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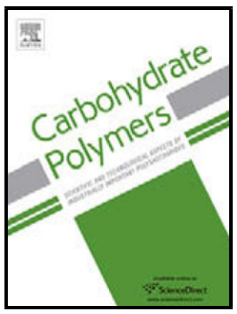

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Molecular brewing: molecular structural effects involved in barley malting and mashing

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Highlights

- Barley starch structural changes during malting are investigated
- Both starch amylose and amylopectin are hydrolyzed during malting
- Protein retards starch hydrolysis in mashing by inhibiting granule swelling
- Barleys with more short-chain amylose molecules release more fermentable sugars
- Starch molecular structure is useful for determining fermentable sugars production

Abstract

Ten barley samples containing varied protein contents were subject to malting followed by mashing to investigate molecular effects of both barley starch and starch- protein interactions on malting and mashing performances, and the underlying mechanism. Starch granular changes were examined using differential scanning calorimetry and scanning electron microscopy. The

molecular fine structures of amylose and amylopectin from unmalted and malted grain were obtained using size-exclusion chromatography. The results showed that both amylose and amylopectin polymers were hydrolyzed at the same time during malting. Protein and amylose content in both unmalted and malted barley significant negatively correlated with fermentable sugar content after mashing. While protein content is currently the main criterion for choosing malting varieties, this study shows that information about starch molecular structure is also useful for determining the release of fermentable sugars, an important functional property. This provides brewers with some new methods to choose malting barley.

Abbreviations

DP, degree of polymerization; DSC: Differential scanning calorimetry; FSs: fermentable sugars; HPLC: High-performance liquid chromatography; SEC: size-exclusion chromatography; SEM: Scanning electron microscopy

Keywords:

Barley (*hordeum vulgare* L); starch; brewing; structural characterization; molecular mechanisms; protein

Chemical compounds used in this study:

DMSO (PubChem CID: 679); sodium azide (PubChem CID: 33557); sodium sulfate (PubChem CID: 24436); Lithium bromide (PubChem CID: 82050); Ethanol

Introduction

The "art of brewing" has become over the decades more and more a rigorous science. The objective of the present paper is to examine the molecular structural aspects of one of the steps in this process: mashing.

Barley (*hordeum vulgare* L.) is one of the main materials for the production of beer. A number of stages are involved in the process of beer production, including malting, mashing, boiling (hopping) and fermentation. Malting is the process whereby barley seed is allowed to germinate for several days and then kilned by subjecting to heat (up to 85 ºC) over 24 h to stabilize the enzymic activities, inducing colour reactions, and to provide a final moisture content around 4 – 4.5% (Wang, Zhang, Chen & Wu, 2004). Mashing is the process whereby ground malted barley is mixed with hot water, leading to starch hydrolysis by malting-activated enzymes. The key change is the degradation of gelatinized carbohydrates (mainly starch) to produce fermentable sugars (Fox, 2016). Mashing is followed by a boiling stage to deactivate amylases, while hops are added for flavour and aroma. The result, termed hopped wort, is then ready for yeast fermentation to produce alcohol from fermentable sugars (FSs) (Fox, 2016).

In brewing, barley varieties are currently chosen to have protein contents of around 8 – 12% (Celus, Brijs & Delcour, 2006) (dry weight basis), as too high or too lower protein content can result in problems (Fox, Kelly, Sweeney & Hocroft, 2011): e.g., a too-low protein content means insufficient enzymes for adequate fermentation and potential issues with foam properties, while high protein can result in lower modification and fermentation problems: e.g. the presence of aggregates containing β-glucan and protein (Zielke, Teixeira, Ding, Cui, Nyman & Nilsson, 2017). Barley protein has been reported previously to reduce *in vitro* starch degradation because it reduces enzyme activity by competitively binding with degradation enzymes and by entrapping starch granules in the protein matrix (Yu, Zou, Dhital, Wu, Gidley, Fox & Gilbert, 2018c). In addition, barley protein has been reported to reduce starch degradation rate (Slack,

Baxter & Wainwright, 1979), but the underlying mechanism is not established.

Although there has been much emphasis on protein in grain choice for brewing, in fact starch is at least as important during malting and mashing processes: it is starch hydrolysis that produces fermentable sugars (Fangel, Eiken, Sierksma, Schols, Willats & Harholt, 2018). In brewing, however, the importance of starch, especially its molecular structure, has been largely ignored. Starch modification starts during malting. Information as to how starch structure changes during this process is desirable with regards to choosing barley variety and for process improvement. Currently in the brewing industry, both RVA and DSC are used to predict the malting and mashing quality of a barley genotype (Zhou & Mendham, 2005); however, RVA and DSC behaviors are determined by starch molecular structure (Cozzolino, Degner & Eglinton, 2016; Srichuwong, Sunarti, Mishima, Isono & Hisamatsu, 2005). Measuring starch structure could give a good indication of process performance and should be useful in brewing. Here we investigate the molecular structural changes in starch, and starch-protein interactions, during malting, using more advanced structural characterization techniques and data interpretation methods than those used previously.

The aims of this study are:

- To reveal any effects of barley protein on starch enzymatic degradation during malting and mashing;
- To study starch molecular structural changes during malting and mashing, including any effects of proteins;
- To find, and to explain mechanistically, statistically valid correlations between changes in barley protein, starch structural parameters and the production of FSs released during mashing.

The results from this study could in future be used to assist the brewing industry in its selection

of optimal barleys and of processing conditions, by including structural characterization as one of the tools to be used in this selection.

1. *Materials and Methods*

1.1. Materials

Ten samples with varied protein contents were used, as shown in Table 1.

1.2. Micro- malting

Micro-malting was carried out in a Phoenix automated micro-malting unit (Queensland Department of Agriculture, Leslie Research Facility, Toowoomba, Australia) to make fully modified malt as follows: 200 g barley grain was firstly steeped with water for 8 h, then 8 h air rest followed by another 8 h steeping, all at 17 °C. Following steeping, germination was allowed to occur by leaving at 17 °C for 4 days, turning barley grains every hour to avoid hot spots. Germination was ceased by kilning: the grain was slowly heated to 50 °C to remove excess moisture, and then further heated (kilning) to 85 °C over 24 h to suspend enzyme activity and to reduce the malt moisture to around 4%.

1.3. Ground of malted barley grain

Grains of both unmalted and malted barley were ground using a Stainless Steel Electric Cereal Mill Machine (FDM) (Guangzhou Itop Kitchen Equipment Co., Ltd, China) and then sieved through 500 μm. Samples were sealed and kept at room temperature for future use.

