Effects of Mash Style on β-Glucan Concentration and β-Glucanase Activity in the Production of Quality Wort for Beer Brewing

By

SOPHIE THERESE HELD

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Food Science

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Glen P. Fox, Chair

Kyria Boundy-Mills

Stephanie Dungan

Committee in Charge

2022

Table of Contents

Abstract

The hydrolysis by β-glucanase enzymes of barley β-glucan is important in the malting process to degrade endosperm cell walls and prepare malt for later sugar extraction during mashing. Excess β-glucan from unmalted adjuncts or unevenly modified malt can have a negative effect on wort and beer quality. This thesis explores how β-glucanase activity during mashing can influence wort β-glucan, viscosity, and filtration. First, a β-glucanase method was adapted for use on a Thermo Scientific BeerMaster Gallery autoanalyzer. This method enabled the simultaneous analysis of β-glucan and β-glucanase throughout the course of two different mash profiles—a European Brewing Congress (EBC) mash starting at 45°C and a modified Institute of Brewing (IoB) mash starting at 65°C. In the lowertemperature EBC mash, malt β-glucanase was able to reduce wort β-glucan contributed from 10-20% additions of unmalted barley. However, the higher temperature of the IoB mash resulted in rapid enzyme denaturation, and therefore a large amount of β-glucan accumulated in the wort. Two thermostable β-glucanases were added at the beginning of the IoB mash with 10-20% barley adjunct to evaluate their impact on β-glucan during the course of the mash. The β-glucanase from *Trichoderma* quickly hydrolyzed β-glucan as it was extracted, resulting in an easily separated wort with low β-glucan. The β-glucanase allozyme from wild barley was not as thermostable as the *Trichoderma* β-glucanase, and thus β-glucan accumulated in the wort as β-glucanase activity decayed. However, the addition of wild barley enzyme was able to reduce β-glucan in 10 and 20% barley mashes by 52 and 47% respectively. This thesis finds that the IoB mash condition is susceptible to high levels of wort β-glucan when the grist includes unmalted barley. Thermostable wild barley β-glucanase is able to reduce the amount of wort β-glucan, but it is not as effective as bacterial-source enzymes that are more thermostable and already commercially available.

Chapter 1

The β-glucan and β-glucanase relationship during malting and mashing: a review

Abstract

The process of malting and mashing beer involves a variety of enzymes hydrolyzing their respective substrates. β-glucanase is responsible for the degradation of β-glucan polymers which are negatively associated with malt, wort, and beer quality. Hydrolysis of the β-glucan in cell walls is also important in making the starch within endosperm cells available for conversion into fermentable sugars. This review covers relevant information related to the structure of β-glucan, its development in the plant, how it is measured, and how it correlates with other barley and malt quality metrics. Next, the nature of the β-glucanase enzyme is discussed as well as its correlation to other quality metrics throughout the malting and mashing process. The interaction between β-glucan and β-glucanase during malting and during different types of mash can provide insights towards the causes for quality problems associated with β-glucan such as high viscosity wort, slow wort separation, and haze. Finally, past work regarding the use of exogenous enzymes added to ameliorate problems from high β-glucan grists and worts will be examined.

Introduction

The primary non-starch polysaccharide in barley is β-glucan which accounts for about 4-5% of the dry mass of the grain (39). Other grains contain β-glucan to varying degrees: oats and barley have similarly high levels of β-glucan while rice, wheat, and other brewing grains have much lower β-glucan

contents. It is proposed that in the cell walls of barley, there is an outer layer of arabinoxylans surrounding the β-glucan bulk protecting the starch and protein reserves within the cell (8).

Malt β-glucanase is a group of enzymes that hydrolyze the β-1,3 and/or β-1,4 linkages in the poly-glucose chain. There is no current guideline for how much β-glucanase activity is necessary in malt because, depending on processing parameters, the heat sensitive enzyme contributes little activity during mashing.

The β-glucan story: transformation of barley to wort

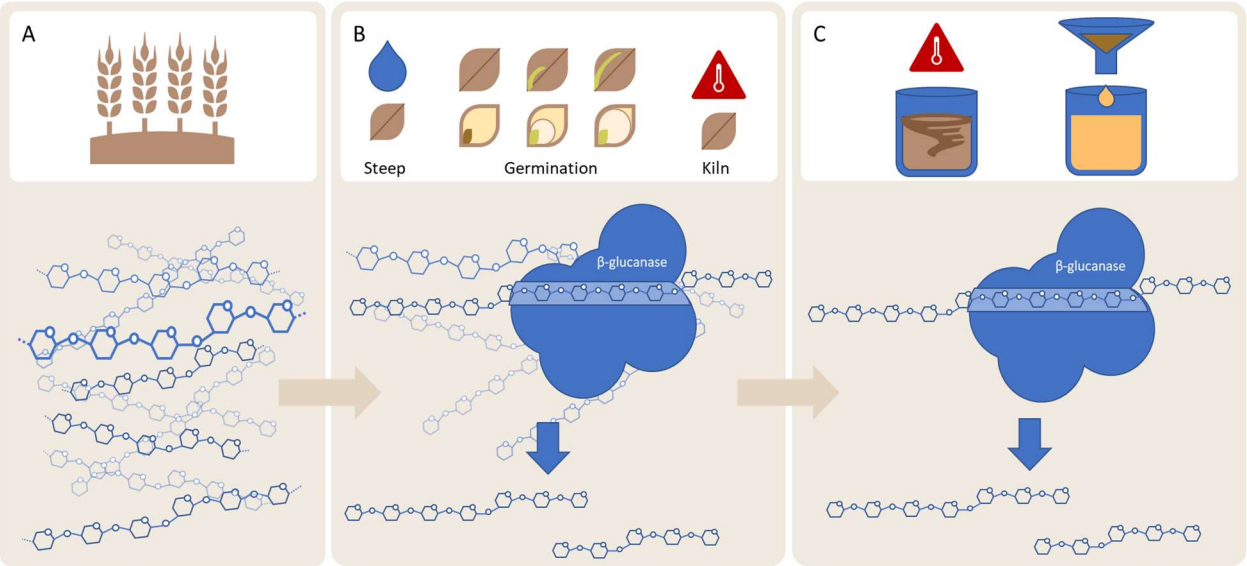

Figure 1.1: A visual description of the progression from barley (A) to malt (B) to wort (C) and how β-glucan is synthesized then hydrolyzed to progressively lower levels by β-glucanase.

