Research Article

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Molecular structure-property relations controlling mashing performance of amylases as a function of barley grain size

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Abstract: In brewing, amylases are key enzymes in hydrolyzing barley starch to sugars, which are utilized in fermentation to produce ethanol. Starch fermentation depends on sugars produced by amylases and starch molecular structure, both of which vary with barley grain size. Grain size is a major industrial specification for selecting barley for brewing. An in-depth study is given of how enzyme activity and starch structure vary with grain size, the impact of these factors on fermentable sugar production, and the underlying mechanisms. Micro-malting and mashing experiments were based on commercial methodologies. Starch molecular structural parameters were obtained using size-exclusion chromatography, and fitted using biosynthesis-based models. Correlation analysis using the resulting parameters showed larger grain sizes contained fewer long amylopectin chains, higher amylase activities and soluble protein level. Medium grain sizes released most sugars during mashing, because of higher starch utilization from the action of amylases, and shorter amylose chains. As starch is the substrate for amylase-driven fermentable sugars production, measuring its structure should be a prime indication for mashing performance, and should be used as an industry specification when selecting barley grains for brewing.

Keywords: amylase; barley; malting; mashing; gel permeation chromatography; starch.

Abbreviations: AC, amylose content; Ap, Amylopectin; Am, Amylose; BSA, bovine serum albumin; BSG, brewer's spent grain; CLD, chain-length distribution; DBE, debranching enzyme; DMSO, dimethyl sulfoxide; DP, degree of polymerization; GBSS, granule bound starch synthase; HPLC, high-performance liquid chromatography; KI, Kolbach index; SBE, starch branching enzyme; SEC, size-exclusion chromatography; SS, starch synthase; SSS, soluble starch synthase.

1 Introduction

Amylases [1] are key enzymes in hydrolyzing barley starch to sugar in the production of beer. Beer brewing involves sequential processes starting with malting of the barley, through to mashing, boiling and fermentation [2-4]. Malting is a three stage process with steeping (~24 h with wet periods and air-rests), then germinating (4-5 days) followed by kilning (~24 h), at a relatively high temperature, to result in a moisture content around 4.0 to 4.5% [5]. At this stage, enzymes present include α -amylase, some β -amylase, limit dextrinase and α -glucosidase, which are either synthesized, activated and/or released from their binding materials [6-8]. The malt is milled and then mixed with hot water to allow starch hydrolysis: mashing. Mashing is the degradation of gelatinized carbohydrate (mainly starch) to produce fermentable sugars at elevated temperatures (the infusion and ramping temperatures). Additionally, proteolysis during mashing produces amino acids and small peptides necessary for yeast metabolism, and the resulting soluble proteins are also important in beer-head retention and stability [9]. Finally, the fermentation process uses sugars (glucose, maltose, maltotriose) and yeast to produce alcohol.

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There are two main types of malting barleys: two-rowed (used in this study) and six-rowed. Two-rowed barley kernels are symmetrical, even in size and thinner husk, whereas six-rowed barley has a symmetrical center, and the two lateral rows of kernels are different in size and with thicker husks [10], thereby resulting in different rates of water uptake, germination and drying [11]. Generally, two-rowed barley has lower protein and enzyme content but more starch, responsible for higher potential extract content; while six-row barley has less starch, but higher enzyme activity and protein content for the high-speed conversion of starch into fermentable sugars [11]. Addition of inexpensive adjuncts, e.g. rice and corn, is used to offset the high protein levels [12].

For brewing, a barley variety is usually chosen by its protein content and/or grain size, since larger grain sizes usually have higher starch content [13]. Barley grain size is controlled by a number of environmental factors, set during the grain filling period. Differences in grain size distribution result in variation in starch quality, amount of starch and starch structure [14-16]. A number of studies have also highlighted the effects of growing environment on starch structural properties of normal and waxy type barleys, but these did not report any grain size data [17-21].

There are inherent varietal differences in barley grain sizes and in starch structure [22,23]. The larger grain size provides increased levels of diastatic power (enzyme activity) but larger grain sizes also have increased pasting properties (measured by the rapid viscoanalyser), which would suggest different starch structures with grain size [24].

While these previous studies explored the variation in barley grain size and or starch quality, there is currently little information on gelatinization and wort quality (extract and fermentable sugars) on different grain size fractions. To examine the effects of barley grain size on potential brewing performance (which is always driven by the activity of various amylases), the following properties were examined: gelatinization temperatures, resultant wort gravity (degree Plato) and fermentable sugar profile from a high temperature mash. Starch structure was characterized using size-exclusion chromatography (SEC, a type of gel permeation chromatography) to observe differences in the amylose chain length distributions between the grain size fractions, in order to provide molecular mechanisms for the observed differences in properties [22].

The general paradigm adopted is as follows: genetics and environment control structure, and structure controls functional properties. The aims here are twofold. (1) Examine effects of grain size (controlled by genetic and environmental effects) on appropriate

structural parameters (specifically starch molecular fine structure), and chemical compositions (the activities of various amylases and other enzymes, together with starch and protein content). (2) Examine, and explain mechanistically, the effects of these structural parameters on functional properties: wort density, fermentable sugars production and gelatinization properties after mashing. This study looks at the impacts of different grain sizes, in terms of bulk size distributions (mixing of various grain sizes) and segregated grain size fractions on the activities of amylases and other enzymes, chemical compositions and starch structural properties.

2 Materials and methods

2.1 Grain size

Barley samples (100 g) were sieved using a Sortimat screening machine (Pfeuffer, Germany) with 2.2, 2.5 and 2.8 mm sieves, sieving ~100 g of grain for 1 min. Grains that passed the 2.2 mm sieve were recorded as screenings and grains that remained above the 2.5 mm sieve were recorded as retention.

2.2 Materials

Eight barleys with varied grain sizes but similar protein contents grown at different locations were from the 2013 National Variety Trials, Australia, as shown in Table 1.

One particular variety (Commander) of barley chosen from Jambin, and the corresponding malt sample, were separated into grain sizes with four different fractions, >2.2 mm, >2.5 mm, >2.8 mm and bulk (mixing of different grain sizes). The chemical composition and starch structure were then characterized, providing data for Aim (1).

2.3 Micro-malting

Micro-malting was carried out in a Phoenix automated micro-malting unit (Phoenix, Adelaide, Australia) to obtain fully modified malt. The malting conditions were as follows. Unmaltered barley grains (200 g) were steeped for 24 h with a combination of 8 h wet, 8 h air rest, 8 h wet, all at 17 °C. Following steeping, the grain was allowed to germinate at 17 °C for 4 days. During germination, the grain was turned every hour to avoid hot spots. After this, germination was terminated by kilning: slowly heating to 50 °C to remove excess moisture, and then further heating to 85 °C to halt enzyme activity and to reduce the malt moisture to around 4%. The whole kilning stage took 24 h.