1.4. Moisture and starch contents of unmalted and malted flour

Moisture content was measured by drying the samples in a vacuum oven at 110 °C overnight and recording the weight loss in triplicates; starch content was measured using a Megazyme Total Starch kit (K-TSTA-1107, Megazyme, Ireland). In this method, a starch-containing

sample is firstly solubilized in dimethyl sulfoxide, incubated at ~ 100°C with thermostable α-amylase and then with amyloglucosidase, which results in complete hydrolysis to D-glucose, the amount of which is measured using a glucose oxidase/peroxidase reagent (GOPOD). Before measuring the starch content, the weighed flour was washed with 2 mL absolute ethanol (2 times) to remove sugars produced during the malting process.

1.5. Soluble and insoluble protein content

For malted barley, 500 mg of flour was mixed with hot water (4 mL) for 10 min to extract the water-soluble protein while the residue was freeze dried and used to measure the total insoluble protein content. The crude total protein content of unmalted and malted barley flour were calculated from the nitrogen content determined using a Leco CNS-2000 analyzer on carbon, nitrogen and sulfur (Seminole, Florida, USA) with a conversion factor of 6.25. The protein hydrolysis during malting was calculated using:

$$Protein\ hydrolysis\ \% = \frac{\text{Total soluble protein content}}{\text{Total protein content of malted barley flour}} \times 100 \quad (1)$$

The amount of protein components solubilized was measured by the Thermo Scientific Pierce™ BCA Protein Assay Kit using bovine serum albumin (BCA) as standard.

1.6. Starch extraction

Barley starch was extracted using the method described previously with minor modifications (Wang, Hasjim, Wu, Henry & Gilbert, 2014). After removing protein by protease, barley β-glucan was removed from the residue by mixing with 0.5 mL lichenase solution (100 µL enzyme mixed with 2 mL sodium phosphate buffer (20 mM, pH= 6.5; Megazyme) and keeping at 40 °C for 1 h. After centrifuging, the residue was re-dissolved overnight in 1.5 mL dimethyl sulfoxide (DMSO; Sigma-Aldrich) containing 0.5% LiBr at 80 °C. For fully branched SEC analysis, purified starches dissolved in DMSO are precipitated using 6 mL absolute ethanol

twice (once for precipitation and once for washing) and then centrifuged for 10 min at 4000 ×g. For debranched analysis, after debranching using the method described previously (Yu, Tan, Zou, Hu, Fox, Gidley & Gilbert, 2017), debranched samples are freeze- dried before being re-dissolved in DMSO/LiBr solution for further SEC analysis. Among other things, this also ensures that there is negligible residual water.

1.7. Size- exclusion chromatography

Starches extracted were analyzed using a Waters SEC-MALLS system (Wyatt Technology), equipped with dual detectors: differential refractive index (DRI) and multiple-angle laser light scattering (MALLS), as shown for example in (Vilaplana & Gilbert, 2010b). With SEC, differential refractive index (DRI) detection gives the distribution of the weight of polymer as a function of molecular size, $w(\log R_h)$: the hydrodynamic radius R_h is the SEC separation parameter. Multi-angle laser light scattering (MALLS) detection gives the overall weight-average molecular weight, $\overline{M}_w$.

Whole starch molecules are highly branched and therefore there is no relation between molecular size and molecular weight. However, there is a unique relationship between size and molecular weight for linear polymers, including the individual chains obtained from whole starch after exposure to a debranching enzyme. The distribution of the number of monomer units in individual chains — the chain-length distribution (CLD), which is the molecular fine structure — was obtained by first debranching the starch with a debranching enzyme, then characterizing the resulting chains by SEC, as described previously (Shi, Fu, Tan, Huang & Zhang, 2017; Tikapunya, Zou, Yu, Powell, Fox, Furtado, Henry & Gilbert, 2017; Yu et al., 2017). Because these are unbranched, the molecular size is directly related to the number of monomer units in the chain (the degree of polymerization, DP, X). This yields the weight CLD as a

function of DP, $w(\log X)$. For a linear polymer such as debranched starch, the relation between the number CLD (the number of chains of a given degree of polymerization X following debranching, $N_{de}(X)$), and the corresponding weight distribution $w(\log X)$ is (Castro, Ward, Gilbert & Fitzgerald, 2005) $w(\log X) = X^2 N_{de}(X)$.

1.8. Amylose content

Based on detailed comparisons between various methods for measuring amylose content, including a highly advanced two-dimensional analysis (Vilaplana & Gilbert, 2010a), the best readily-implemented way of determining amylose content is to use either the Concanavalin-A binding method (Matheson & Welsh, 1988), or by finding the ratio of the area under the amylose region divided by the total area in the debranched SEC result (Vilaplana & Gilbert, 2010a), which is at the degree of polymerization (~100) where a clear separation of amylose and amylopectin chains is seen.

1.9. Model fitting to amylose and amylopectin CLDs

To find statistically valid correlations between starch structure and functional properties, the debranched data (the weight CLD) were fitted to biosynthetic models for amylose and for amylopectin. These reduce the data to a small number of biosynthetically-meaningful parameters. Although the starch structure after malting is no longer that of the native starch, the implicit functional forms in the models are sufficiently flexible that they can often fit data for modified starches (e.g. after malting). While the resulting parameters are no longer biologically meaningful, they serve the purpose of reducing the data to a small number of parameters which provide an excellent fit to the data, so these parameters can be used for subsequent statistical analysis.

The fit to SEC CLDs of barley starch before and after malting in the amylopectin region was

implemented with the model of (Wu & Gilbert, 2010; Wu, Morell & Gilbert, 2013) using publicly available code (https://sourceforge.net/projects/starchcldfit/?source=directory). In brief, this model assumes that the overall CLD is the sum of CLDs from a number of enzyme sets. The model parameters are the ratio of the activities of starch branching enzyme to that of starch synthase in enzyme set i, $\beta_{\mathrm{Ap},\,i}$, and the relative contribution of that set to the overall CLD, $h_{\mathrm{Ap},\,\mathrm{i}}$. The fits of amylose CLDs of both unmalted and malted barley starch were implemented with a new method, which also uses publicly available code (Nada, Zou, Li & Gilbert, 2017; Yu, Li, Zou, Tao, Zhu & Gilbert, 2018a; Yu, Tao & Gilbert, 2018b). The amylose fitting parameters, $\beta_{i,\mathrm{Am}}$ and $h_{i,\mathrm{Am}}$, have the same meaning as those for amylopectin.

1.10. Enzyme activity of amylases and limit dextrinase in malted barley

The α- and β- amylase activities of malted barley flour were measured using a Malt Amylase Assay Kit (Megazyme International Ireland, Ltd). Limit dextrinase was measured using a Pullulanase/Limit-Dextrinase Assay Kit (PullG6 Method; Megazyme International Ireland, Ltd). Duplicate measurements were performed.