β-glucan is synthesized in the barley seed where it forms cell walls in the endosperm of the grain (Figure 1.1A). To malt the grain, it is steeped in cool water to hydrate the endosperm (Figure 1.1B). The fraction of soluble β-glucan increases. The steeping process initiates the enzyme synthesis in the grain that begins the process of germination. The goal of malting and germination is to modify the endosperm of the grain—breaking down the "packaging" around the starch reserves. β-glucanases are synthesized in the scutellum and embryo (81). They diffuse through the hydrated grain and hydrolyze β-glucan polymers in the cell walls. Cell wall hydrolysis is important so that the protein and starch matrix within

can be accessed by proteinases during malting and diastases during mashing. When the grain is killed, enzyme activity is arrested, and much of the enzyme denatures due to high heat (11,60).

The malt is ground and mixed with warm water to begin the process of mashing (Figure 1.1C). The goal of mashing is to convert the starches in the endosperm to fermentable sugars via hydrolysis with native enzymes. Any residual β-glucanase activity is once again able to resume its work hydrolyzing the remaining β-glucan (11). Since β-glucanase is very heat labile, it contributes some activity during mashing, but the enzyme is denatured by the end of the process. At the end of the mash, the liquid wort is separated from the spent grains, and some of the remaining unhydrolyzed β-glucan is swept into the wort fraction.

This review will examine β-glucan and β-glucanase separately at first, and then discuss how they interact throughout malting and mashing and what the processing and quality concerns are regarding excess β-glucan in wort and beer.

β-Glucan

Structure

Barley 1,3-1,4-β-glucan is made up of glucose units joined from carbons one and three or carbons one and four (Figure 1.2). The stereochemistry around those linkages is in a β, or axial, position to the six-carbon ring, distinct from the α-1,4 linkages found between glucose in starch.

The β-glucan content of a grain or liquid is an important metric in brewing, but the size of β-glucan polymers, their structure, and possible intermolecular interactions are likely even more important in beer quality. Unhydrolyzed barley β-glucans typically have a molecular weight (MW) between 200 to 300 kDa and are comprised of 1,200 to 1,850 glucose units (12). Barley and malt β-glucan has water non-soluble and water-soluble fractions (7). Both the size of the polymer and its ability to interact with other β-glucan polymers influences its solubility (61).

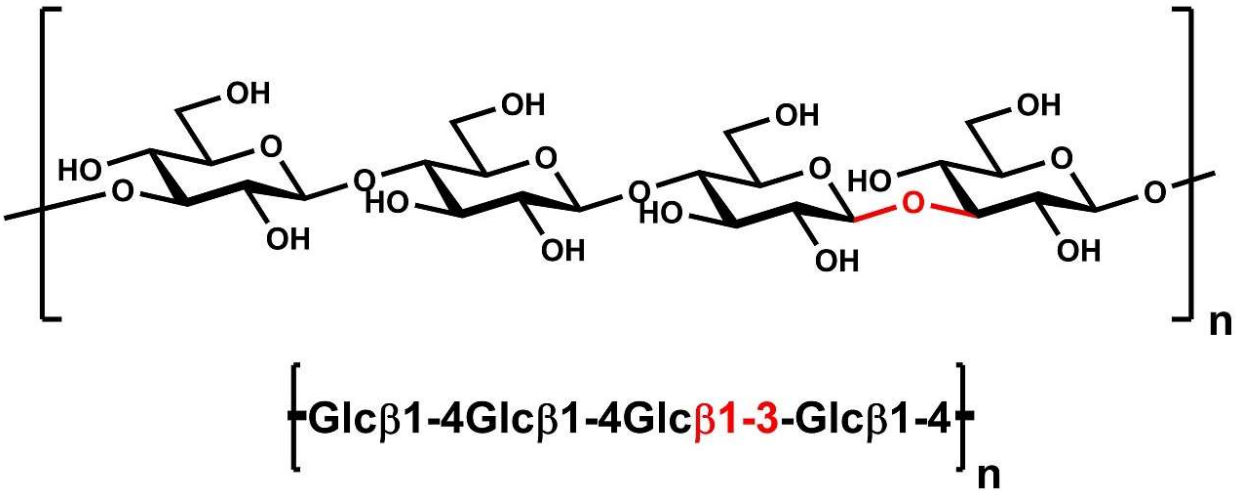

Figure 1.2: Molecular structure of a 1,3-1,4-β-glucan segment displaying the geometry of both the 1,3 and 1,4 linkages of glucose subunits in the polymer (64).

Key in the intermolecular interactions of β-glucan is the pattern of β-1,3 and β-1,4 linkages. In fact, 1,3-β-glucanase hydrolyzed the β-glucans extracted from green malt at 40°C differently than the β-glucans extracted at 65°C (15). Since the total ratio of β-1,3 to β-1,4 linkages was the same, the authors predicted that the arrangement of the β-glucan polymer may also play a role in its solubility. Bamforth (12) describes the more readily water-soluble β-glucans as having short series of three to four glucose units bound by β-1,4 linkages and interrupted with single β-1,3 linked units. Runs of β-1,4 linkages can be as long as 14 units, but without the disordering effect of regular β-1,3 linkages, the polymer becomes less water soluble (12,47). Beyond this primary carbohydrate structure, β-glucan polymers can interact with one another via hydrogen bond cross linkages that can impact the apparent size of the β-glucan in solution.

Research on β-glucans from other foods can shed light on the character of β-glucan in beer. Arcangelis et al. (24) found that more series of four 1,4 linked glucose molecules compared to series of three 1,4 linked glucose molecules was associated with more water soluble β-glucan. They describe that for the purposes of baking bread, β-glucan from shrunken endosperm barley tends to have reduced solubility, better inter-molecular aggregation, and more resistance to undesired hydrolysis in the dough.

β-glucan and barley

In the plant

β-glucan plays a variety of roles in the barley plant. Barley β-glucan content and cell wall thickness are positively correlated (3). Barley with thinner endosperm cell walls is modified more quickly during malting. However, reducing barley synthesis is not a quick answer to reducing cell wall mass and increasing the proportion of starch in each grain. When CRISPR was used to knockout several β-glucan synthase genes, the barley plants responded differently to the lack of each synthase (34). The effect ranged from differences in polysaccharide distribution and starch packing in the grain, differences in DP3:DP4 ratios, as well as reduced plant height, vigor, and spike development.

Barley β-glucan and the environment

While plant genetics are important in controlling the β-glucan content of malt, wort, and beer, the growing location often has a larger impact on β-glucan in malting and brewing (27). Additionally, Evans et al. found that cell-wall degrading enzymes vary considerably in malts across growing and malting locations (32).