Table 1. Chemical compositions of barley (unmalted) and corresponding malt samples with bulk size distribution (mixing).^a

ID	Variety	Grain size			Barley (unmalted)					Malt					
		>2.2 mm (%)	>2.5 mm (%)	>2.8 mm (%)	CP (%)	SP (%)	Starch (%)	KI (%)	CP (%)	SP (%)	Starch (%)	Loss of starch after malting (%)	KI (%)	α-Amylase (U/g)	β-Amylase (U/g)
1	Commander 1	4	4	95	11	3 ± 0 ^b	54	26 ± 0 ^a	10	6 ± 0 ^a	50	8	57 ± 0 ^a	96 ± 2 ^b	397 ± 7 ^a
2	Gairdner 4	25	25	71	12	2 ± 0 ^h	54	15 ± 0 ^e	12	5 ± 0 ^c	46	14	38 ± 0 ^b	61 ± 1 ^e	212 ± 14 ^d
3	Gairdner 60	36	36	4	14	3 ± 0 ^{bc}	48	19 ± 0 ^d	14	4 ± 0 ^a	46	5	26 ± 0 ^e	58 ± 2 ^e	270 ± 6 ^c
4	Commander 10	18	18	72	12	2 ± 0 ^d	53	21 ± 0 ^{bc}	14	4 ± 0 ^a	43	17	28 ± 0 ^d	58 ± 4 ^e	226 ± 4 ^d
5	Gairdner 2	23	23	75	14	2 ± 0 ^g	52	14 ± 0 ^e	11	4 ± 0 ^a	47	10	35 ± 0 ^c	71 ± 1 ^d	271 ± 9 ^c
6	Commander 25	34	34	41	11	2 ± 0 ^f	53	20 ± 0 ^c	13	4 ± 0 ^a	41	22	30 ± 0 ^d	68 ± 1 ^d	268 ± 15 ^c
7	Commander 4	14	14	82	12	2 ± 0 ^e	60	19 ± 0 ^d	13	4 ± 0 ^a	45	25	29 ± 0 ^d	108 ± 5 ^a	327 ± 12 ^b
8	Commander 3	11	11	86	12	3 ± 0 ^a	47	26 ± 0 ^a	14	5 ± 0 ^b	44	7	38 ± 0 ^b	89 ± 3 ^c	342 ± 27 ^b

^a All data on dry basis and based on duplicate measurements except for crude protein and total starch content; the “> 2.2” represents barley grains larger than 2.2 mm but smaller than 2.5 mm, etc.; SP, soluble protein; CP, crude protein. Growing locations of sample from 1 to 8 are as follows: Jambin, Westmar, Macalister, Jambin, Westmar, Brookstead, Brookstead. Values with different letters in the same column are significantly different at p < 0.05.

2.4 Milling of grains

Barley grains (50 g) were ground to flour using a stainless-steel electric cereal mill machine (FDM; Guangzhou Itop Kitchen Equipment Co., Ltd, China) followed by screening through a Sortimat machine (Pfeuffer, Germany). Flour samples were sealed in plastic bags and kept in a freezer for future analysis.

2.5 Protein and moisture contents

The crude protein of both barley and malt flour was obtained from the nitrogen content determined using a LECO CNS2000 auto-analyzer using a conversion factor of 6.25.

Soluble protein content was measured based on the bovine serum albumin (BSA) method, using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit with BSA as a reference standard. A 0.1 mL volume of each standard or sample was added into 2.0 mL of BCA working reagent and incubated at 37 °C for 30 min. The samples were then allowed to cool to room temperature and the absorbance measured by a UV-1700 Pharma Spectrophotometer (Shimadzu) at 562 nm. A series of BSA dilutions of known concentration were prepared, and the soluble protein concentration of barley samples determined based on the standard curve. The results were then used to calculate the Kolbach index (KI), which is the soluble protein content as a percentage of the total nitrogen content measurable in the malt.

Moisture content was measured by drying the samples in a vacuum oven (Laboratory Equipment Pty, Ltd., Australia) at 110 °C overnight and recording the weight loss of moisture in triplicate measurements.

2.6 Enzyme activities of α - and β -amylase in malted flour

The enzyme activity analysis is required for Aim (1). The activities of α -amylase and β -amylase were measured using a Malt Amylase Assay Kit (K-MALTA, Megazyme International Ireland, Ltd.). Five mL of Betamyl-3 buffer A was first added into a 0.5 g of flour and vortexed briefly. The enzymes were then allowed to dissolve by placing the sample in a thermomixer for 1 h at room temperature, followed by centrifugation at $2,000 \times g$ for 10 min. The supernatant was then added to 4 mL of diluted Betamyl-3 buffer B and this solution (Extract A) was used to measure both α - and β -amylase activities.

2.7 Assay of β -amylase

Extract A and Betamyl-3 substrate solution were incubated at 40 °C for 5 min, then 0.2 mL of the substrate solution was mixed with 0.2 mL of Extract A and further incubated at 40 °C for another 10 min. The reaction was terminated and colour developed by the addition of stopping reagent; the absorbance of the reaction solution and reagent blank was then read at 400 nm against distilled water. The activity of β -amylase was calculated using:

$$\text{units of } \beta\text{-amylase} - \text{amylase/g flour} = 19.7 \Delta E_{400} \quad (1)$$

where ΔE_{400} is the difference of absorbance at 400 nm between the flour sample and blank.

2.8 Assay of α -amylase

The amount of 0.2 mL of Extract A was diluted with 3 mL of Ceralpha Buffer A to make Extract B. Extract B and Ceralpha substrate solution were incubated at 40 °C for 5 min, and 0.2 mL of the substrate solution was mixed with 0.2 mL of Extract B and further incubated at 40 °C for another 10 min. The reaction was terminated and colour was developed by the addition of Stopping Reagent; the absorbance of the reaction solution and reagent blank was then read at 400 nm against distilled water. The activity of α -amylase was calculated using:

$$\text{units of } \alpha\text{-amylase} - \text{amylase/g flour} = 315.6 \Delta E_{400} \quad (2)$$

Each unit of enzymatic activity (U/g) indicates the release of a micromole of the restored sugar per minute under the defined assay conditions.

The reagent blank was made by first mixing Stopping Reagent with substrate solution only, then adding to diluted malt extract.

2.9 Micro-scale mashing

Both experiments 1 and 2 of mashing were required for Aim (2).

Experiment 1. Prior to mashing, the eight malt samples (Table 1) were milled in a disc mill with a coarse setting 0.8 (Buhler Miag, Germany). The mashing of the eight malts were conducted using an IEC Mash Bath (Industrial Equipment & Control Pty, Ltd., Melbourne, Australia) based on the method of the Institute of Brewing and Distilling (high-temperature infusion) using 50 g grist with 200 mL of hot liquor (65 °C) and mashed constantly for 1 h. After this, the wort was cooled to room temperature. Filtration was through Whatman No. 507 1/2

filter paper with the first 100 mL of wort returned back. Specific gravity was measured on 10 mL of wort in a DMA 5000 (Anton Paar, Germany). The specific gravity SG (g/mL) was then expressed in degrees Plato ($^{\circ}P$), which gives an indication of the original extract content using the formula from the American Society of Brewing Chemists (ASBC):

$$\text{degree Plato} = -616.868 + (1111.14 SG) - (630.272 SG^2) + 135997 SG^3 \quad (3)$$

One degree Plato corresponds to 1 g extract per 100 g of liquid solution. Wort density and maltose content in barley samples with bulk size fractions after mashing is shown in Figure S1.

Experiment 2. Mashing of one particular variety of malt sample with four different grain-size fractions was based on the method of the Institute of Brewing and Distilling (high-temperature infusion) with exactly 5 g grist (the crushed malt) with 20 mL of distilled water incubated in a water bath for exactly 60 min. Following this, the mixed mashing liquid was centrifuged at $4,000 \times g$ for 10 min. The resultant supernatant (wort) sample was kept in boiling water for another 60 min to inactivate the enzymes.

2.10 Starch contents

Starch contents of barley, malt and brewer's spent grain (BSG) samples were measured using a Megazyme Total Starch Kit (K-TSTA-1107, Megazyme, Ireland). Before measuring the starch content, weighed flour was washed with 4 mL of absolute ethanol followed by centrifuging to remove small sugars such as glucose and maltose produced during malting.

2.11 Starch utilization during mashing

The starch solubilization or utilization during mashing was calculated as starch utilization (fraction) = (total dry weight of starch for mashing – total dry weight of starch in BSG)/total dry weight of starch for mashing. The result for Commander malt samples with segregated grain size is shown in Figure S2.