1.11. Micro-mashing

Mashing was conducted with 5 g grist with 20 mL of distilled water incubated in a water bath for 60 min at 65 °C , based on a method of the Institute of Brewing (high- temperature infusion (Fox, 2016)). After this, the mixed mashing liquid was centrifuged at 4000 × g for 10 min. The supernatant (wort) portion was kept in boiling water for another 60 min to deactivate the enzyme and then stored at –20 °C.

1.12. Measurement of fermentable sugars

Wort samples were diluted 20× prior to fermentable sugar analysis. Standard mixes of glucose, sucrose, maltose and maltotriose were made in the range of 1-27 μg/mL, dissolved in water.

Samples are analysed on an Agilent 1100 HPLC with an Evaporative Light Scattering Detector (ELSD). The HPLC solvent was 75% acetonitrile in water, with a 1 mL/min flow rate using an Alltech Carbohydrate column, 4.6 mm × 250 mm. The ELSD was set to a nitrogen flow rate 2 mL/min, temperature 87 °C. Samples were based on duplicate measurements.

Starch consumption during mashing was calculated using:

$$\underset{\text{solubilization}}{\text{starch}} = 100\,\frac{\text{total dry weight of starch for mashing - total dry weight of starch in barley spent grain}}{\text{total dry weight of starch for mashing}} \quad (2)$$

1.13. Differential scanning calorimetry

Thermal properties of malted barley flour were evaluated as described by (Patindol, Mendez-Montealvo & Wang, 2012) with some modifications, using a TA differential scanning calorimeter (DSC25, TA). Ground malted barley flour (5 mg dry basis) was weighed into an aluminium pan with 15 µL distilled water being added (1:3, m:v). The pan was hermetically sealed and equilibrated at 4 °C for 1 h before running. Thermal scans involved equilibrating at 20 °C for 3 min and then heating the sample from 20 °C to 100 °C at 5 °C /min. An empty pan was used as a reference. Enthalpy, onset (T_o), peak (T_p) and conclusion (T_p) temperatures were calculated using TA software from the endothermic transition peaks corresponding to starch gelatinization.

1.14. Scanning electron microscopy (SEM)

For SEM characterization, flour sample was mixed with water to a concentration of ~ 6 mg/mL, and 2- 3 µL of this was put onto double-sided adhesive tape mounted on an aluminium stub, dried and then platinum-coated in a sputter coater for preparing samples. A scanning electron microscope (JEOL 6460, JEOL Ltd., Tokyo, Japan) under an accelerating voltage of 15 kV was used to take images of the starch granules.

1.15. Data analysis

Means and standard deviations were calculated for duplicate measurements. Two-tail tests were carried out to determine significant differences between two different factors, and $p \leq 0.05$ and ≤ 0.01 were used as thresholds of significance and extreme significance respectively. Analysis of variance (ANOVA) of means was performed with SPSS univariate (version 16.0). Multiple-means comparisons were determined with Duncan's multiple range test at the $p< 0.05$ confidence level. The PCA analysis was conducted using XLSTAT 2018.

2. Results

2.1. Chemical composition of unmalted and malted barley flour

The change of chemical composition with malting is shown in Table 1 for the various barley samples, as well as the growing locations in various regions in Australia. As expected, kilning decreased the moisture content of malted barley samples. Malting resulted into a loss of starch, which can also be seen by the existence of pinholes on the surfaces of starch granules (Figure 1). Few if any pinholes were seen with small (B- type) granules (Figures 1 and S1 in Supporting Information). This is consistent with earlier findings by MacGregor and Ballance (1980) that in normal barley, as used here, differences in the physical structure of the two types of granules result in different α- amylolysis patterns. In large granules, this proceeds through pinholes from the inside out: the enzyme tends to attack at discrete points on the surface, and subsequently from pores into the granule interior, leading to pinholes. For smaller starch granules, the rough texture is quite different, leading to the hydrolysis pattern being surface erosion without pinholes. This is also consistent with reports (Myllärinen, Autio, Schulman & Poutanen, 1998) that during malting, larger granules are hydrolysed from inside, whilst no pinholes were observed in small granules, which were hydrolysed by surface erosion. No significant correlations between starch and protein contents was found, as seen previously (Yu et al., 2017).

Table 1. Compositions and provenances of unmalted and malted barley samples [*]

Genotype	Location	No.	Protein Content/% [b]		Insoluble protein content [b]	Moisture content/ %		Starch content/ % [b]		Amylose content/ %	
			Unmalted	Malted	Malted	Unmalted	Malted	Unmalted	Malted	Unmalted	Malted
Commander	Charlick, SA	70	(5.9 ± 0.0)^{a}	(4.7 ± 0.1)^{a}	(3.4 ± 0.0)^{a}	(9.9 ± 1.2)^{de}	(2.9 ± 0.3)^{a}	(51.5 ± 3.0)^{bc}	(48.2 ± 1.1)^{e}	(31.5 ± 0.6)^{ab}	(31.0 ± 0.2)^{a}
Commander	Macalister, Qld	73	(11.0 ± 0.0)^{f}	(12.1 ± 0.1)^{f}	(10.5 ± 0.0)^{ef}	(9.2 ± 0.1)$^{b-d}$	(3.7 ± 1.9)^{a}	(47.5 ± 2.2)^{a}	(44.2 ± 0.1)^{c}	(30.2 ± 2.8)^{a}	(33.2 ± 1.5)$^{b-d}$
Commander	Westmar, Qld	75	(9.8 ± 0.0)^{d}	(11.7 ± 0.0)^{e}	(9.8 ± 0.3)^{d}	(9.3 ± 0.2)$^{b-d}$	(3.3 ± 0.2)^{a}	(48.8 ± 0.4)^{ab}	(46.2 ± 0.2)^{d}	(33.2 ± 1.5)^{ab}	(34.7 ± 0.2)^{de}
Commander	Mungindi, Qld	78	(11.8 ± 0.1)^{h}	(12.7 ± 0.1)^{g}	(11.2 ± 0.1)^{g}	(9.8 ± 0.3)$^{c-e}$	(2.1 ± 0.2)^{a}	(53.0 ± 2.6)^{cd}	(43.4 ± 0.9)^{c}	(33.9 ± 0.0)^{b}	(34.1 ± 1.3)^{d}
Gairdner	Charlick, SA	82	(7.1 ± 0.1)^{b}	(6.6 ± 0.2)^{b}	(4.6 ± 0.2)^{b}	(9.6 ± 0.1)$^{b-e}$	(2.6 ± 0.3)^{a}	(50.6 ± 1.1)$^{a-c}$	(41.7 ± 0.4)^{b}	(31.2 ± 0.2)^{ab}	(32.1 ± 0.3)$^{a-c}$
Gairdner	Jambin, Qld	86	(9.15 ± 0.1)^{c}	(8.4 ± 0.1)^{c}	(8.1 ± 0.1)^{c}	(10.5 ± 0.4)^{e}	(1.9 ± 0.9)^{a}	(60.9 ± 1.2)^{f}	(44.5 ± 0.8)^{cd}	(31.1 ± 1.7)^{ab}	(33.7 ± 1.3)^{cd}
Gairdner	Wongan Hills, WA	87	(12.3 ± 0.1)^{j}	(10.8 ± 0.1)^{d}	(9.7 ± 0.2)^{d}	(8.6 ± 0.4)^{bc}	(1.8 ± 1.0)^{a}	(55.7 ± 1.8)^{de}	(44.0 ± 0.2)^{c}	(30.0 ± 0.0)^{a}	(33.1 ± 0.3)$^{b-d}$
Gairdner	Roseworthy, SA	89	(10.0 ± 0.1)^{e}	(11.8 ± 0.1)^{e}	(10.3 ± 0.0)^{e}	(9.0± 0.3)$^{b-d}$	(3.6 ± 0.8)^{a}	(59.0 ± 2.8)^{ef}	(44.3 ± 0.4)^{cd}	(30.9 ± 1.9)^{ab}	(31.6 ± 0.1)^{ab}
Gairdner	Mungindi, Qld	92	(11.49 ± 0.1)^{g}	(12.3 ± 0.1)^{f}	(10.7 ± 0.0)^{f}	(8.5 ± 0.4)^{ab}	(2.6 ± 1.1)^{a}	(48.4 ± 1.3)^{ab}	(39.6 ± 0.8)^{a}	(33.8 ± 1.0)^{b}	(36.2 ± 0.3)^{e}
Gairdner	Macalister, Qld	93	(12.14 ± 0.1)^{i}	(13.1 ± 0.2)^{h}	(11.7 ± 0.1)^{h}	(7.5 ± 0.5)^{a}	(2.6 ± 1.2)^{a}	(54.3 ± 2.5)^{cd}	(41.4 ± 0.6)^{b}	(34.0 ± 0.8)^{b}	(34.8 ± 0.5)^{de}