There is a clear link between hotter and drier conditions during grain development and reduced malting quality for barley. Heat and stress during grain filling and high fertilization rates lead to grains with increased percentages of both protein and β-glucan compared to grain starch content (70). Short periods of heat stress are followed by periods of reduced grain growth, resulting in lower yields and a shift in the ratio of A and B-type granules towards B-granules (75). This study noted that heat stress without drought conditions during grain filling did not affect β-glucan content. When drought conditions were introduced alongside heat stress, a further decrease in grain weight and starch content was observed (74). Environmental stress has varied impacts on the grain and other tissues due to the source-sink relationship of nutrients in the plant (14).

Conversely, rainfall during grain development is associated with decreased percentage of β-glucan, both soluble and insoluble, and decreased extract viscosity (2). These changes in composition were measured to a greater extent in endosperm than the whole grain, indicating that environment affects endosperm development more than it does the husk. Rain did not appear to affect the structure of β-glucan so much as it reduced the synthesis of β-glucan relative to other grain components (2). Beyond climate effects, the rate of nitrogen application also appears to affect barley β-glucan (14,68,70). However, it appears that this relationship is somewhat different for high amylose and high amylopectin barleys compared to conventional or husk-less varieties (68). Overall, nitrogen application rates and environment appear to have a synergistic effect on barley quality—high amounts of fertilizer coupled with lack of rainfall during grain filling yields barley with higher β-glucan and protein and lower starch (68).

A key takeaway from experiments looking at the effects of genetics and environments is that barley can be bred to have lower β-glucan content, but that environmental pressures (nitrogen, rainfall, heat) can override those efforts. Climate change is affecting rainfall patterns and heat levels in barley growing regions (17). In light of this, it is important to consider how enzymes degrade cell walls and proteins during malting and mashing and their ability to correct for elevated levels of protein and fiber.

Barley quality correlations

In barley, β-glucan content is correlated with other metrics of quality (Table 1.1). Barley β-glucan correlates positively with protein content, malt β-glucan, wort β-glucan, beer β-glucan, and starch B- and C-type granules. Conversely, barley β-glucan is negatively correlated with starch content, average grain weight, rapid starch digestion, A-type starch granules, and amylopectin α-1,4 series length. Generally, higher barley β-glucan is associated with other traits that would indicate poor malt quality. In the case of starch digestibility, it was proposed that the β-glucan polymer is perhaps directly responsible for inhibiting the enzyme's access to the starch substrate (5). In the case of increased protein content

and decreased starch content, it is more likely that similar biological and environmental factors drive differences in grain ripening and composition (14,75).

Table 1.1: Correlation values (R) between barley β-glucan content and other quality metrics.

Correlated Quality Metric	R	β-glucan Method	Source
A-granule	-0.62	Calcofluor	(5)
A-granule	-0.66	Megazyme	(5)
Beer β-glucan by Calcofluor	0.44	Calcofluor	(27)
β-glucan by Calcofluor	0.93	Megazyme	(5)
B-granule	0.63	Megazyme	(5)
C-granule	0.69	Megazyme	(5)
C-granule	0.67	Calcofluor	(5)
Amylopectin chain length	-0.74	Megazyme	(5)
Amylopectin chain length	-0.72	Calcofluor	(5)
Malt β-glucan by Calcofluor	0.48	Calcofluor	(27)
Protein content	0.92	Megazyme	(5)
Protein content	0.91	Calcofluor	(5)
Rapidly digested starch	-0.61	Calcofluor	(5)
Rapidly digested starch	-0.62	Megazyme	(5)
Starch content	-0.95	Calcofluor	(5)
Starch content	-0.95	Megazyme	(5)
Thousand grain weight	-0.73	Calcofluor	(5)
Wort β-glucan by calcofluor	0.46	Calcofluor	(27)

Measuring β-glucan

There are a number of ways to estimate the β-glucan content of barley, malt, or wort. In the past the viscosity of an acid extract, β-glucan precipitation, colorimetric assays, and enzymatic hydrolysis have been used to quantify β-glucan (53). The precipitation, color, and enzyme measurements all use a spectrophotometer for the end measurement, making these assays accessible for most labs. In colorimetric analysis, the fluorescent dye, calcofluor, can bind to β-glucan polymers larger than 10–50 kDa and the absorbance or fluorescence at 405 nm can be related to β-glucan concentration (53). Congo Red also can bind to β-glucan, but this is a less commonly used reagent. In enzymatic analysis, β-glucan

is extracted and converted into reducing sugar form to be measured by a glucose oxidase peroxidase (GOPOD) color reaction (63). Wort β-glucan measured by calcofluor correlates well (R = 0.93) with measurements by enzyme, but calcofluor tends to overestimate β-glucan by 0.7-3.4% (5). Besides the fact that Calcofluor seems a little less accurate, Asare (5) et al did find correlation with both the Calcofluor and Megazyme methods and other barley quality metrics (Table 1.1). B-type starch granules could only be correlated with Megazyme β-glucan, and thousand grain weight could only be correlated with Calcofluor β-glucan (Table 1.1). These results show that while the methods are not completely interchangeable, they both could impact a researcher's ability to declare a result significant.

β-glucan can be quantified with instruments other than the spectrophotometer. Reverse phase high performance liquid chromatography can be used to separate β-glucan from other polymers. The β-glucan can be detected by refractive index to quantify the total content of β-glucan in the sample (71). This method correlates well with the colorimetric and enzymatic methods for quantifying β-glucan. However, using size exclusion chromatography followed by enzymatic hydrolysis and quantification could provide yet more detail on the size distribution of the β-glucan (11,61). It may be yet more impactful to measure polymer gyration radius instead of MW distribution (53).

Beyond conversations of β-glucan content vs composition, other researchers have suggested that barley and malt homogeneity is important to track for β-glucan as well as other metrics (1). Single grain analysis of nitrogen content was used to show that a commercial malt that produced beer with unwanted haze and wort separation problems was indeed a combination of two malts (69). While the average nitrogen content was within specification, the lack of homogeneity in malt quality caused problems. Single grain analysis was used again to show how the pattern of β-glucan hydrolysis in two barley varieties differed during malting (72). The inhomogeneity of the β-glucan distributions indicate that an average value may be insufficient in predicting the modification level of malt.

In summary, the method used to measure β-glucan impacts the accuracy of the result. β-glucan content can be measured as it decreases during the malting process and as it is extracted into wort during mashing. High β-glucan is almost always *associated* with negative quality outcomes. However, for that metric to be *predictive* of other quality traits like viscosity, more information on polymer size, composition, and homogeneity should be investigated.

β-glucanase

Enzyme characteristics

Muller et al. (65)examined the crystal structure of barley β-glucanase EII in comparison with a bacillus β-glucanase. Besides their function, these two different enzymes have entirely distinct folding patterns and active sites. One notable feature of isoenzyme EII's active site is that it tightly binds four central glucose units with one less tightly bound glucose unit on either side. This geometry suggests that the enzyme needs at least four β-1,4 connected glucoses in order for proper assembly of the enzyme-substrate complex.