2.12 Extraction of barley starch for structural analysis by SEC

This is required for Aim (2). Starches were extracted from four different grain sizes fractions of the Commander sample and the corresponding malt, and the starch molecular structure characterized by SEC. Each milled

flour sample was treated with 0.5 mL of protease (2.5 units/mL) in tricine buffer (pH 7.5, 250 mM) and incubated at 37 °C for 30 min followed by centrifugation at $4,000 \times g$ for 10 min. The precipitate was then mixed with 0.5 mL sodium bisulfite solution (0.45%, w/w) and again incubated followed by centrifugation at the defined conditions. Afterwards, lichenase in sodium phosphate buffer (20 mM, pH 6.5) was added to the precipitate and incubated at 40 °C for 1 h and then centrifuged for 10 min at $4,000 \times g$. To dissolve the starch, the precipitated starch was suspended in dimethyl sulfoxide (DMSO) solution containing 0.5% lithium bromide (1.5 mL DMSO/LiBr) and heated in a thermomixer at 80 °C for 24 h. Following this, the mixture was centrifuged at the defined conditions and the supernatant was precipitated using ethanol to remove the non-starch soluble components. This step was repeated to remove residual DMSO/LiBr [25].

For SEC of the whole starch, the precipitated starch was dissolved in 1 mL of DMSO and incubated at 80 °C for 3 h followed by centrifugation at $4000 \times g$ for 10 min. The supernatant was then collected for SEC analysis.

The starch chain-length distribution (CLD) can be presented either as the number distribution $N_{de}(X)$ (the number of chains with degree of polymerization X) or the weight distribution $w(\log X)$, with $w(\log X) = X^2 N_{de}(X)$ [26]. SEC with refractive index detection on debranched starch gives $w(\log X)$. Experimentally, the precipitate (extracted starch) from the previous procedure was dispersed in 0.9 mL of hot deionized water and heated in a boiling water bath until all precipitate was dispersed, forming a clear solution. The sample was debranched using isoamylase after addition of 5 μ L sodium azide solution (0.04 g/mL) and 0.1 mL of acetate buffer (0.1 M). The whole mixture was then incubated at 37 °C for 3 h and then adjusted to pH 7 by adding 0.1 M NaOH. Lastly, the debranched starch dispersion was heated at 80 °C for 2 h, freeze-dried, and then redissolved in DMSO/LiBr solution prior to SEC analysis [27].

2.13 Size-exclusion chromatography

Starches extracted from flour samples were separated using an Agilent 1100 SEC-MALLS system (Wyatt Technology, USA) equipped with a refractive index detector (DRI), using ASTRA software (version 6.1, Wyatt Technology, USA). SEC separates molecules based on molecular size, specifically the hydrodynamic radius, R_h [28]. The DRI detector gives the SEC weight distribution, $w(\log R_h)$, the relative weight (not molecular weight) of molecules as a function of size. For complex branched molecules such as whole starch, there is no relationship between

size and molecular weight. However, for linear polymers such as debranched starch, there is a unique relationship between R_h and molecular weight, and hence with degree of polymerization (DP) X . Details, including parameter values used here, are given elsewhere [29]. GRAM 100 and GRAM 3000 columns (PSS, Mainz, Germany) were used for branched starches; GRAM 100 and GRAM 1000 columns were used for debranched starches. Columns were set at 80 °C with a flow rate of 0.3 mL/min for branched starch samples and 0.6 mL/min for debranched starch samples.

Two common averages of the CLD are the degree of branching and amylose content (AC), which were found from the $w(\log X)$ of the debranched starch, as described elsewhere [29,30].

2.14 Fitting debranched data with biosynthesis-based models

There are different models for amylose and amylopectin. The CLD for both types of molecules is assumed to be governed by a number of enzyme sets. For amylopectin, each set contains one isoform of each of starch synthase (SS; chain growth), starch branching enzyme (SBE; chain stoppage and the formation of two new growing chains, including the requirement that there are minimum chain lengths for the remnant and snipped chains [31,32]) and debranching enzyme (DBE; chain removal). For amylose, again several enzyme sets are assumed; growth is mainly by granule-bound starch synthase (GBSS; elongation of amylose chains) but perhaps also by a soluble starch synthase (SSS; synthesis of long chains). Amylose has a small but significant number of long-chain branches, and chain stoppage is by one or more SBEs [33]. Both models assume that enzyme activities for these processes are independent of chain length over the DP range of interest. The solutions of the equations describing the kinetics of chain growth [34,35] give the CLD over the range of chain length of interest. Although the expressions for the CLDs are complex, publicly-available software [35,36] is available to fit data (which includes, for amylose chains, compensating for the effect of SEC band broadening). The models are in terms of two parameters for each enzyme set. As stated above, the parameter β is the ratio of the activity of the SBE and that of the SS (either as GBSS or SSS) in that set; h is the relative activity of the SS in that set. Although the amylopectin model explicitly takes DBEs into account, the mathematics shows [34] that the ratio of the DBE/SS activity for a given enzyme set is, in fact, related to that for SBE/SS (which is β), so DBE activity is not an independent variable.

2.15 Measurement of composition of fermentable sugars using HPLC

The measurement of sugar profiles is required for Aim (2). Wort samples from mashing experiment 2 were diluted 20× prior to analysis. Standard mixes of glucose, sucrose, maltose and maltotriose were made in the range of 1–27 µg/mL by dissolving in water. Samples were analyzed on an Agilent 1100 HPLC (Agilent Technologies, Germany) with an evaporative light scattering detector (ELSD). The HPLC solvent was 75% acetonitrile in water, with a 1 mL/min flow rate. An Altech Carbohydrate column (4.6 mm × 250 mm) used. The ELSD set to a nitrogen flow rate of 2 mL/min, temperature at 87 °C.

2.16 Differential scanning calorimetry

The measurement of starch gelatinization properties is required for Aim (2). The thermal properties of starches were measured with a TA differential scanning calorimeter (DSC25, TA). The sample (unmalted or malted barley flour, ~5 mg, dry basis) was weighed into an aluminium pan and 15 µL of distilled water (1:3 w/v) added. The pan was then hermetically sealed. The sample was equilibrated at 20 °C for 3 min and then heated from 20 °C to 100 °C at 5 °C/min. An empty pan was used as a reference. Enthalpy and gelatinization temperatures were calculated using TA software from the endothermic transition peaks corresponding to starch gelatinization [37]. The results of the thermal properties of raw and malted barley flour are shown in Table S1.

2.17 Data analysis

A two-tail test was carried out to determine significant differences between two different factors; $p \leq 0.05$ and ≤ 0.01 were used as thresholds of significant and highly significant, respectively. The univariate analysis of variance (ANOVA) of means, Pearson and Spearman rank correlations were performed using IBM SPSS software (version 16.0). Pearson correlation was used to determine the linear relationship between four different fractions of grain sizes and the wort density, as well as other physicochemical properties of barley. Spearman correlation, with lower sensitivity to outliers and no assumptions required towards the data distribution, was also used to determine the strength and direction of the relationship between the two defined variables. The means of duplicate measurements were used for the correlation analysis. Multiple means comparisons were determined with the Duncan's multiple range test at the

$p < 0.05$ confidence level. Principle component analysis (PCA) analysis was conducted using XLSTAT.

3 Results

3.1 Chemical composition

The chemical compositions of barley (unmalted) and corresponding malt samples, for different grain sizes (bulk fractions) are shown in Table 1. Malting resulted in a dramatic change in enzymatic activities. Starch content was seen to vary with protein content. Samples with more, larger grains had a higher starch content. In addition, larger grain sizes had higher soluble protein content, leading to a higher KI. The loss of starch during malting varied strongly among the samples.