[*] **Data from duplicate measurements; results from dry basis; samples with different letters in the same column are significantly different at $p < 0.05$.**

It has been reported (Bertoft & Henriksnäs, 1983; Kano, Kunitake, Karakawa, Taniguchi & Nakamura, 1981) that after malting, amylose content increased as a result of the preferential enzyme hydrolysis of amylopectin, as there were no intermediate polymers created after malting. In our study, however, no significant changes in amylose content was observed after malting, as also reported for rice germination (de Guzman, Parween, Butardo, Alhambra, Anacleto, Seiler, Bird, Chow & Sreenivasulu, 2017). Now, it could be postulated that, during malting, amylopectin molecules may have been hydrolyzed into very low molecular polymers that might not be seen in measuring the amylose content. However, this possibility can be precluded because, as shown in Figure S3, no significant change was observed in the CLDs. Degradation into chains not detected by SEC with the present method (DP $\lesssim$ 6) would be detected in the FS analysis. Thus, it is concluded that, in the malting samples and process used here, both amylose and amylopectin molecules were hydrolysed together; this also explained why no new starch polymers with intermediate sizes, those between amylose and amylopectin of native unmalted barley starches, were created during malting.

As shown in Figures 1 and S1, after malting, the surface of barley starch granules become rough compared with native barley starch granules (confocal images of brewers spent grain, with starch granules in colour, are shown in Fig. S2). This indicates that during malting, modifications of both starch and protein have occurred, consistent with the change of protein content after malting (Table 1).

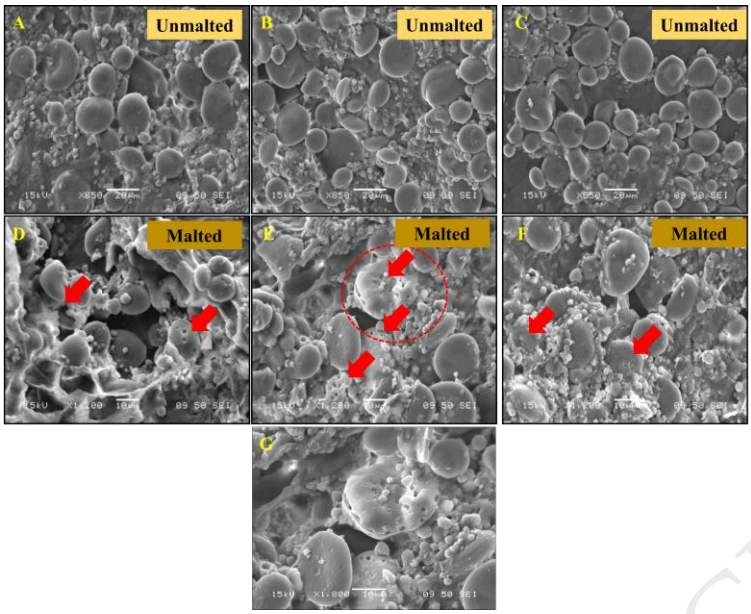

Figure 1. SEM micrographs of granule surfaces before and after malting. A, B, C, unmalted; D, E, F, malted barley; G: enlargement of E. A, D, sample 82; B, E, sample 86 and C, F, sample 89. All other samples in Figure S1. Red arrows indicate pinholes.

Table 2 shows that malting resulted in a significant decrease in the weight-average molecular weight of all starch polymers while the average size (R_g) does not change significantly. This indicates a molecularly sparser distribution resulting from this process. Because it will be seen that malting does not significantly change the CLD, this implies that malting removes whole chains throughout the whole starch molecule.

Table 2. Overall $\overline{M}_W$ and R_g of fully branched starch polymers from both unmalted and malted barley [a].

Sample ID	$\overline{M}_W / 10^8$		R_g	
	Unmalted	Malted	Unmalted	Malted
70	(4 ± 1) [a]	(3± 1) [ab]	(161 ± 19) [a]	(160 ± 0) [a]
73	(5 ± 1) [a]	(4 ± 1) [b]	(164 ± 13) [ab]	(186 ± 5) [a]
75	(4 ± 0) [a]	(3± 1) [ab]	(151 ± 0) [a]	(188 ± 40) [a]
78	(4 ± 0) [a]	(2± 0) [a]	(148 ± 14) [a]	(162 ± 14) [a]
82	(5 ± 0) [a]	(2 ± 1) [a]	(188 ± 2) [b]	(170 ± 13) [a]
86	(5 ± 0) [a]	(2 ± 0) [a]	(166 ± 10) [ab]	(162 ± 18) [a]
87	(4 ± 1) [a]	(2 ± 0) [a]	(153 ± 12) [a]	(166 ± 19) [a]
89	(4 ± 1) [a]	(2 ± 0) [a]	(252 ± 7) [c]	(171 ± 17) [a]
92	(5 ± 0) [a]	(2 ± 0) [a]	(162 ± 10) [ab]	(165 ± 13) [a]
93	(5 ± 1) [a]	(2 ± 1) [a]	(160 ± 1) [a]	(164 ± 4) [a]

[a], Data from duplicate measurements; samples with different letters in the same column are significantly different at $p < 0.05$.