Barley malt β-glucanase is known to be less heat tolerant than diastase enzymes. In a 50°C mash, the enzyme has a half-life of 10 minutes. The half-life is reduced to 2 minutes at 60°C (11). Similar analysis performed by Loi et al (60) indicated that after 30 minutes at 45°C, approximately half of the original enzyme activity was left while the enzyme activity declined rapidly at 55°C and 65°C . In both the study from Bamforth and Martin (11) and from Loi et al. (60), the researchers measured enzyme activity viscometrically.

β-glucanase quality correlations

Malt β-glucanase activity is positively correlated with apparent attenuation limit (AAL) for three mashes with different temperature profiles (29,31). Because this correlation exists in a mash that does not allow for much β-glucanase activity, malt β-glucan is likely associated with other positive indicators

for AAL in the malt. The positive correlation between β-glucanase and friability, protein, free amino nitrogen (FAN), and xylanase is also likely because of good overall malt quality (23,32).

β-glucanase activity is negatively correlated with European Brewing Congress (EBC) mash viscosity and β-glucan (23). It is likely that β-glucanase activity during mashing is responsible in part for this relationship since the EBC mash is characterized by a lower initial temperature at 45°C that allows for enzyme survival.

Table 2: Other quality metrics correlated (R) with β-glucanase activity.

Correlated Quality Metric	R	Source
Diastatic power	0.41	(29)
EBC AAL	0.51	(29)
Old 65 AAL	0.6	(29)
Final 65 AAL	0.58	(29)
Final 65 extract	0.45	(31)
Final 65 AAL	0.39	(31)
Final 65 lauter efficiency	0.34	(31)
EBC viscosity	-0.58	(23)
EBC β-glucan	-0.544	(23)
Friability	0.405	(23)
Protein	0.28	(32)
FAN	0.422	(32)
Xylanase	0.489	(32)

Solubilase

Historically, there has been debate about the existence of a second hydrolase called malt β-glucan solubilase and its possible role in brewing research. Solubilase is defined by its ability to free long, relatively insoluble β-glucan from the endosperm cell wall matrix likely through carboxypeptidase or esterase mechanisms (9). It is relevant here to summarize a few of the arguments for and against the consideration of the enzyme—both to legitimize my choice to not invoke it in the discussion of my research results and because it is an illuminating example of the way the science community takes time to arrive at a consensus/truce.

The enzyme was first suggested when it was observed that if a malt enzyme extract was heated for 1.5 minutes at 65°C prior to addition to a mash, more wort β-glucan was produced than if the enzyme extract had been boiled prior to its addition in mashing (79). Scott (79) determined that 1.5 minutes was enough time to stop the activity of β-glucanase enzymes that are capable of releasing β-glucan from cell walls (7). Thus, the existence of a different, more thermostable enzyme active in releasing β-glucan was proposed (79).

The existence of a solubilase enzyme and its apparent improved thermostability could have many implications for how we approach the reduction of β-glucan in malt and wort. It was proposed that the lack of correlation between initial malt β-glucan and final wort β-glucan may be related to the solubilase activity in the mash (10). Solubilase has been suggested to have potential esterase or carboxypeptidase activity (9,10). When another group performed its own purification of the enzyme, they observed that solubilase activity lacked the carboxypeptidase activity previously described and was conspicuously associated with the barley husk (96).Authors have suggested that this observed solubilase activity may be due to dilute cellulase concentrations from *Trichoderma* fungus on the outer husk of barley (90). This seems to support earlier observations that the level of β-glucan solubilase activity varies based on climatic conditions and crop maturity (95).

In response to suggestions of enzyme being specific to the husks or to grain microflora, an experiment was designed where barley was dehusked using sulfuric acid and then the resulting barley flour was denatured by boiling in ethanol. Dehusked and denatured barleys released less β-glucan over time at all temperatures compared with native and dehusked barley (49). However, the same paper notes that harsh acid and heat pre-treatments may alter the properties of the β-glucan polymers. Palmer and Agu observed that less β-glucan was released from heat pre-treated malts, perhaps due to a change in solubility (69). Both enzymatically inactivated and native malts released more β-glucan into solution at 65C than at 45C, indicating that higher mash temperatures can extract more β-glucan (69).

Research into glass transition behavior of oat β-glucan films illustrates how water temperature plays a role at increasing water-glucan interactions compared to glucan-glucan interactions (94).

No notable papers have been published on solubilase since 2001, although the solubilase hypothesis has continued to appear in reviews and to be used to explain results (46,59,77). The objective of this thesis was to observe the change in wort β-glucan over time compared to the persistence of β-glucanase activity during mashing. There were no experimental parameters put in place to test for the presence or lack of solubilase, and thus the discussion will not speculate as to the implications of solubilase in mashing.

Malting: Development of β-glucanase and hydrolysis of β-glucan

Development of β-glucanase

Malting is a process by which the natural mechanisms of grain germination are used to modify the grain to make the energy stored in the starch more accessible. First grains are steeped to hydrate the grain to 40% moisture content. Then the grains are allowed to germinate for 3-4 days. During this time cell wall hydrolases like β-glucanase and proteinases work to break down the "packaging" surrounding the starch (12). Finally, the germinated grain is kilned—arresting hydrolysis of the grain storage molecules and reducing the moisture content to approximately 5%.

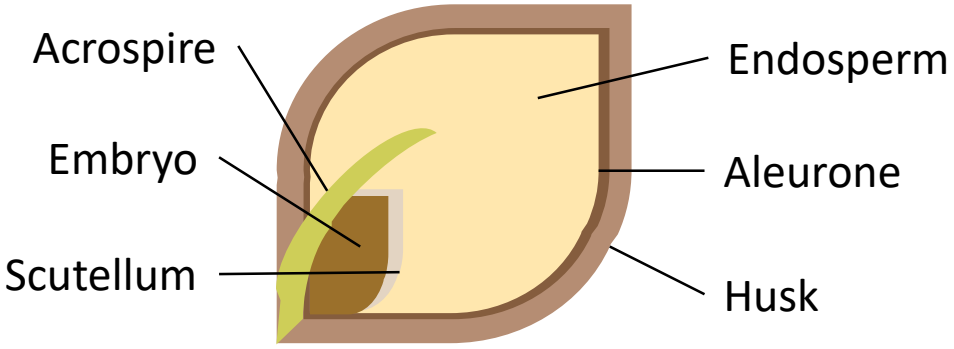

Figure 1.3: A simplified illustration of a barley grain during germination to offer a visual definition of barley corn structures.