3.2 CLDs of debranched starches

While the starch weight CLDs from SEC for unmalted and malted starches with bulk size fractions (mixing of different grain sizes) are shown in Figure 1 and Table 2. The amylopectin fitting results of eight barley and malt samples with bulk-mixed grain sizes are shown in Figure S3 and Figure S4, respectively. Figure S5 shows the amylopectin fitting results of Commander samples with segregated grain size fractions. The amylose fitting results of barley and malt samples with bulk-mixed grain sizes are

depicted in Figure S6 and Figure S7, respectively. Figure S8 illustrates the amylose fitting results of Commander samples with segregated grain size fractions. Showing data for all grain sizes together is of interest to brewers because of industry practice. The CLDs for the barley (unmalted) samples shows the structural features seen in many publications in the literature, e.g. [22,23]. One sees short chains from amylopectin, with the smaller of the features being for chains confined to a single crystalline lamella ($X \lesssim 34$) (set 1) and the second to chains crossing more than one lamella (trans-lamella chains), $34 \lesssim X \lesssim 70$ (set 2), and between $76 \lesssim X \lesssim 95$ (set 3). The five amylopectin model fitting parameters are as follows: the relative enzyme activity ratios of SBE to SS for each set ($\beta_{Ap,i}$, $\beta_{Ap,ii}$ and $\beta_{Ap,iii}$), and the relative contribution of SS in set 2 and 3 to set 1 ($h_{Ap,ii/i}$ and $h_{Ap,iii/i}$ respectively) [38]. The amylopectin chains show no noticeable variation with grain size, for biosynthetic reasons which have been established [34]. Significant differences were observed in $\beta_{Ap,iii}$, $h_{Ap,ii/i}$ and $h_{Ap,iii/i}$ between each sample before and after malting, as well as between different samples with various grain size distributions.

The amylose chains, which are DP $X \gtrsim 100$, show two discernible features below and above $X \sim 1000$. The amylose model fitting parameters are $\beta_{Am,i}$, $\beta_{Am,ii}$, $h_{Am,i}$ and $h_{Am,ii}$.

As shown in Table 2 and Figure 1, after malting, the AC increased significantly ($p < 0.05$) in samples 2, 3, 4 and 6. While samples 1, 5 and 7 did not show major

Table 2. Starch structural parameters from fitting with the biosynthesis models for amylose and amylopectin from debranched SEC data for barley (unmalted) and malt samples with bulk size fractions (mixing of different grain sizes).^a

Sample	Amylopectin fitting parameters					Amylose fitting parameters					
	$\beta_{Ap,i}/10^{-2}$	$\beta_{Ap,ii}/10^{-2}$	$\beta_{Ap,iii}/10^{-2}$	$h_{Ap,ii/i}/10^{-2}$	$h_{Ap,iii/i}/10^{-4}$	AC (%)	$\beta_{Am,i}/10^{-3}$	$\beta_{Am,ii}/10^{-4}$	$h_{Am,i}/10^{-2}$	$h_{Am,ii}/10^{-1}$	
Barley 1	8.0 ± 1 ^b	5.5 ± 0.1 ^b	3.0 ± 0.5 ^d	2.4 ± 0.0 ^c	8.9 ± 0.6 ^d	31 ± 2 ^{bc}	8.4 ± 0.1 ^{ab}	8.4 ± 0.0 ^{bcd}	5.9 ± 0.4 ^{abc}	1.8 ± 0.1 ^{ab}	
Malt 1	8.1 ± 0.0 ^b	5.5 ± 0.1 ^b	3.7 ± 0.0 ^{bc}	2.5 ± 0.0 ^{bc}	9.4 ± 1.7 ^{bcd}	30 ± 0 ^{bc}	8.2 ± 0.8 ^{ab}	7.7 ± 0.0 ^{cd}	5.9 ± 0.1 ^{abc}	1.7 ± 0.0 ^b	
Barley 2	8.0 ± 0.1 ^b	5.6 ± 0.3 ^b	3.5 ± 0.2 ^{bc}	2.5 ± 0.0 ^{bc}	8.9 ± 0.3 ^d	29 ± 0 ^c	8.5 ± 0.5 ^{ab}	8.9 ± 0.0 ^b	5.8 ± 1.0 ^{abc}	1.7 ± 0.0 ^b	
Malt 2	7.9 ± 0.2 ^b	5.4 ± 0.1 ^b	3.5 ± 0.1 ^{bcd}	2.7 ± 0.2 ^b	12.0 ± 1.3 ^{abcd}	31 ± 1 ^{abc}	8.5 ± 1.1 ^{ab}	7.7 ± 0.1 ^{cd}	7.2 ± 0.7 ^a	1.8 ± 0.1 ^{ab}	
Barley 3	8.1 ± 0.1 ^b	5.6 ± 0.3 ^b	3.6 ± 0.0 ^{bc}	2.6 ± 0.0 ^{bc}	10.3 ± 2.2 ^{bcd}	31 ± 1 ^{bc}	6.6 ± 0.0 ^b	8.2 ± 0.0 ^{bcd}	5.1 ± 0.7 ^{bc}	1.7 ± 0.1 ^b	
Malt 3	8.0 ± 0.1 ^b	5.3 ± 0.1 ^b	3.4 ± 0.0 ^{cd}	2.7 ± 0.0 ^b	12.2 ± 1.2 ^{abc}	32 ± 2 ^{abc}	8.7 ± 0.2 ^{ab}	7.6 ± 0.0 ^{cd}	7.4 ± 0.3 ^a	1.8 ± 0.2 ^{ab}	
Barley 4	8.0 ± 0.1 ^b	5.5 ± 0.1 ^b	3.4 ± 0.0 ^{cd}	2.5 ± 0.0 ^c	9.1 ± 0.0 ^{cd}	31 ± 1 ^{abc}	8.4 ± 0.2 ^{ab}	7.8 ± 0.1 ^{cd}	6.2 ± 0.0 ^{abc}	1.8 ± 0.1 ^{ab}	
Malt 4	8.1 ± 0.1 ^b	5.4 ± 0.1 ^b	3.4 ± 0.1 ^{bcd}	2.6 ± 0.0 ^b	13.5 ± 0.6 ^a	34 ± 4 ^a	7.1 ± 2.1 ^{ab}	7.4 ± 0.2 ^d	6.5 ± 0.4 ^{ab}	2.1 ± 0.4 ^a	
Barley 5	8.0 ± 0.2 ^b	5.5 ± 0.3 ^b	3.5 ± 0.3 ^{bc}	2.6 ± 0.0 ^{bc}	9.6 ± 0.7 ^{bcd}	30 ± 1 ^{bc}	8.7 ± 0.6 ^{ab}	8.5 ± 0.7 ^{bc}	6.1 ± 0.7 ^{abc}	1.8 ± 0.0 ^{ab}	
Malt 5	8.1 ± 0.0 ^b	5.6 ± 0.3 ^b	3.8 ± 0.2 ^{bc}	2.7 ± 0.1 ^b	11.5 ± 2.6 ^{abcd}	31 ± 1 ^{bc}	6.3 ± 2.6 ^b	7.7 ± 0.8 ^{cd}	4.8 ± 1.0 ^c	1.7 ± 0.1 ^b	
Barley 6	8.5 ± 0.1 ^a	6.1 ± 0.1 ^a	4.4 ± 0.1 ^a	3.3 ± 0.1 ^a	13.6 ± 0.6 ^a	24 ± 0 ^d	8.8 ± 0.2 ^{ab}	11.5 ± 0.5 ^a	6.6 ± 0.1 ^{ab}	1.2 ± 0.0 ^c	
Malt 6	8.1 ± 0.2 ^b	5.6 ± 0.1 ^b	3.9 ± 0.1 ^b	2.6 ± 0.1 ^{bc}	11.7 ± 0.1 ^{abcd}	32 ± 0 ^{abc}	9.0 ± 0.8 ^{ab}	8.0 ± 0.3 ^{bcd}	6.3 ± 0.6 ^{abc}	1.9 ± 0.0 ^{ab}	
Barley 7	8.0 ± 0.0 ^b	5.6 ± 0.0 ^b	3.5 ± 0.1 ^{bc}	2.4 ± 0.0 ^c	9.3 ± 0.4 ^{bcd}	31 ± 2 ^{bc}	9.5 ± 0.3 ^a	8.5 ± 0.9 ^{bcd}	6.7 ± 0.4 ^{ab}	1.8 ± 0.2 ^{ab}	
Malt 7	8.1 ± 0.1 ^b	5.5 ± 0.1 ^b	3.8 ± 0.2 ^b	2.6 ± 0.0 ^{bc}	12.3 ± 3 ^{ab}	31 ± 2 ^{bc}	6.1 ± 2.0 ^b	7.7 ± 0.0 ^{cd}	4.8 ± 1.5 ^c	1.8 ± 0.1 ^{ab}	
Barley 8	7.9 ± 0.1 ^b	5.6 ± 0.1 ^b	3.4 ± 0.3 ^{cd}	2.5 ± 0.1 ^{bc}	9.5 ± 0.6 ^{bcd}	33 ± 1 ^{ab}	6.4 ± 1.7 ^b	8.3 ± 0.2 ^{bcd}	4.9 ± 0.5 ^c	1.9 ± 0.0 ^{ab}	
Malt 8	8.1 ± 0.2 ^b	5.5 ± 0.2 ^b	3.7 ± 0.1 ^{bc}	2.5 ± 0.0 ^{bc}	10.6 ± 0.6 ^{abcd}	32 ± 0 ^{abc}	8.0 ± 0.5 ^{ab}	7.77 ± 0.6 ^{cd}	6.2 ± 0.2 ^{abc}	1.8 ± 0.0 ^{ab}	