A typical weight CLD, $w(\log X)$, of starch from unmalted, malted barley, wort and the post-mashing undegraded material, which is called brewer's spent grain (BSG), is shown in Figure 2; results for all samples are in Fig. S3. No significant changes are observed with malting in the CLDs, and the concomitant fitting parameters (Tables 1, S2 & S3). The reason that this needed confirmation by fitting with model parameters is this would bring out differences in particular regions of the CLD which might not be apparent from visual inspection of the CLDs themselves.

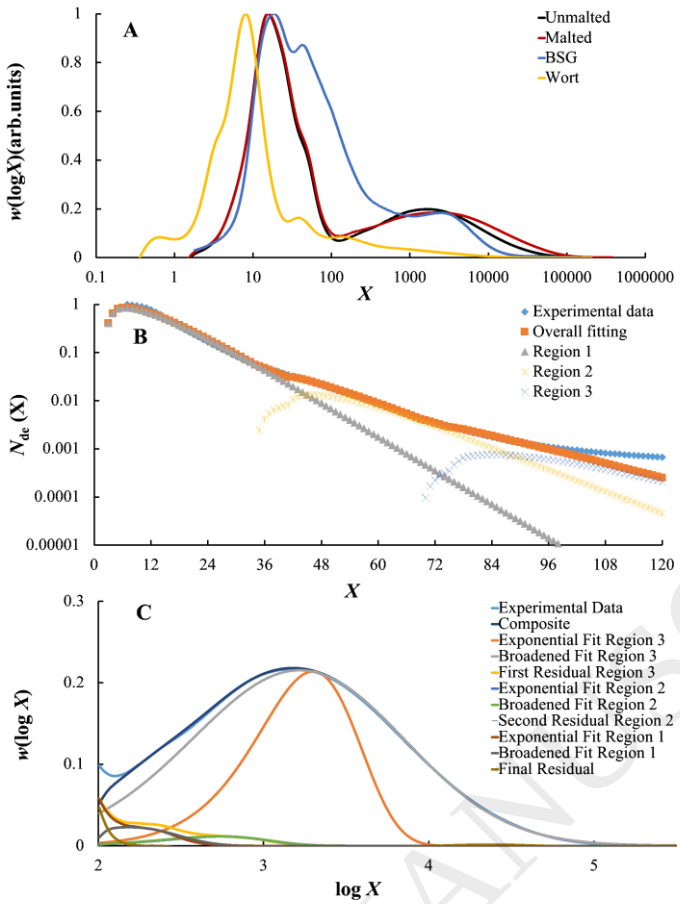

Figure 2. A representative SEC weight CLD (A) of debranched starches (from the sample with protein content 10.77, and the amylopectin (B) and amylose figures (C) fitting. Results for all samples in Fig. S3. All distributions normalized to the height of the first peak.

2.2. Correlations among grain structural properties in both unmalted and malted barley

The objective of this paper is to determine, and explain mechanistically, how structural parameters in the parent grain control functional properties in the malted barley. The structural parameters of the barley are determined by genetic and environmental conditions, and are not the subject of this investigation, although discussed in previous work (Gous, Hasjim, Franckowiak, Fox & Gilbert, 2013; Yu et al., 2017). Table 3 shows no significant correlations between starch structural parameters and protein content, except for average R_g and $\overline{R}_{h,Am}$,

which is trivial as they are both measures of the size of starch polymers.

Table 3. Correlations between starch structural parameters and protein content for parent (unmalted) and malted barley (n=10).

		1. Unmalted					
		Total protein content [a]	Amylose content	$\overline{M}_W$	R_g	$\overline{R}_{h,Am}$	
Amylose content		0.29	1				
Branched data	$\overline{M}_W$	-0.14	0.04	1			
	R_g	-0.20	-0.37	0.09	1		
	$\overline{R}_{h,Am}$	-0.34	-0.57	-0.14	0.72*	1	
Amylopectin fitting data	$\beta_{Ap,\,1}$	-0.61	-0.33	-0.17	-0.42	0.02	
	$\beta_{Ap,\,3}$	-0.58	-0.11	0.13	-0.38	-0.10	
	$h_{Ap,\,1}$	-0.11	-0.17	0.02	0.32	-0.06	
	$h_{Ap,\,3}$	0.36	0.29	0.23	0.35	-0.03	
Amylose fitting data	$h_{Am,\,1}$	0.55	0.44	0.13	-0.05	-0.13	
	$h_{Am,\,2}$	0.29	0.74*	0.15	-0.67*	-0.78**	
	$h_{Am,\,3}$	-0.25	0.31	0.24	0.15	0.08	
	$\beta_{Am,\,1}$	0.10	0.01	-0.42	-0.40	-0.24	
	$\beta_{Am,\,2}$	0.00	-0.29	-0.28	-0.06	0.10	
	$\beta_{Am,\,3}$	0.23	-0.19	-0.25	-0.23	-0.12	
		2. Malted					
		Total protein content	Insoluble protein content	Amylose content	$\overline{M}_W$	R_g	$\overline{R}_{h,Am}$
Amylose content		0.66*	0.66*				
Branched parameters	$\overline{M}_W$	-0.12	-0.18	-0.35			
	R_g	0.31	0.22	0.08	0.67*		
	$\overline{R}_{h,Am}$	-0.42	-0.42	-0.66*	-0.29	-0.26	
Amylopectin fitting data	$\beta_{Ap,\,1}$	-0.69*	-0.70*	-0.66*	0.21	0.00	0.31
	$\beta_{Ap,\,3}$	-0.71*	-0.73*	-0.50	0.06	-0.19	0.24
	$h_{Ap,\,1}$	0.00	-0.07	-0.10	0.83**	0.42	-0.42
	$h_{Ap,\,3}$	0.61	0.55	0.59	0.22	0.34	-0.52
Amylose fitting data	$h_{Am,\,1}$	-0.53	-0.49	0.014	0.07	-0.14	-0.34
	$h_{Am,\,2}$	-0.41	-0.45	-0.15	0.34	0.07	-0.40
	$h_{Am,\,3}$	-0.21	-0.31	-0.03	0.19	0.09	-0.32
	$\beta_{Am,\,1}$	0.89**	0.94**	0.57	-0.20	0.24	-0.21
	$\beta_{Am,\,2}$	0.76*	0.83**	0.52	-0.15	0.19	-0.21
	$\beta_{Am,\,3}$	0.30	0.39	0.14	-0.20	-0.07	0.24

a, data is on dry basis; * Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level.