In a germinating barley grain, there are two forms of 1,3-1,4-β-glucanase that are active. Isoenzyme EI is generated in the aleurone and scutellum (Figure 1.3) and becomes the primary isoenzyme in young shoots and leaves (81). Isoenzyme EII β-glucanase activity is generated mostly in the aleurone layer and to a greater extent than isoenzyme EII (81).

During malting, the grain embryo releases hormones and enzymes that begin the process of germination for the new plant. β-glucanase synthesis increases starting from 12-24 hours until leveling off at about 6 days after steeping (11,51). However, it should be noted that the commercial germination process ends far earlier than 132 hours, meaning that β-glucanase activity will likely never reach its potential peak (60).

The expression of enzymes is not consistent throughout the grain. The distribution of β-glucanase favors the proximal end of the grain nearer the embryo (51). Application of gibberellic acid, a hormone secreted by the embryo is known to spur β-glucanase isoenzyme EII activity to higher levels and a more uniform distribution (51,81). It has been observed that application of the abscisic acid hormone alone inhibits β-glucanase synthesis, but when combined with gibberellic acid, enzyme expression increases (51,82).

The β-glucanase generated in malt must be able to survive kilning in order to be of use in mashing. Bamforth and Martin (11) found that 34% of activity remained after gentle kilning regimes, and that at higher kiln temperatures, less than 10% of total β-glucanase activity remained. Loi et al. (60) observed that 38% of isoenzyme EII's activity was preserved after an 80C kilning regime.

Hydrolysis of β-glucan

It has been noted that initial barley β-glucan is not a strong predictor of finished malt β-glucan (27,40). This is in part because β-glucanase activity during malting can vary, allowing some barley with high initial β-glucan to "catch up" (27).Throughout the course of a 10-day germination, barley β-glucan can be reduced from roughly 4% to 1% by weight as measured by hydrolysis of the remaining polymer

into glucose units detectable with a GOPOD reaction (1). However, by day 4, the malt would be considered fully modified based on acrospire length. At that point, β-glucan would only be reduced to 3% by weight (1). More recent authors have observed sufficient malt β-glucan reduction by day 4, at which point the malt is kilned (97). The fraction of soluble β-glucan increases then decreases (1,59). The decrease in soluble β-glucan is due to hydrolysis by β-glucanases until approximately 90% of the β-glucan is degraded by day 5 after steeping. The soluble fraction decreases again when malt is kilned (59).

A difference in the distribution of various enzymes may limit the predictability of one malt quality metric for another. For example, the Kolbach Index (the ratio of soluble amino nitrogen to total protein) is often used to evaluate endosperm modifications, but one study showed no relationship between KI and malt β-glucan for 60 malt samples (27).Concluding whether malt β-glucan correlates with malt modification is dependent on the methods used to evaluate both β-glucan and modification. Henry (40) noted that percent β-glucan by enzymic hydrolysis correlated well with endosperm modification by Calcofluor staining. However, β-glucan hydrolysis did not have a significant relationship with endosperm modification or β-glucanase activity by single grain analyses (72). It could be that structural differences between individual grains, like cell wall thickness, could obfuscate the connection between β-glucan, β-glucanase, and modification (3). It could also be that uneven distribution of β-glucanase synthesis in individual grains results in uneven β-glucan hydrolysis only detectable in a single grain analysis (51).

Grain storage and dormancy may affect malt β-glucan and β-glucanase (91). As germination index increased throughout the year, so too did β-glucanase activity measured at the end of malting. Wort viscosity and wort β-glucan decreased throughout the year. After 28 weeks β-glucan and β-glucanase levels both had reached a plateau, indicating that further storage would not impact the enzyme-substrate relationship in malting.

Hydrolysis during malting changes the molecular weight (MW) distribution of β-glucan. Initially, barley has lots of very high MW β-glucan and some very low MW β-glucan with not much in the middle (11). After malting, there are still peaks at high and low MWs, but there is a higher frequency of β-glucan polymers at middle weights (11). A description of this effect of malting on β-glucan MW distribution is provided by Marconi et al. (61).

Mashing: Survival of β-glucanase and Extraction of Malt

The primary goal of mashing is to extract fermentable sugars from malt. Saccharification of starch nears completion after 50-60 minutes at 65°C, and thus most mash regimes include this key temperature and time parameter. Other temperatures rests are often implemented to encourage activity from other enzymes. Ideally, mashing conditions optimize sugar extraction while limiting the extraction of undesirable compounds (i.e., excess protein or β-glucan) and make efficient use of time and energy. .

Types of mash

In industry, the temperature and malt/grain combination (grist) of mashes vary greatly based on style and equipment. Brewing scientists have long discussed how best to perform test mashes to both approximate industry scale-up and to compare between labs. Two methods have been used historically in research settings: one from the European Brewing Congress (EBC) and one from the Institute of Brewing (IoB).

The two test mash methods vary in a number of parameters; but most importantly for the conversation on β-glucan and β-glucanase, they vary in temperature (Figure 1.4). The EBC mash is the more common method to prepare worts for standard malt analyses. This mash starts at 45°C before ramping to a saccharification rest at 65°C. It mimics the lower initial temperature often employed in the brewing of lagers, allowing for proteinase and β-glucanase activity. The Institute of Brewing (IoB) favors using a test mash that starts at 65°C which is more relevant for ale styles. Modern brewing scientists are

interested in making these test mashes even more analogous to industrial mashes. Evans et al. (29) suggested that what they called the "Final 65" mash could be a better alternative than the EBC or the IoB mashes. A modified IoB mash was proposed that importantly includes 3.0 mM $CaSO_4$ (28). Even so, comparison with new test mashes and legacy test mashes shows that new mashes provide better resolution between malts, but do not affect the quality rank of malts (29). When mashes start at temperatures higher than the 45°C used in the EBC mash, β-glucan levels will remain consistently high because there is no β-glucanase activity to counteract extraction (28). Additionally, at higher mash temperatures more β-glucan is able to be dissolved or dispersed in the liquid fraction, wort (59).