^a AC is the amylose content. Values with different letters in the same column are significantly different at $p < 0.05$. All data based on dry basis and on duplicate measurements.

Figure 1. CLDs of debranched starches extracted from eight different barleys (A) and malts (B) with various grain size distributions (bulk size fractions), for a representative amylopectin (C) and amylose (D) fitting result. All distributions are normalized to the highest amylopectin peak. Fits for all samples are given in Figures S3–S8.

changes in the amylose CLD after malting, sample 8 was noticeably modified. All amylose fitting parameters varied significantly among the barley (unmalted) and malt samples.

3.3 Correlation between grain sizes and other barley and malt parameters

The correlations between barley grain size, chemical compositions and structural attributes are shown in Table 3A. There were no significant correlations between

chemical composition and grain size fractions. The starch structural parameters $\beta_{Ap,i}$ is related to the distribution of shorter amylopectin chains: a smaller value gives a lower proportion of short amylopectin chains. There were significant positive correlations between the amount of barley with size larger than 2.5 mm and $\beta_{Ap,ii}$, $\beta_{Ap,iii}$ and $h_{Ap,ii/i}$. Amylose content was significantly and negatively correlated to the medium size of barley. The amount of largest grain sizes (>2.8 mm) was significantly and negatively correlated to $\beta_{Ap,ii}$ and $\beta_{Ap,iii}$: the higher the proportion of grain sizes >2.8 mm, the lower the

Table 3. Correlations between barley grain sizes (bulk distribution), chemical compositions of barley (A) and malt (B).^a

(A) Barley (unmalted)						
	Pearson correlation			Spearman correlation		
	>2.2	>2.5	>2.8	>2.2	>2.5	>2.8
Starch	-0.416	-0.242	0.377	-0.192	-0.262	0.238
Crude Protein	0.471	0.378	-0.466	0.108	0.190	-0.119
Soluble Protein	0.169	-0.434	0.047	-0.132	-0.524	0.452
KI	-0.074	-0.577	0.268	-0.132	-0.548	0.429
Amylose content	-0.237	-0.615	0.394	-0.323	-0.738*	0.643
$\beta_{Ap,i}$	0.332	0.588	-0.449	0.639	0.707	-0.707
$\beta_{Ap,ii}$	0.361	0.608	-0.476	0.611	0.810*	-0.762*
$\beta_{Ap,iii}$	0.421	0.768*	-0.578	0.752*	0.855**	-0.819*
$h_{Ap,ii/i}$	0.305	0.597	-0.434	0.407	0.786*	-0.667
$h_{Ap,iii/i}$	0.445	0.642	-0.546	0.551	0.643	-0.571
$\beta_{Am,i}$	-0.426	-0.062	0.331	0.084	0.190	-0.143
$\beta_{Am,ii}$	-0.480	-0.115	0.373	-0.120	0.262	-0.143
$h_{Am,i}$	-0.293	0.011	0.197	0.132	0.000	-0.024
$h_{Am,ii}$	-0.356	-0.675	0.498	-0.635	-0.833*	0.857**
(B) Malt						
	Pearson correlation			Spearman correlation		
	>2.2	>2.5	>2.8	>2.2	>2.5	>2.8
Starch	-0.175	-0.418	0.277	-0.647	-0.238	0.357
Crude Protein	0.579	0.464	-0.572	0.743*	0.333	-0.452
Soluble Protein	-0.460	-0.707*	0.582	-0.874**	-0.762*	0.786*
KI	-0.507	-0.685	0.605	-0.826*	-0.619	0.690
Amylose content	0.269	0.317	-0.304	0.826*	0.500	-0.643
α -Amylase	-0.507	-0.732*	0.623	-0.707	-0.762*	0.833*
β -Amylase	-0.255	-0.697	0.437	-0.635	-0.714*	0.786*
$\beta_{Ap,i}$	-0.359	-0.377	0.388	-0.275	-0.357	0.429
$\beta_{Ap,ii}$	-0.640	-0.331	0.564	-0.536	-0.299	0.419
$\beta_{Ap,iii}$	-0.437	-0.252	0.395	-0.271	-0.216	0.311
$h_{Ap,ii/i}$	0.443	0.807*	-0.608	0.536	0.838**	-0.814*
$h_{Ap,iii/i}$	0.289	0.511	-0.390	0.695	0.405	-0.524
$\beta_{Am,i}$	0.493	0.401	-0.489	0.491	0.571	-0.595
$\beta_{Am,ii}$	0.009	0.225	-0.091	-0.343	-0.180	0.263
$h_{Am,i}$	0.569	0.443	-0.557	0.671	0.643	-0.738*
$h_{Am,ii}$	0.220	0.261	-0.249	0.707	0.357	-0.500

^a * Correlation is significant at the 0.05 level (2-tailed); ** correlation is significant at the 0.01 level (2-tailed).

proportions of short amylopectin chains. The amount of medium grain size was significantly and negatively correlated to $h_{Am,ii}$, while a significant positive correlation was seen between the proportions of largest size of barley and $h_{Am,ii}$. This implies that the higher the amount of medium sizes, the lower the proportion of long amylose

chains, while higher fractions of largest grain size resulted in longer amylose chains.

Table 3B shows significant correlation between grain size and chemical composition: e.g. negative correlation between the medium size of malt (>2.5 mm) and both soluble protein and α -amylase activity. The relative amount

of medium-size amylopectin chains ($34 \lesssim X \lesssim 70$), $h_{Ap,ii/i}$, was significantly and positively correlated to the amount of medium size grains in malt. Several significant negative correlations were observed between the proportions of smallest size malt (> 2.2 mm) and soluble protein and KI. In addition, the amount of the smallest size of malt also significantly and positively correlated to the protein and AC. There were no significant correlations between the amount of the smallest size of malt and amylopectin as well as amylose fitting parameters. The proportion of the largest malt (> 2.8 mm) showed a several significant positive correlations with soluble protein content, α -amylase and β -amylase activities, and also significant negative correlations with $h_{Ap,ii/i}$ and $h_{Am,i}$.

3.4 Correlations between malting parameters and mashing performance

The correlations of barley malting parameters and starch structural attributes to wort density and maltose content are shown in Table 4. There was a significant negative correlation between $h_{Ap,iii/i}$ and wort density: shorter amylopectin chains result in higher wort density. Protein content of malt was significantly and negatively correlated to both wort density and maltose content. A significant positive correlation was found between both soluble protein and KI to wort density. While there were no significant correlations between amylose and amylopectin fitting parameters in malt to wort density and maltose content, strong negative associations were seen between $h_{Ap,ii/i}$ and $h_{Ap,iii/i}$ to wort density.