Significant positive correlations between amylose content and protein content was found for malted barley, consistent with the correlations between both $\beta_{Am,\,1}$ and $\beta_{Am,\,2}$ and protein content (these β's are factors controlling the chain lengths and amounts of amylose with DP < 2000).

For malted amylopectin, protein is significantly and negatively correlated with $\beta_{Ap,1}$, $\beta_{Ap,3}$, which are factors controlling the amount of amylopectin chains with DP< 67.

2.3. Change in gelatinization characteristics of barley flour after malting

The gelatinization temperature for malted barley starch used in brewing is usually assumed to be at 65 ºC (Fox, 2016). However, Table 4 shows that though the onset temperature of malted barley flour was below 65 ºC, the offset temperatures of malted barley flours were all above 65 ºC, except samples 70 and 82, which have a noticeably lower protein content. This means that the mashing temperature in brewing can depend on the malted barley sample. Samples with gelatinization temperatures close to or greater than 65 °C are at risk of reduced starch hydrolysis and thus lower amounts of fermentable sugars (higher amounts of non-fermentable sugars).

Table 4. Gelatinization characteristics of unmalted and malted barley flours.

No.	T_o/ °C		T_p/ °C		T_c/ °C		Retrogradation enthalpy / kJ mol^{-1}	
	Unmalted	Malted	Unmalted	Malted	Unmalted	Malted	Unmalted	Malted
70	$(54.9 \pm 0.3)^{a}$	$(55.8 \pm 0.2)^{a}$	$(60.6 \pm 0.0)^{a}$	$(60.8 \pm 0.3)^{a}$	$(68.9 \pm 0.8)^{a}$	$(64.9 \pm 0.3)^{a}$	$(5.3 \pm 0.4)^{a-c}$	$(4.1 \pm 0.3)^{c-e}$
73	$(55.9 \pm 0.7)^{bc}$	$(59.4 \pm 0.2)^{c}$	$(62.4 \pm 0.4)^{c}$	$(64.0 \pm 0.4)^{c}$	$(71.6 \pm 0.3)^{d}$	$(68.1 \pm 0.8)^{bc}$	$(5.6 \pm 1.5)^{a-c}$	$(4.2 \pm 0.1)^{de}$
75	$(55.4 \pm 0.1)^{b}$	$(59.7 \pm 0.2)^{cd}$	$(61.9 \pm 0.1)^{b}$	$(64.5 \pm 0.4)^{c-e}$	$(70.4 \pm 0.3)^{bc}$	$(69.1 \pm 0.4)^{cd}$	$(4.6 \pm 0.0)^{a}$	$(3.4 \pm 0.6)^{a-d}$
78	$(58.8 \pm 0.6)^{de}$	$(59.7 \pm 0.2)^{cd}$	$65.0 \pm 0.0)^{e}$	$(64.8 \pm 0.2)^{de}$	$(73.7 \pm 0.7)^{f}$	$(69.6 \pm 0.1)^{d}$	$(4.9 \pm 0.2)^{ab}$	$(2.6 \pm 0.2)^{a}$
82	$(54.0 \pm 0.2)^{a}$	$(55.2 \pm 0.2)^{a}$	$(60.5 \pm 0.1)^{a}$	$(60.45 \pm 0.0)^{a}$	$(69.9 \pm 0.4)^{ab}$	$(64.8 \pm 0.0)^{a}$	$(4.5 \pm 0.1)^{a}$	$(4.5 \pm 0.3)^{e}$
86	$(59.7 \pm 0.0)^{f}$	$(60.3 \pm 0.7)^{d}$	$(65.6 \pm 0.0)^{f}$	$(65.3 \pm 0.3)^{e}$	$(71.3 \pm 0.1)^{cd}$	$(69.9 \pm 0.5)^{d}$	$(6.3 \pm 0.8)^{bc}$	$(3.9 \pm 0.4)^{b-d}$
87	$(56.3 \pm 0.2)^{c}$	$(57.1 \pm 0.1)^{b}$	$(62.6 \pm 0.1)^{c}$	$(62.8 \pm 0.3)^{b}$	$(72.1 \pm 0.4)^{de}$	$(67.9 \pm 0.3)^{b}$	$(6.6 \pm 0.1)^{c}$	$(3.3 \pm 0.1)^{a-c}$
89	$(56.2 \pm 0.1)^{c}$	$(56.6 \pm 0.4)^{b}$	$(62.6 \pm 0.1)^{c}$	$(62.3 \pm 0.3)^{b}$	$(73.1 \pm 0.3)^{ef}$	$(67.9 \pm 0.6)^{b}$	$(6.1 \pm 0.8)^{a-c}$	$(2.9 \pm 0.1)^{a}$
92	$(59.2 \pm 0.1)^{ef}$	$(59.8 \pm 0.4)^{cd}$	$(65.2 \pm 0.2)^{ef}$	$(65.2 \pm 0.2)^{e}$	$(73.7 \pm 0.8)^{f}$	$(69.9 \pm 0.2)^{d}$	$(5.5 \pm 0.3)^{a-c}$	$(3.0 \pm 0.2)^{ab}$
93	$(58.2 \pm 0.4)^{d}$	$(59.4 \pm 0.3)^{c}$	$(64.2 \pm 0.2)^{d}$	$(64.3 \pm 0.2)^{cd}$	$(73.4 \pm 0.4)^{f}$	$(69.0 \pm 0.1)^{cd}$	$(6.0 \pm 0.5)^{a-c}$	$(3.1 \pm 0.2)^{ab}$

a. **Data based on duplicate measurements; samples with different letters in the same column are significantly different at $p < 0.05$. DSC results in Figure S4.**

2.4. Enzyme activity in malted barley and the fermentable sugars in wort after mashing.

The enzyme activities of α- and β- amylases and limit dextrinase after malting are shown in Figure 3. Subsequent to malting, these enzymes hydrolyze starch to release fermentable sugars. After malting, no significant correlation was found between α- amylase activity and total protein content of malted barley, nor was there any significant correlation between limit dextrinase activity and total protein content. There was a significant positive correlation between β- amylase activity and total protein content, as also seen by Arends, Fox, Henry, Marschke and Symons (1995). Linear regression and principal component analyses of these relations are shown in the Supporting Information.

After mashing, maltose was the most abundant sugar released during mashing, followed by

maltotriose and then glucose, as shown in Figure 3D. As reported by Maeda, Kiribuchi and Nakamura (1978), during mashing, the action of both α- and β- amylases would preferentially hydrolyse starch to release maltose and maltotriose. Surprisingly, for samples with higher enzyme activities (β- and α- amylases, sample 92, with protein content of 12.27), no increased maltose content was observed. This means that other factors including both protein content and barley starch molecular structures can also affect the release of FSs.