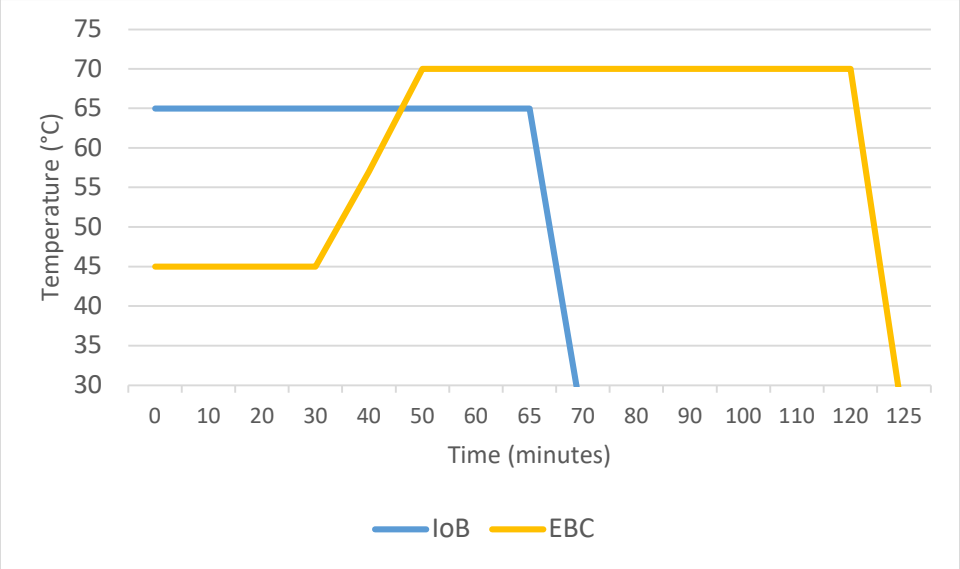

Figure 1.4: Temperature profile of the modified IoB and EBC methods' programmed temperature progression over the duration of the mash (6,29).

Besides variation in temperature, mash conditions may vary in their grist to liquor ratio. It has been observed that the initial 1:4 grist to liquor ratio in an EBC mash offers less protection to enzymes during thermal stress compared to the thicker 1:3 ratio more common in industry (29). Thicker mashes may also resist β-glucan accumulation in the wort since the liquid phase is saturated with other solutes (41). However, increasing mash thickness has its limits. At grist to liquor ratios larger than 1:3, the risk of clumping increases (29). Other researchers have seen that in thicker mashes, addition of purified β-

glucan caused a 20% decrease in starch digestibility (66). This effect was observed at a variety of β-glucan molecular weights.

The last two differences of note in mash conditions are stirring frequency and grist size. Coarser grist (0.7mm mill gap) stirred more infrequently tends to release less β-glucan into the wort (41). Interestingly, β-glucanase activity in wort seems unaffected by grist particle size (41).

Mashes can also vary in the types of grains used. While the two aforementioned test methods were designed with malt analysis in mind, many brewers incorporate non-barley adjuncts into their brews (22,25,77). Brewing with unmalted barley has also been investigated as a way to reduce costs and the carbon footprint associated with malting (26). It was found that native malt enzymes were able to hydrolyze excess β-glucan contributed by up to 20% additions of barley, as long as the initial temperature remained below 50°C (41). Brewing trials with unmalted quinoa and tritordeum have been analyzed both by infusion and EBC mash to understand how mash protocol affects wort quality (25).

Wort quality correlations

Similar to barley, levels of β-glucan in wort are associated with other beer quality indicators (Table 1.3). Higher wort β-glucan is often associated with lower quality parameters. Since wort preparation affects wort β-glucan, EBC mash β-glucans could correlate differently with quality parameters compared with IoB/infusion mash β-glucan. These correlations have been analyzed more frequently for the standard EBC style mash than the infusion mash, but there are still a few opportunities to compare the two mashes' effect on β-glucan and wort quality. For both an infusion mash and EBC mash, there was a positive correlation between wort β-glucan and viscosity (23,32). It is possible that the β-glucan polymer has a direct impact on wort viscosity, but it is also true that viscosity can be affected by pentosans and starch (73). For example, xylanase activity was mildly associated with decreased wort β-glucan in both mash styles as well (23,32). In this case, high xylanase activity may be

working in parallel with other cell-wall degrading enzymes, specifically β-glucanase, throughout malting and mashing to yield a low final wort β-glucan content (32).

Table 1.3: Correlation values (R) for wort quality metrics associated with wort β-glucan measured in different types of wort preparations.

Correlated Quality Metric	Wort Type	R	Glucan Method	Source
AAL	EBC	-0.572	EBC	(23)
AAL	EBC	-0.23	Calcofluor	(31)
α-amylase	EBC	-0.461	EBC	(23)
β-amylase	EBC	0.391	EBC	(23)
Beer β-glucan	EBC	0.95	Calcofluor	(27)
Coarse extract	EBC	-0.399	EBC	(23)
Congress AAL	EBC	-0.52	Segmented flow	(29)
DP	EBC	-0.61	Segmented flow	(29)
FAN	EBC	-0.5	Calcofluor	(31)
Filtration rate	Infusion	-0.43	Enzyme hydrolysis	(10)
Final 65 AAL	EBC	-0.44	Segmented flow	(29)
Fine-coarse difference	EBC	0.83	Calcofluor	(27)
Friability	EBC	-0.814	EBC	(23)
β-glucanase	EBC	-0.544	EBC	(23)
Hartong index	EBC	-0.509	EBC	(23)
Infusion AAL	EBC	-0.668	EBC	(23)
Infusion SWIFT	EBC	-0.494	EBC	(23)
Kolbach index	EBC	-0.433	EBC	(23)
Lauter Efficiency	Modified Infusion	-0.239	Congo red, EBC	(32)
Malt protein	Modified Infusion	-0.456	Congo red, EBC	(32)
Wort protein	EBC	0.419	EBC	(23)
Viscosity	EBC	0.632	EBC	(23)
Viscosity	Modified Infusion	0.544	Congo red, EBC	(32)
Wort SWIFT	Modified Infusion	-0.32	Congo red, EBC	(32)
Xylanase	EBC	-0.528	EBC	(23)
Xylanase	Modified Infusion	-0.271	Congo red, EBC	(32)

The strongest associations observed with wort β-glucan were the positive correlation with fine-coarse extract difference (R = 0.83) as well as the negative correlation with friability (R = -0.814). A large fine-coarse extract difference is an indication of lack of uniformly well-modified malt, a condition accompanied by high malt and wort β-glucan (72). Friability is also a measure of malt modification that increases with the softening of the endosperm as cell walls and proteins are hydrolyzed. When grain is

not fully modified, the distal end of the grain may still have intact cell walls, yielding reduced extract, reduce apparent attenuation limits (AAL), and high β-glucan (23,29,31,56).

Besides associations with malt and wort quality parameters, wort β-glucan itself can have an impact on downstream processes. High wort β-glucan positively correlates with high beer β-glucan (27). Infusion worts saw a negative correlation between wort filtration/lauter efficiency (LE) although this pattern was not observed for EBC worts (10,32). Infusion wort filtration measured by a small-scale wort filtration test (SWIFT) was negatively correlated with wort β-glucan content of EBC wort (23). Beer filtration by SWIFT was also negatively correlated with wort β-glucan when both measurements were generated from infusion mashes (32). With regards to beer filtration, post-mash operations like boiling, cold crashing, and filtration can continue to separate wort β-glucan, limiting the ability of wort β-glucan or wort viscosity to predict final beer characteristics (53).