3.5 Chemical composition: dependence on grain size

The chemical compositions of Commander barley and malt samples are shown in Table 5. The starch and protein contents of barley and malt varied significantly with grain size, in accordance to previous studies [8,22], in which the largest size (> 2.8 mm) had the highest starch content. They contained statistically the same level of starch after malting, except for the medium malt size (> 2.5 mm). The smallest size of barley and malt (> 2.2 mm) had the highest protein content. An inverse relationship between protein and starch contents was observed in the malt samples (this last not always with $p < 0.05$). All barley samples had undergone endosperm modification with a decreased protein and total starch contents. After malting, the

smallest grain size had the lowest starch loss whereas the largest size of grain had the highest protein loss, while the medium size of grain had the lowest protein loss. In contrast to the α -amylase activity, the β -amylase activity varied significantly among the different size of samples, in which the largest size of malt had the lowest β -amylase activity whereas the smallest size of malt had the highest enzymatic activity.

3.6 Grain-size dependence of CLDs from barley and malt

The weight CLDs of debranched starches from Commander barley and malt samples with segregated size fractions is shown in Table 6 and Figure 2. The range above the minimum in $w(\log X)$, close to $X = 100$, was considered as amylose and the area under the curve used to calculate the AC. AC did not vary significantly amongst the barley and malt samples with different grain sizes. There are significant grain-size differences in $\beta_{Ap,i}$, $\beta_{Ap,ii}$, $h_{Ap,ii/i}$ and $h_{Ap,iii/i}$ for both barleys and malts. There was no significant variation with grain size in the amylose chains except for the degradation of short chains in set 1 ($DP \lesssim 1000$), as indicated by the decrease in $h_{Am,i}$ after malting.

3.7 Correlation between chemical properties and fermentable sugars released

As shown in Figure 3A, β -amylase activity showed a strong correlation to the production of sugars, ascribed to the lower sugars released for the largest grain size (> 2.8 mm). The amount of starch utilized by the hydrolytic enzymes (Fig. 3B) also indicates a strong positive association with fermentable sugars released during mashing.

3.8 Correlation between starch structural attributes and mashing performance

As shown in Figure 4, there were no significance differences in glucose and sucrose content, but a noticeable variation was observed in maltose content with the β_{Am} parameters. A significant difference was seen in maltotriose content with $\beta_{Am,i}$ and $\beta_{Am,ii}$ in malts. As seen in Figure 4A and 4B, the β_{Am} parameters were positively correlated to the fermentable sugars content: the amount of short amylose chains was strongly associated with the sugars released during mashing.

Table 4. The correlation between chemical compositions and structural attributes of barley (A) and malt (B) to the mashing properties.^a

(A) Barley (unmalted)				
	Pearson correlation		Spearman correlation	
	Wort density	Maltose content	Wort density	Maltose content
Starch	0.349	-0.053	0.371	0.262
Crude Protein	-0.558	-0.469	-0.347	-0.571
Soluble Protein	0.050	-0.177	0.072	-0.286
KI	0.322	0.080	0.192	0.048
Amylose content	0.218	-0.407	0.263	-0.500
$\beta_{Ap,i}$	-0.313	0.289	-0.428	0.359
$\beta_{Ap,ii}$	-0.338	0.270	-0.491	0.310
$\beta_{Ap,iii}$	-0.602	-0.019	-0.661	-0.036
$h_{Ap,ii/i}$	-0.367	0.250	-0.527	0.119
$h_{Ap,iii/i}$	-0.494	0.135	-0.790*	-0.262
$\beta_{Am,i}$	0.299	0.087	-0.144	-0.024
$\beta_{Am,ii}$	-0.167	0.335	0.180	0.452
$h_{Am,i}$	0.089	0.005	-0.252	-0.143
$h_{Am,ii}$	0.326	-0.375	0.455	-0.524
(B) Malt				
	Pearson correlation		Spearman correlation	
	Wort density	Maltose content	Wort density	Maltose content
Starch	0.639	0.478	0.578	0.500
Crude protein	-0.817*	-0.718*	-0.683	-0.690
Soluble protein	0.837**	0.524	0.934**	0.333
KI	0.920**	0.685	0.886**	0.476
Amylose content	-0.563	-0.287	-0.707	-0.310
α -Amylase	0.531	-0.254	0.587	-0.143
β -Amylase	0.546	0.064	0.395	-0.143
$\beta_{Ap,i}$	0.014	-0.420	-0.084	-0.619
$\beta_{Ap,ii}$	0.355	0.099	0.175	0.204
$\beta_{Ap,iii}$	0.239	-0.086	0.090	-0.156
$h_{Ap,ii/i}$	-0.652	-0.060	-0.446	0.108
$h_{Ap,iii/i}$	-0.687	-0.540	-0.575	-0.500
$\beta_{Am,i}$	0.054	0.299	0.343	0.383
$\beta_{Am,ii}$	-0.509	-0.233	-0.587	-0.357
$h_{Am,i}$	-0.088	0.556	-0.252	0.571
$h_{Am,ii}$	-0.279	0.290	-0.371	0.238

^a* Correlation is significant at the 0.05 level (2-tailed); ** correlation is significant at the 0.01 level (2-tailed).**Table 5.** Chemical compositions of Commander barley (unmalted) and malt samples with segregated grain size distribution (n=8).^a

Segregated grain size fractions	Starch (%)		Loss of starch after malting (%)	Protein (%)		Loss of protein after malting (%)	α -Amylase (U/g)	β -Amylase (U/g)
	Barley	Malt		Barley	Malt		Malt	Malt
> 2.2 mm	54 $\pm$ 0 ^c	53 $\pm$ 1 ^a	2 $\pm$ 1 ^b	13 $\pm$ 0 ^a	11 $\pm$ 0 ^a	17 $\pm$ 2 ^b	121 $\pm$ 16 ^a	327 $\pm$ 12 ^a
> 2.5 mm	55 $\pm$ 0 ^{ab}	52 $\pm$ 1 ^b	6 $\pm$ 1 ^a	11 $\pm$ 0 ^c	10 $\pm$ 0 ^b	3 $\pm$ 0 ^d	112 $\pm$ 13 ^a	265 $\pm$ 17 ^b
> 2.8 mm	56 $\pm$ 1 ^a	54 $\pm$ 0 ^a	4 $\pm$ 1 ^{ab}	13 $\pm$ 0 ^b	8 $\pm$ 0 ^d	39 $\pm$ 2 ^a	102 $\pm$ 6 ^a	240 $\pm$ 1 ^b
Bulk	54 $\pm$ 0 ^{bc}	53 $\pm$ 0 ^a	2 $\pm$ 1 ^b	10 $\pm$ 0 ^d	9 $\pm$ 0 ^c	9 $\pm$ 0 ^c	111 $\pm$ 7 ^a	267 $\pm$ 10 ^b

^a All data based on duplicate measurements and on dry basis; standard deviation given in parenthesis. Values with different letters in the same column are significantly different at $p < 0.05$. Bulk: from mixing three different grain size fractions, which acts as a control.

Figure 2. The CLDs of debranched starches extracted from Commander barleys and malts with segregated size fractions (A), the enlarged version of amylose CLD from DP 100 to 10000 (B), for a representative amylopectin (C) and amylose (D) fitting result, showing the components from each enzyme set and the overall fit. All distributions were normalized to the highest amylopectin peak. The deviation between fitted and experiment at lower amylose DPs is because of overlap with longer amylopectin chains. All fits are given in Figure S5 and Figure S8.

