2.1.1	Endo, hydrolysing α1 – 4 linkages	Dextrins	pH optimum 5.5 Ca^{2+} stabilizes
β-Amylase	3.2.1.2	Exo, hydrolysing alternate α1 – 4 linkages commencing at the non-reducing end	Maltose, branched dextrins	pH optimum 5.2
Limit dextrinase (pullulanase)	3.2.1.41	Endo, hydrolysing α1 – 6 linkages	Linear dextrins	pH optimum 5.5 Released from inhibitor pH is lowered from pH 5.5 to 4 or when the mash temperature exceeds 55 – 60 °C
α-Glucosidase	3.2.1.20	Exo, hydrolysing successive α1 – 4 linkages commencing at the non-reducing end.	Glucose	Far more activity on dextrins than on high molecular weight starch pH optimum 3.5 – 4.5 Low thermal stability

rogate methods that provide more applicable information to look at. Barley texture is a relatable trait as its common in the wheat industry for classifying quality for specific baked goods. Soft barley grain is related to higher malt friability which is positively related to higher extract and fermentable. Thus, soft barley is the better quality and grain hardness is easily measured.

Gelatinization temperature is driven by several factors. Early studies identified temperature including high night-time temperatures as a risk of increasing gelatinization temperature [66-70]. But regardless of the drivers of gelatinization, however a gelatinization temperature lower that 65 °C is best for the optimal hydrolysis of starch by enzymes in the mash.

9 Starch-degrading enzymes

There are four enzymes that are significant in the degradation of starch in cereals (Table 2).

9.1 α-Amylase

α-Amylase is unlikely to be in limiting quantities in a mash unless the malt is substantially replaced by adjuncts [71]. Furthermore, it is extremely heat-tolerant if calcium is present. Even so, there had been the pursuit of increased α-amylase expression in barley. The α-amylase gene families in barley have been identified and characterized [72].

α-Amylase has an endogenous inhibitor in grain named barley amylase-subtilisin inhibitor (BASI) [73]. Its level in raw grain is correlates directly with the level of α-amylase developed during germination. There are significantly higher levels in malting varieties than in feed varieties. It is important to note the inhibitory activity of BASI is lost in the mash between 50 °C and 70 °C, so it is not considered to have a substantial effect on starch hydrolysis during mashing [74].

Muller [75] and Muller & *Canterranne* [76] studied the impact of mashing at very-high gravities (1.2 – 1.7 : 1 water to grist). In very thick mashes, the enzyme system becomes limited for water. It was shown that α-amylase from different sources display different abilities to function at these very low water: grist ratios, partly because they vary in the extent of product inhibition by the sugars generated Muller [77] does observe that the starch-degrading enzymes are more tolerant of heat in thicker mashes.

9.2 β-Amylase

β-Amylase is more sensitive to heat than is α-amylase, although the product maltose boosts its stability [78]. It is unusual amongst malt enzymes in being present in barley unmalted, where it is attached to an inhibitor protein. The serine proteinase inhibitor (serpin) Protein Z may interact with β-amylase [79, 80].

Barley contains two active forms of β-amylase: Bmy1 is the endosperm-specific enzyme, and Bmy2, the less prevalent so-called “ubiquitous β-amylase” [81]. The latter enzyme is the more tolerant of heat.

Various factors may impact the activation of the enzyme during malting [82, 83]. β-Amylase is released from its binding to a serpin during germination. There appears to be an initial cleavage of -S-S- bridges between proteins, the disruption of hydrophobic interactions and finally proteolysis by enzymes that require -SH groups for their activity [84]. Serine proteinases may be involved [85], releasing a fragment of approximately 4,000 in molecular weight from the carboxy terminal [86] thereby increasing the enzyme’s relative thermostability [87]. Specifically, Bmy2 increased thermotolerance is due to a deletion of the4 kDa C-terminal tail, resulting in a ~ 3 – 4 °C difference in thermotolerance. Although free Bmy1 is reduced in size form 60 to 56 kDa during malting and mashing, it has a similar level of thermotolerance to Bmy2 [87].

Ma et al. [88] note the higher affinity and catalytic activity of β-amylase with higher molecular weight starch as opposed to the dextrins produced by α-amylase. Barley contains four allelic forms of β-amylase that vary slightly in their thermotolerance [89]. Recombinant substitution of seven amino acids in β-amylase boosts its ability to withstand heat [90]. Ma et al. [91] have explored this further. There is much less of this enzyme in other cereal employed in brewing, such as wheat and sorghum, than there is in barley [92, 93]. The importance of β-amylase as the limiting enzyme in starch degradation during the early stages of mashing has been highlighted [94]. Vinje et al [95] indicate that it may be possible to boost the levels of β-amylase expression in barley without increasing levels of storage proteins, despite the prior belief that they could not be separated. The rationale is that this is on account of de novo expression of the Bmy2 gene during germination. One key factor, particularly for grists of relatively low β-amylase level, is the extent to which protein impedes attack on starch [96].