Exoglucanase and fermentable sugars

Since mashing is regarded as a process by which complex carbohydrates are converted to fermentable sugars, researchers have asked if those complex carbohydrates are limited to starch. It has been proposed that fully hydrolyzed β-glucan may provide additional fermentable sugars in the form of D-glucose (42,50,56). Exo-glucanases do exist in barley and do display some heat tolerance, but they are expressed in such small quantities that β-glucan does not currently contribute fermentable sugars to brewing (50).

β-glucan problems

Viscosity

The viscosity of wort and beer is approximately 1-3 mPa·s (37,73). Higher viscosity is associated with an excess of large, non-hydrolyzed malt polymers like dextrins, glucans, or pentosans (73). This high viscosity is undesired because it would lead to lengthier lauter or filtration (73). Increase in β-glucan

concentration is correlated with an increase in wort viscosity as seen in Table 1.3 (23,32). It has been observed that viscosity increases approximately linearly with β-glucan concentration allowing one to determine a value for the intrinsic viscosity (46,73). The authors evaluated the Mark-Houwink exponents to find that β-glucans adopt a more compact confirmation in wort or beer compared to that in water, weakening their ability to affect viscosity in the brewing process comparatively. Marconi et al. (61) confirmed the findings of Jin et al. (46) that the intrinsic viscosity of a malt β-glucan solution is related to molar mass distribution via the Mark-Houwink equation. It appears that as long as wort β-glucan is less than 800 mg/L, thus below its overlap concentration $C^* = 1.08/[\eta]_{\beta\text{-glucan}}$, it will be a secondary factor in determining wort viscosity compared to other components (46).

As β-glucan is an important component of other cereal crops, research on the β-glucan structures from other cereals, like oats, can provide insight into the structure and function of β-glucan in barley. When viscosities varied for oat β-glucan extracts at the same β-glucan concentration, it was determined that β-glucan MW distribution affected the viscosity (21). The difference in MW distribution in oats appeared to be cultivar dependent. While it is possible that cultivar has an effect on barley β-glucan polymer size, enzyme activity during malting changes the MW distribution (11,61). Wort viscosity is higher when made with malt germinated for shorter periods of time (78). Additionally, wort or beer viscosity may change over time as a result of aggregation of low MW β-glucan during storage (92). Brewing with adjunct grains can impact wort viscosity (37). Shearing wort at 20°C increased wort viscosity (47). Shearing wort at 48°C or 76°C also increased wort viscosity, but not to the extent observed at 20°C. Mashing in at higher temperatures is also associated with higher wort viscosity (78).

Wort separation and filtration

Wort separation is a unit operation that can happen through a traditional lauter process or through mash filtration. The process of separating wort from the spent grains during lautering can be thought of from a chromatographic viewpoint (38). From this perspective, unhydrolyzed β-glucan has a

greater affinity for the husk “column” components than the elution fluid (wort or sparge water). Smaller β-glucans exist in equilibrium between interacting with the elution fluid or the column media. Overextraction with high temperature or extended time can extract small MW β-glucan and other undesired compounds from the husk “column” into the wort (38). To have a successful separation, brewers need to set up a consistent grain bed “column” and elution fluid gradient to extract only desired components.

In the lab, a lauter is approximated by filtering cooled wort through a funnel with a filter paper. Evans et al. (32) found that lautering efficiency (LE) was moderately correlated with SWIFT (0.584), and minorly negatively correlated with wort viscosity (-0.255). While the root causes of a slow lauter, slow filtration, and high viscosity have significant overlap, it is likely that the complex interactions of enzyme activity, polymer content, and polymer structure produce slight differences in worts that reduce the correlation between measurements. While information on lauter performance can be inferred by wort viscosity, having a low number on a malt certificate of analysis (CoA) does not guarantee an effortless lauter. Analysis of the “Final 65” mash proposed by Evan et al. (29) showed that lautering efficiency (LE) is a good predictor for industrial scale lautering problems. The inclusion of β-glucanase improved LE (29).

It has been proposed that barley malt microflora has an impact on wort separation (55). Injured kernels are more susceptible to bacterial infection. By measuring the wort β-glucanase activity at 60°C, the proportion of β-glucanase activity from microbial sources becomes observable. If about 12% of the kernels have husk injuries, rate of separation increases by 25% and extract decreases by 10% (55). These results are paralleled by higher wort β-glucan from the added β-glucanase activity of injured kernels. This trend can be ameliorated if malt steeping water contains a *Lactiplantibacillus plantarum* strain that is able to keep *Leuconostoc* species in check (88).

Filtration is also used to improve the clarity of finished beer. A small-scale wort filtration test (SWIFT) can be used to predict filtration performance using easy to find equipment. The SWIFT correlates with the Esser test previously used by the brewing industry (84). High MW β-glucan has a large impact on membrane filtration (52). Membrane filtration can separate fluids using a smaller pore size than conventional cellulose or diatomaceous earth filters. It has applications for seltzer and other flavored malt beverages where minimal malt character is desired.

Haze and precipitate

Intentionally hazy beers have a permanent haze made up of protein-polyphenol complexes. Chill haze is an unwanted effect in beers intended to be bright. One of the earlier characterizations of chill haze found that it was 80-96% carbohydrate compared to permanent haze that only contained 16% carbohydrate (86). The precipitate could be digested with β-1,4- or β-1,3-glucanase and had an infrared absorption pattern more similar to 1,3-1,4-β-glucan than starch. The chill haze β-glucans had ratios of 1:3 to 1:4 β-1,3 linkages compared to β-1,4 (44). This ratio is roughly the same as for β-glucan in malt or wort (12). If branch structure does not impact the tendency for β-glucans to form hazes, the hydrolysis products of β-glucanase activity may be a factor (93). The hydrolysis products of an enzyme that showed β-1,4-glucanase activity at 60°C were found to produce the most precipitate (93).

Addition of a xylanase-glucanase blend did reduce the permanent haze of a highly hazy beer, but it did not bring the haze below the 20 NTU moderate haze threshold unlike the action of pepsin on the same beer (43). Here, the nephelometric turbidity unit (NTU) quantifies the intensity of light scattered by dispersed particles. Unexpected results for the high MW β-glucan concentration observed in hazy and normal beers lead the authors to hypothesize that stress-induced changes to yeast cell wall polysaccharides could be involved in unfilterable haze in beer (43). Flake-like precipitates are more likely to form when beer is repeatedly exposed to freezing temperatures and then rewarmed (86). Shaking the beer in the package increases the carbohydrate content of the particles from 12% to 20% (89).