Table 6. Starch structural parameters from debranched SEC data for barley (unmalted) and malt samples with segregated grain size fractions: amylose content and fitting values to the amylose and amylopectin component of the CLD with the biosynthesis models [35,38,39].^a

Sample	Amylopectin					Amylose				
	$\beta_{Ap,i}/10^{-2}$	$\beta_{Ap,ii}/10^{-2}$	$\beta_{Ap,iii}/10^{-2}$	$h_{Ap,ii/i}/10^{-2}$	$h_{Ap,iii/i}/10^{-4}$	AC (%)	$\beta_{Am,i}/10^{-3}$	$\beta_{Am,ii}/10^{-4}$	$h_{Am,i}/10^{-2}$	$h_{Am,ii}/10^{-1}$
> 2.2 mm Barley	8.6 ± 0.1 ^d	4.8 ± 0.0 ^b	4.6 ± 0.1 ^a	1.7 ± 0.0 ^a	9.4 ± 0.3 ^a	33 ± 1 ^a	3.4 ± 0.1 ^b	6.8 ± 0.5 ^{abc}	8.7 ± 1.4 ^a	1.7 ± 0.1 ^{ab}
> 2.2 mm Malt	8.8 ± 0.0 ^{cd}	4.9 ± 0.0 ^{ab}	4.7 ± 0.0 ^a	1.6 ± 0.0 ^{ab}	8.2 ± 0.0 ^{ab}	32 ± 1 ^a	6.5 ± 0.1 ^{ab}	8.0 ± 0.0 ^{ab}	5.4 ± 0.2 ^b	1.7 ± 0.1 ^{ab}
> 2.5 mm Barley	8.7 ± 0.0 ^{cd}	4.9 ± 0.1 ^{ab}	4.8 ± 0.1 ^a	1.6 ± 0.0 ^{ab}	8.2 ± 0.9 ^{ab}	33 ± 1 ^a	3.6 ± 0.1 ^b	6.8 ± 0.9 ^{abc}	7.8 ± 1.7 ^{ab}	1.7 ± 0.1 ^{ab}
> 2.5 mm Malt	8.9 ± 0.1 ^{bc}	4.8 ± 0.1 ^b	4.9 ± 0.1 ^a	1.6 ± 0.0 ^{ab}	9.1 ± 0.8 ^a	35 ± 4 ^a	9.1 ± 1.4 ^a	8.3 ± 0.1 ^a	5.0 ± 0.7 ^b	2.0 ± 0.3 ^a
> 2.8 mm Barley	8.7 ± 0.1 ^{cd}	5.0 ± 0.1 ^{ab}	4.9 ± 0.2 ^a	1.6 ± 0.0 ^{ab}	7.6 ± 0.3 ^{ab}	34 ± 0 ^a	3.1 ± 0.1 ^b	6.1 ± 0.0 ^{bc}	9.2 ± 0.7 ^a	1.7 ± 0.0 ^{ab}
> 2.8 mm Malt	8.9 ± 0.2 ^{abc}	5.3 ± 0.4 ^a	5.3 ± 0.7 ^a	1.5 ± 0.1 ^b	5.2 ± 2.8 ^b	31 ± 2 ^a	6.1 ± 4.6 ^{ab}	7.3 ± 1.8 ^{abc}	5.1 ± 2.0 ^b	1.7 ± 0.2 ^{ab}
Bulk Barley	9.1 ± 0.1 ^{ab}	4.9 ± 0.2 ^{ab}	4.7 ± 0.3 ^a	1.6 ± 0.1 ^{ab}	7.9 ± 1.7 ^{ab}	33 ± 4 ^a	7.3 ± 0.2 ^{ab}	6.1 ± 0.1 ^c	8.7 ± 1.8 ^a	1.6 ± 0.3 ^{ab}
Bulk Malt	9.1 ± 0.0 ^a	5.0 ± 0.0 ^{ab}	4.8 ± 0.1 ^a	1.6 ± 0.0 ^{ab}	6.9 ± 0.6 ^{ab}	31 ± 1 ^a	3.8 ± 0.0 ^b	6.2 ± 0.1 ^{bc}	7.8 ± 0.7 ^{ab}	1.6 ± 0.1 ^b

^a All data based on dry basis and on duplicate measurements. Values with different letters in the same column are significantly different at $p < 0.05$. AC is the amylose content.

Figure 3. The fermentable sugars released in wort as a function of β -amylase activity (A) and starch utilization (B). All data based on duplicate measurements. All data measured based on dry basis.

4 Discussion

4.1 Effects of grain size on chemical composition and starch structure

Previous studies showed that smaller grains generally had higher protein levels and lower starch content [8,40]. The results in this study (Table 1) are consistent with this. These effects are due to environmental conditions, such as higher temperature during grain filling, resulting in cessation of grain size growth followed by the reduction of capacity to convert sucrose into starch due to the irreversible loss of the activity of SSS [14,41]. Protein accumulation within the grains was less influenced by the heat stress compared to starch, thereby leading to a higher protein and lower starch level in smaller grains [42]. After malting, the soluble protein and KI for all the malt samples were in the lower range, except for sample 1 with a higher fraction of larger grain size, which could result from higher proteolytic activities hydrolyzing the insoluble protein components during germination, causing a higher content of nitrogen nutrition [8,43,44].

Grain size has a significant impact on the activity of starch-degrading enzymes. The main difference between

Figure 4. Dependences of fermentable sugars content in wort after mashing on amylose structural parameters ($\beta_{Am,i}$ and $\beta_{Am,ii}$) in malts. All data based on duplicate measurements. All data measured based on dry basis.

α -amylase and β -amylase in mashing is that α -amylase undergoes *de novo* synthesis in the aleurone layer and is regulated by gibberellins secreted from the scutellum during germination [45,46]. Larger grain size will result in larger aleurone layers, which can then synthesize higher amounts of α -amylase and endoproteases. The high activities of endoproteases can then lead to significant degradation of disulfide linkages and insoluble protein components in the protein matrix, releasing β -amylase and increasing the nitrogen nutrition. This is consistent with our observation that larger grain size fractions had higher enzymatic activities and KI. The differences in amount of starch after malting would be influenced by genetic and environmental influences [17,18]. For instance, sample 1 had a higher fractions of larger grain size compared to sample 7, but the starch loss in sample 1 was much lower than sample 7, despite having similar enzymatic activities; this can be ascribed to the different growing sites: Jambin and Brookstead.

It was found that samples with higher fractions of largest grain size, such as sample 1, contained lower amount of long amylopectin chains ($h_{Ap,iii/i}$), whereas samples with higher fractions of medium grain size would contain higher ratio of longer amylopectin chains ($h_{Ap,ii/i}$), with significant positive correlation as shown

in Table 3A. The significant negative correlations of $\beta_{Ap,ii}$ and $\beta_{Ap,iii}$ to largest grain size suggesting that the amount of short chains in region 3 was lower compared to smaller grain size. It appears that higher fractions of medium grain size would contain lower AC, potentially due to lower amount of long amylose chains, such as in sample 6. After malting, an increase in $\beta_{Ap,iii}$ was observed in samples with larger grain size, i.e. relatively more short chains, probably from higher activity of α -amylase cleaving the long amylose chains during imbibition and germination of barley [23,47]. Additionally, there were increases in $h_{Ap,ii/i}$ and $h_{Ap,iii/i}$ in all malt samples (except sample 6); this is attributed to preferential enzymatic attack on shorter amylopectin chains, resulting in a significant increase of the ratio of longer amylopectin chains ($34 \lesssim X \lesssim 95$) to shorter ones ($X \lesssim 34$). There was an increase in AC in all samples (except those with higher fractions of largest grain size such as samples 1 and 8), and a concomitant increased fraction of amylose chains ($h_{Am,i}$ and $h_{Am,ii}$) after malting, which is ascribed to the preferential enzymatic hydrolysis of amylopectin over amylose [6,48]. This result suggests that the crystalline lamellae formed by the short, external chain segments of amylopectin are more exposed and situated at the outer regions of starch granules, thereby increasing the accessibility of enzymatic hydrolysis [49,50]. Except for samples 3 and 6 with smaller grain size fractions and lower enzymatic activities, there were significant decreases of $\beta_{Am,i}$ and $\beta_{Am,ii}$ in all samples after malting, simply because the enzymatic degradation of starch is not confined to the amylopectin chains. Increases of $\beta_{Am,i}$ and $\beta_{Am,ii}$ in sample 3 and 6 simply be because the degradation of amylopectin chains perforce increases the ratio of amylose to amylopectin [51,52].