9.3 Limit dextrinase

There is a single copy of the gene for limit dextrinase in the barley genome [97]. Its transcription maximized after 5 days of germination, somewhat later than that of a-amylase and indicating that prolonged germination is needed for more fermentable worts. In a lager malt kilning regime with a maximum grain temperature of 75 °C during a 24-h period, some 75 % of the limit dextrinase survived [98]. For an ale regime (maximum grain temperature of 95 °C), there was 13 % survival of the enzyme. Contrary to earlier beliefs, it has now known that limit dextrinase is of comparable thermostability to β-amylase and that the earlier supposition that it was particularly heat-sensitive was on account of purified enzyme having been used in the investigations [99] but that its action is limited during mashing because it is in an inactive bound form [99, 100] and not freed until higher mash temperatures ie. 55 °C to 60 °C and active at 65 °C [101]. The enzymes can be released at low pH [98, 102]. Any factor that increases the extractability of the total quantity of enzyme (free and blocked) as well as factors that promote the release of the enzyme from its inhibition, will therefore, increase fermentability.

Hammer milling of grain increases extract yield and enhances the release of all enzymes, including limit dextrinase [97]. MacGregor et al. [103] showed that this enzyme can be greatly freed from the limit dextrinase inhibitor (LDI) by the reducing agent dithiothreitol (DTT). This may explain why “anaerobic” malting enhances levels of limit dextrinase. It has also been suggested that proteinases might eliminate the inhibitor [104]. This may be a cysteine proteinase. *Cho* et al [105] showed that the level of limit dextrinase expressed in barley is enhanced if the barley overexpresses thioredoxin. This suggests that there may be a relatively specific reducing action occurring during germination that leads to the release activation of limit dextrinase from its binding. *Stenholm* and *Home* [98] showed that there was a much closer correlation between free limit dextrinase activity and fermentability than there was with total limit dextrinase. In turn, fermentability correlated rather less well with α- and β-amylase. *Bathgate* [106] discusses limit dextrinase in the context of whisky fermentations. Of course, in these it is the case that the fermentation is of worts that have not been boiled and therefore the enzymes that survive mashing maintain their activity. During fermentation, the drop in pH leads to an activation of limit dextrinase [107]. In a practical brewery context it is likely that limit dextrinase makes a significant contribution to the production of fermentable sugars [101, 108]. Clearly much will depend on the degree of branching in the starch. It is also relevant to note that the enzyme has much greater affinity for the polymer pullulan (solely α1 – 6 linkages) than for amylopectin [109, 110]. Equally, another area that requires further investigation is the observation that limit dextrinase is activated by maltodextrins [109]. It is now recognized that limit dextrinase certainly contributes to DP [63, 111] and *Evans* and co-workers have delivered predictive models [63, 112-116]. When the enzyme is released from its inhibitor it is more susceptible to acid conditions and to heat, meaning that there is a relatively narrow window of opportunity for the enzyme to act [106, 108].

In their modelling work, MacGregor et al. [117] showed that the addition of limit dextrinase causes a large increase in fermentability and that limit dextrinase acts synergistically with β-amylase. They concluded that commercial malts contain an excess of β-amylase but are limited for limit dextrinase

9.4 α-Glucosidase

α-Glucosidase has attracted the least attention of the starch-degrading enzymes, although it has been claimed to be second only to α-amylase for its significance in starch degradation during malting [118, 119]. This enzyme thermolabile and is unlikely to be of significance during mashing [120]. α-Glucosidase may improve the efficiency of β-amylase action by removing maltose as a product inhibitor. *Andriotis* et al [121] calls it maltase. *Duke* et al [88] indicated that the enzyme works synergistically with α-amylase when hydrolysing ungelatinized starch granules, such that (in view of its sensitivity to heat) it would only be of relevance in the initial stages of mashing, prior to gelatinization.

The proportion of fermentable sugars in varying in wort based on several factors including malt quality, grist size, mashing conditions and enzyme level. However glucose will be around 20 % of total fermentable sugars. The low level of glucose suggests a relatively minor role for a-glucosidase [2, 113].

9.5 Exogenous enzymes and yeast

Goode et al [122] report detailed investigations of exogenous enzymes to promote starch degradation in mashing. Glucoamylases are extensively reviewed by *James* and *Lee* [123]. Not all Brewers want to (or are allowed to) use exogenous enzymes – and Evans et al. [63, 113] present relevant information on maximizing diastatic power in malts and mashes

Krogerus and *Gibson* (124] discuss diastatic yeasts, highlighting the over-simplicity of classifying them uniformly as spoilage organisms. The world's first approved genetically modified yeast featured the inclusion of a gene for glucoamylase [125].

10 Concluding remarks

Very clearly there is a considerable body of information concerning the structure of starch, the factors that impact it, its relevance of starch degradation and also on the battery of enzymes that are involved both in the synthesis of starch in grain filling but also on the degradation of starch in malting and, primarily, following gelatinisation in mashing. There remain, however, some observations that warrant further investigation, particularly from the aspect of optimising starch degradation in the brewhouse. Inter alia these include further study of the original observation of *MacWilliam* [126] that the severity of kilning of malt greatly impacts the susceptibility of starch to enzyme attack. Secondly there remains a need for a better understanding of the impact of malt modification on the gelatinization properties of starch granules, as highlighted before (2, 3).

11 Literature

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