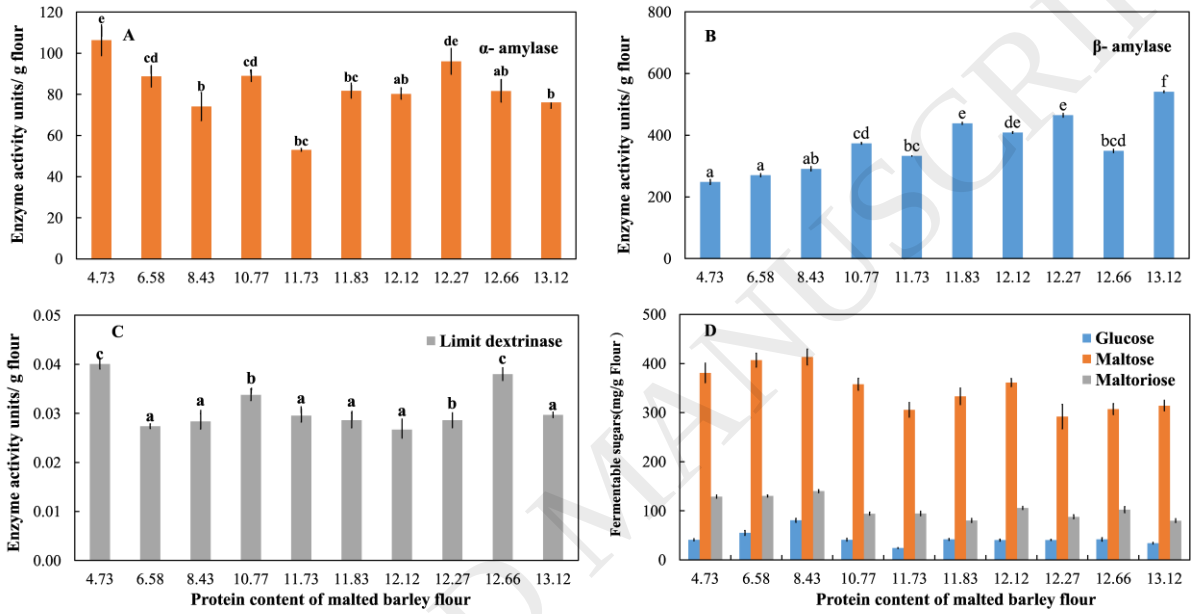

Figure 3. Enzyme activity of α- (A), β- (B), limit dextrinase (C) in malted barley flour and content of fermentable sugars (D) after mashing as functions of protein content (dry basis). Samples with different letters are significantly different at $p < 0.05$.

2.5. Correlations among unmalted protein content, starch structure and functional properties.

For unmalted barley, total protein content significantly negatively correlated with both maltose and maltotriose contents after mashing, while the amylose content showed significant negative correlation with only maltose content after mashing. There was a significant negative correlation between $h_{Ap,3}$ (controlling the amount of longer amylopectin chains, ~ DP 37 – 64)

and maltotriose content after mashing. Both $\beta_{Ap,\ 1}$ and $\beta_{Ap,\ 3}$ (involved in the amount of amylopectin chains with DP $\lesssim$ 67) in the parent barley also showed significant positive correlation with maltotriose content after mashing. For the DSC results, there was only a significant negative correlation between $\beta_{Ap,\ 1}$ and the conclusion gelatinization temperatures (Table 5).

In our study, maltose and maltotriose both correlated with different parameters, except for that between maltotriose and some amylopectin/ amylose fitting parameters (Table 5). For example, unlike what is seen for maltose, there was significant positive correlation between maltotriose content and $\beta_{Am,\ 1}$ for malted barley, where this parameter controls the amount of shorter-chain amylose. Our hypothesis is that during the first stage of mashing, maltotriose is produced by α-amylase and then further hydrolyzed to maltose and glucose by other enzymes, including β-amylase and amyloglucosidase (Hall & Manners, 1978). However, at the mashing temperature (65 °C), β-amylase can rapidly lose its enzyme activity (MacGregor, Bazin, Macri & Babb, 1999) and therefore the main enzyme would be α-amylase, which would lead to a higher accumulation of maltotriose at the last stage of mashing. This would explain the differences between the correlations of maltose and maltotriose with starch structural parameters.

No significant correlation between glucose content and starch structural parameters or protein content was found (Table 5). It is possible because that during mashing, significant β-glucanolysis took place, and the resulting glucans further be hydrolyzed by different enzyme(s) to disaccharides and glucose (Bamforth & Martin, 1983), thereby losing any correlation.

2.6. Correlations among barley protein content, starch structural parameters and functional properties of malted barley flour

As shown in Table 5 (and in Figures S6 & S7), amylose content significantly and positively

correlated with gelatinization temperatures of malted barley flour while $\overline{R}_{h,Am}$ had a significant and negatively correlation with gelatinization temperatures. This is reflected in the significant negative correlation between amylose content and starch solubilization content. The significant positive correlations between $\beta_{Am,1}$, $\beta_{Am,1}$ and the gelatinization temperature merely indicates the well-known result (e.g. (Tao, Li, Yu, Gilbert & Li, 2018)) that short amylose chains are important in gelatinization temperatures because of the way they affect crystallinity.

The significant positive correlation between $h_{Am,1}$ (the amount of shorter amylose chains) and maltotriose content indicates that the amount of amylose with shorter chains (Region 1, DP 100~ 500) significantly and positively correlated with maltotriose content released during mashing (Table 5).

Table 5. Correlations between fermentable sugars and starch structural parameters of both unmalted and malted barley.

1. Unmalted

		Fermentable sugars			AM	PH	Gelatinization properties			
		Glucose	Maltose	Maltotriose			Onset	Peak	Endset	Enthalpy
	Total protein	-0.30	-0.64*	-0.73*						
Amylose content		-0.37	-0.72*	-0.36	1.00					
Gelatinization properties	Onset	0.33	-0.34	-0.20	0.45	-0.63*	1.00			
	Peak	0.31	-0.36	-0.21	0.46	-0.65*	1.00**	1.00		
	Endset	-0.14	-0.66*	-0.71*	0.41	-0.78**	0.75*	0.76*	1.00	
	Enthalpy	0.30	0.12	-0.23	-0.40	-0.38	0.42	0.41	0.40	1.00
Amylopectin fitting data	$\beta_{Ap,\,1}$	0.37	0.63*	0.76*	-0.33	0.69*	-0.27	-0.28	-0.74*	0.02
	$\beta_{Ap,\,3}$	0.44	0.58	0.72*	-0.11	0.66*	-0.06	-0.08	-0.57	0.06
	$h_{Ap,\,3}$	-0.42	-0.43	-0.66*	0.29	-0.36	0.07	0.07	0.48	0.14
Amylose fitting data	$h_{Am,\,1}$	-0.12	-0.48	-0.40	0.44	-0.64*	0.39	0.39	0.51	-0.19
	$\beta_{Am,\,1}$	-0.65*	-0.27	-0.27	0.01	0.21	-0.37	-0.36	-0.10	-0.02