Current industrial brewing standards emphasize larger grain size as a primary quality parameter [15]. The present study examines this practice by examining both size-fractionated and bulk-mixed grain sizes. It is seen (Table 5) that the largest size had the highest starch content but did not achieve the highest starch loss after malting, probably because of lower enzymatic activities; the smallest size had the lowest starch loss despite having the highest enzymatic activities. This is ascribed to the higher protein content: the protein matrix protects starch granules from enzyme hydrolysis [53]. The bulk control sample had lower starch content and enzyme activities, and thus lowest starch loss after malting with a higher starch content. Lastly, the highest β -amylase activity was observed in the smallest size of malt, which could be because of the elevated levels of nitrogen in the endosperm [54].

The smallest size of barley had relatively more longer amylopectin chains, and a significant increase in $\beta_{Ap,i}$ and $\beta_{Ap,ii}$ after malting for the smallest and largest size of grain. This is ascribed to the cleavage or trimming of longer chains by α -amylase, or to the hydrolysis of branches by DBE such as limit dextrinase [47], as indicated by the decrease in $h_{Ap,ii/i}$ and $h_{Ap,iii/i}$ and thus an enrichment of short amylopectin chains. The decrease in $\beta_{Ap,ii}$ and increase in $h_{Ap,iii/i}$ in the medium grain size means the enzymes preferentially attacked shorter amylopectin chains [6]. For amylose, the increase in short chains ($\beta_{Am,i}$) in all three segregated grain size samples after malting is ascribed to the longer chains in this set being cleaved by α -amylase.

4.2 Effects of chemical composition and starch structure on mashing performance

Raw barley undergoes endosperm modification during malting in order to secrete enzymes, which result in hydrolysis of starch and protein into fermentable sugars and amino acids, respectively. One therefore expects no correlations between the chemical compositions of unmalted barley and mashing properties, such as wort density and maltose content (Table 4A). Our starch structural results are consistent with a previous study [22], which found a significant negative correlation between $h_{Ap,iii/i}$ and wort density. All the barley samples listed in Table 1 had higher $h_{Ap,iii/i}$ values than sample 1: those samples had a higher proportion of long amylopectin chains, increasing the crystallinity and molecular order of starch granules, and thereby limiting the amylolysis and release of free sugars [55,56]. Higher gelatinization temperatures and enthalpy change are then required during mashing process due to higher crystallinity; longer amylopectin chains also require longer hydrolysis times.

After malting, we observed significant correlations between chemical composition and mashing properties, e.g.: Table 4B showing significant negative correlations of crude protein content with wort density and maltose content. This is because higher protein level results in the hordein network entrapping larger amounts of starch granules, giving a physical barrier limiting the accessibility of enzymes to starch, and hence reducing the rate of starch digestion, which in turn decreases the maltose sugars production and wort density [57,58]. A higher level of protein matrix would also inhibit the swelling of starch granules during mashing, influencing starch gelatinization and enzymatic degradation of starch into soluble sugars [59]. This is probably why samples 3 and 4 produced lower maltose content and wort density,

because of the higher malt protein content. The significant positive correlations between soluble protein and KI with wort density are ascribed to the supplementation of high nitrogen to hydrolytic enzymes in sample 1, providing more energy to degrade the starch into fermentable sugars, increasing the wort density [60,61]. There were no significant correlations of either amylose and amylopectin structural parameters to mashing performance. However, the values of $h_{Ap,ii/i}$ and $h_{Ap,iii/i}$ in malted starch were negatively associated with wort density, implying that shorter amylopectin chains were advantageous in releasing a higher amount of fermentable sugars.

Generally, it is believed that the higher the starch content in barley, the higher the fermentable sugars released later in the mashing stage [8]. However, in Table 5, it is seen that the largest size of barley (>2.8 mm) with the highest starch content did not contribute to the highest fermentable sugars production, which could be due to having the lowest β -amylase activity, thereby reducing the efficiency of starch digestion to sugars [62]. As shown in Figure 3A, it appears that the smallest size of barley did not produce the highest fermentable sugars despite having the highest β -amylase activity. The determining factor is perhaps the high protein content, as reported in previous studies: firstly, a higher level of protein matrix could act as a physical barrier, embedding the available starch granules and impact the enzymatic digestion [59]; secondly, there could be effects of non-catalytic binding of amylolytic enzymes on protein [63], which means that excess protein will competitively bind to the α -amylase, thereby reducing its activity; lastly, higher levels of surface proteins could also retard the catalytic binding of enzyme on the starch granule surface [64,65]. In addition, it was found [66] that the higher the degree of starch-protein interactions, the lower the starch hydrolysis and thus the poorer the quality of malting. The medium malt size produced the highest fermentable sugars in wort during mashing, which could be due to the highest level of starch utilization by the amylolytic enzymes during mashing (Fig. 3B), together with a lower protein content, which will not result in the hindering of starch hydrolysis [53], and thereby producing the highest fermentable sugars.

The molecular structure of starch also plays an important role in determining the fermentable sugars production. Figures 4A and 4B indicated that the medium malt size with the highest amount (h) of short amylose chains in sets 1 and 2 produced the highest fermentable sugars. This is because during mashing, starch granules will first swell and gelatinize, followed by leaching of amylose molecules into the mashing solution; longer

amylose chains will entangle and co-crystallize more easily with amylopectin chains in the crystalline lamellae, resulting limited swelling of starch granules, which in turn causes a significant decline of the rate of enzymatic diffusion to the available starch, hence decreasing the starch digestion into fermentable sugars [67,68].

4.3 Conclusions

In the study of bulk-mixed grain sizes, the results indicated the samples with more larger grains would have higher starch content and lower protein level. Higher enzymatic activities were observed in the corresponding malts with more larger grains, which is attributed to the supplementation of high nitrogen providing more energy to enzymes for amylolysis. Samples with higher fractions of larger grain size were identified with lower proportions of long amylopectin chains ($h_{Ap,iii/i}$). Additionally, an inverse relationship was observed between starch and protein content in the segregated grain size study. Higher protein level is suggested to result in higher enzymatic activities in the smallest size of malt. Besides, the medium grain size was found to contain the highest amount of short amylose chains.

High levels of protein resulted in lower extract content in wort, ascribed to the protein matrix protecting the starch granules, which limit the degradation into free sugars. Furthermore, higher enzymatic activities could increase the breakdown of starch into sugars, increasing the wort density. In respect to starch structure, higher proportions of long amylopectin chains ($h_{Ap,iii/i}$) was shown to reduce the wort density. A possible reason for this is the increased crystallinity and molecular order of starch granules resulting in the reduction of amylolysis and release of soluble sugars. These are most probably the reasons of sample 1 with more larger grains had the highest maltose content and wort density. It was found that medium grain size with the highest proportion of starch being utilized during brewing resulted in highest fermentable sugars production. Finally, a higher amount of short amylose chains was found to release more sugars in wort, owing to the absence of limited granular swelling and suppression of starch gelatinization during mashing.

As starch (not protein) is the main substrate for amylase-driven fermentable sugars production, measuring its structure should be a better indication for the mashing performance. Therefore, it is essential to take into consideration of starch structure as an industry specification when selecting barley grains used for beer brewing.

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