2. Malted

		Glucose	Maltose	Maltotriose	PC	Insoluble PC	Am	PH	Starch utilization	$\bar{R}_{h,Am}$	Onset	Peak	Endset	Enthalpy
Total protein content		-0.45	-0.79**	-0.83**	1.00									
Insoluble protein content		-0.33	-0.73*	-0.77**	0.99**	1.00	1.00							
Amylose content		-0.18	-0.63*	-0.41	0.66*	0.66*	1.00	1.00	1.00					
Starch utilization		-0.40	0.28	0.01	-0.43	-0.51	-0.70*	0.54	1.00					
Gelatinization properties	Onset	0.01	-0.46	-0.23	0.65*	0.71*	0.81**	-0.72*	-0.80**	-0.82**	1.00			
	Peak	0.03	-0.51	-0.30	0.70*	0.77**	0.84**	-0.78**	-0.84**	-0.74*	0.98**	1.00		
	Endset	0.01	-0.57	-0.43	0.77**	0.84**	0.80**	-0.86**	-0.83**	-0.60	0.92**	0.97**	1.00	
	Enthalpy	0.34	0.80**	0.73*	-0.72*	-0.75*	-0.48	0.62	0.47	0.26	-0.42	-0.53	-0.68*	1.00
Amlopectin fitting data	$\beta_{Ap,\,1}$	-0.23	0.50	0.48	-0.69*	-0.70*	-0.66*	0.66*	0.56	0.31	-0.54	-0.61	-0.62	0.44
	$\beta_{Ap,\,3}$	-0.33	0.46	0.50	-0.71*	-0.73*	-0.50	0.72*	0.37	0.24	-0.47	-0.55	-0.59	0.45
Amylose fitting data	$h_{Am,\,1}$	0.38	0.31	0.72*	-0.53	-0.49	0.01	0.43	-0.28	-0.34	0.14	0.09	-0.02	0.25
	$\beta_{Am,\,1}$	-0.13	-0.48	-0.66*	0.89**	0.94**	0.57	-0.97**	-0.45	-0.21	0.64*	0.71*	0.79**	-0.57
	$\beta_{Am,\,2}$	-0.03	-0.36	-0.54	0.76*	0.83**	0.52	-0.88**	-0.44	-0.21	0.64*	0.71*	0.77**	-0.45

*** Correlation is significant at the 0.05 level; **. Correlation is significant at the 0.01 level; Am, amylose content; PH, protein hydrolysis.**

3. Discussion

In beer brewing, malting is a process involving enzymatic degradation of barley starch (Figure 1, Table 1). Protein modification is currently an important criterion used to evaluate malted barley mashing performance, but an important functional property involves the post-mashing formation of ethanol from fermentable sugars derived from the starch. For the samples chosen here (varieties typical of those used for malting, with a significant range of protein contents), it is shown that the malting does not result in significant changes in any region of the starch CLDs of both amylopectin and amylose, and consistently no significant change in amylose content (Figures 2 and S3, Tables 1, S1, S2 and S3). The CLD and $\overline{M}_W$ data show that the malting process removes whole chains throughout the starch molecules.

The correlations among malted starch structural parameters and protein content showed differences after malting (Table 2), simply ascribed to the enzymes involved removing whole chains throughout the starch molecule.

The negative correlation between $h_{Ap,\,3}$ in the parent barley and maltotriose content along with the positive correlation between FSs contents and both $\beta_{Ap,\,1}$ and $\beta_{Ap,\,3}$ (DP < 67) (Table 5) suggests that for amylopectin, an increase in short chains tends to increase the release of fermentable sugars after mashing. This is probably because this reduces the efficiency of packing within the crystalline region of starch granules, resulting in lower gelatinization temperature (Table 5), as also reported by (Noda, Takahata, Sato, Suda, Morishita, Ishiguro & Yamakawa, 1998) and giving the enzymes more time to hydrolyze these chains. For malted barley, no significant correlation between amylopectin parameters and FSs was found.

In our previous study, barley protein was found to competitively bind with amylolytic enzymes in an *in vitro* starch digestion environment (Yu et al., 2018c), explaining the observed reduced

starch degradation rate. Here, our results show that in addition, both amylose content and protein content have significant positive correlations with gelatinization temperatures and have significant negative correlation with the FS content. Two alternative mechanisms are consistent with the results, as follows.

(1) During mashing, starch will be firstly gelatinized, when amylose leaches out into the mashing liquid, thereby slowing enzyme diffusion and thus reducing starch enzymatic hydrolysis. In addition, amylose molecules may entangle and/or co-crystallize with amylopectin chains in the crystalline lamellae, thereby causing limited starch swelling (Tester & Morrison, 1990). Because of this, the enzymatic hydrolysis of starch by malt amylases (mainly α- amylase) is limited, leading to a significantly lower content of maltose after mashing (Table 5). The negative correlations between amylose content and starch solubilization during mashing supports this hypothesis (Table 5). By parameterizing the starch structural data with our biosynthesis-based models, it was found that barleys with a higher amount of short-chain amylose molecules produce more FSs after mashing, probably as a result of more small amylose molecules leaching out and bother becoming available for enzymatic degradation to release fermentable sugars and also loosening the structure of the remaining starch. This is one possible explanation of why both amylose content and chain-length distribution affect the release of fermentable sugars.

(2). The second possible explanation is as follows. Protein can inhibit the swelling of barley starch granules (Figure S4), thereby affecting the enzymatic hydrolysis of starch, particularly for those starch molecules bigger than 100 nm (Figs 5D & E). This is in accordance with a previous study reporting that in wheat, gluten can inhibit the hydrolysis of wheat starch with R_h > 100 nm (Zou, Sissons, Warren, Gidley & Gilbert, 2016). This reduces the amount of both maltose and maltotriose produced during mashing.

4. Conclusion

Various starch structural parameters for the parent barleys were found to be correlated with the final FSs. The present study has established the importance of barley starch structural properties, particularly with its structure (amylose content, the CLDs of both amylose and amylopectin) in the malting of barley, in addition to the one mainly in current use: barley protein content. The observed correlations all have reasonable mechanistic explanations. Our results also showed that for barleys with higher protein content, higher mashing temperatures are needed to acquire a higher FS content as well as the amounts of different FSs. Overall, barley cultivars with lower protein and amylose contents, smaller amylose molecular sizes, lower amount of amylopectin long branches tend to release more FSs after mashing.

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