Raman spectroscopy has been proposed as a method to analyze the various beer components that may create haze (48). Individual analysis of protein, polyphenol, and β-glucan solutes in a model solution are routinely correctly identified. However, when analyzing a more complex force-aged beer, there was a level of fluorescence high enough to obscure the analysis of any one compound.

Exogenous enzymes as a solution

When exogenous β-glucanase has been added to mashes, it reduces the β-glucan content of the wort. Commercial enzyme blends can successfully reduce the β-glucan contributions from raw barley to levels below an all-malt, no enzyme control (22,83). Brewing with malt and a 10-20% addition of oats does not have a profound effect on mash and wort quality (77). Only when the adjunct exceeds a normal level would exogenous enzymes be necessary. In fact, mashes with 40-50% unmalted barley and exogenous enzyme addition have outperformed all-malt mashes on a number of quality metrics (22). One hurdle to using exogenous enzymes and raw grain is that the enzymes' ideal temperatures are different than that of the native barley malt enzymes, necessitating the re-design of mash temperature profiles (36). Another impact is that quality wort can be created from even low-quality barley not suitable for malting (31). Altogether, mashing with unmalted grains and enzymes can produce beer that is acceptable to consumers from a sensory perspective (83). However, attitudes about the craft of beer may provide resistance for the acceptability of this brewing method (13).

Brewing with exogenous enzymes does successfully reduce high molecular weight β-glucan contributed by raw grains (77). Improvements in β-glucan hydrolysis can reduce raw barley viscosity, but it has no impact on wort separation (31,36). A beer made with unmalted barley and enzymes had lower flux and higher permanent haze after microfiltration compared with an all-malt beer, despite the fact that the exogenous enzyme beer had lower β-glucan and arabinoxylan contents (20). It seems that tanno-protein colloids (a result from insufficient protein hydrolysis) overrode the effects of the

polysaccharides, indicating that enzyme blends need to be comprehensively analyzed throughout the brewing process before deeming them effective.

When evaluating commercial enzyme blends, brewer's should consider the types of enzymes in the blend as well as dosage rates. Scheffler and Bamforth (76) found that enzyme blends with higher xylanase activity than β-glucanase activity increased mash viscosity because the digestion of xylanase released more β-glucanase into solution (76). Additionally, determining the proper dosage as demonstrated by other authors makes sure that the costs of enzyme addition stay low (16,18,36).

Mutagenesis can be used to enhance both heat tolerance and catalytic activity (67). Inclusion of this recombinant enzyme in mashes reduced filtration time and viscosity compared to native enzyme controls. Improved enzymes can be added exogenously or incorporated into the barley genome. The successful incorporation of an engineered enzyme appears to have no other effects on the germination and growth of the barley, while extending the β-glucanase half-life to over 4 hours at 70°C (45).
Wild barley can be a useful source of new genetic material that can be traditionally bred into the barley genome. Lauer et al. (57) predicted that a number of genetic sequences for a wild barley β-glucanase would confer thermostablity advantages. In a mash, these wild barley enzymes had 3.4-8% higher activity than the control conventional enzyme. Alternatively, another approach is to improve enzyme efficiency rather than thermostability. Enzyme chimeras have the ability to improve kinetics and substrate binding (33).

While most exogenous enzyme studies have focused on the addition of enzymes in the mash, it is also possible to add β-glucanase during fermentation to slowly degrade β-glucan before filtration (18). It has been proposed to engineer yeast to secrete β-glucanase during fermentation to control haze (87). Since β-glucan levels can cause wort separation problems prior to fermentation, addition of β-glucanase earlier in the process could be more impactful.

Summary

1,3-1,4-β-glucan is the primary cell wall polymer in barley, and endo-1,3-1,4-β-glucanase isoenzyme EII is the enzyme responsible for β-glucan's hydrolysis during malting and mashing. High barley and malt β-glucan are negative quality indicators. Barley β-glucan can be determined somewhat by genetics, but environmental stress can increase β-glucan content. β-glucanase can hydrolyze elevated barley β-glucan during malting. Malt β-glucanase is more temperature sensitive than other malt enzymes, leading to quick denaturation during thermal processing operations like kilning and mashing.

Malt and wort β-glucan have been correlated in a number of studies with other quality parameters. Since the methods used to produce wort can impact the wort β-glucan, more comparisons of EBC and IoB/Infusion mashes could be useful. In literature, β-glucanase has an impact, albeit a limited one, on wort β-glucan.

Besides association with other quality parameters, wort β-glucan can be directly associated with processing challenges like slow filtrations and lauters and quality problems like precipitates and haze. Understanding native substrate-enzyme interactions could be useful in determining if exogenous enzyme interventions are needed.

Chapter 2

Method validation for Gallery analysis of β-glucan and β-glucanase

Abstract

In the malting and brewing process, β-glucan content and β-glucanase activity can have an impact on quality. In the quantification of β-glucan content, enzymatic analysis produces more accurate results, but the method is more complicated to perform than the somewhat less-accurate colorimetric Calcofluor analysis. Therefore, adapting an enzymatic 1,3-1,4-β-glucan kit for analysis on the BeerMaster Plus Gallery (Gallery) could help make the method more feasible. Additionally, adapting a β-glucanase method for the Gallery could help to analyze β-glucanase activity when evaluating malt quality. When the Gallery and manual results were plotted against each other, the slope was 1.11 for the β-glucanase and 0.99 for β-glucan. The ability to transfer reagent mixing, incubation, and final absorbance measurement to the Gallery reduced researcher active time needed to analyze β-glucanase activity and β-glucan content in malt. The two methods were successfully adapted to the Gallery and will be able to be useful to improve throughput in future quality evaluations throughout the malting and brewing process.

Introduction

β-glucan is a polysaccharide found in the cell walls of barley and other grains in the brewing of beer. Barley β-glucan is reduced by β-glucanase hydrolysis during malting. To be considered quality barley malt, β-glucan must be lower than 250ppm, according to American Malting Barley Association standards. Excess malt β-glucan is associated with lower rates of endosperm modification during germination and inferior starch content and quality (5,12,47).

During mashing, a considerable fraction of β-glucan will become soluble in the wort. Higher β-glucan extraction into the wort can increase the viscosity of the wort (23,31). Especially for worts with considerable amounts of high molecular weight (MW) β-glucan, high viscosity is associated with slow lauters and filtration (73). B-glucan can also form undesired chill haze in the final product (93).

It is possible to measure β-glucan by enzymatic hydrolysis or by binding to a colorimetric or fluorometric dye. In the enzymatic method, heat is used to denature any native enzyme activity (63). Then, the β-glucan is separated from the rest of the grain or malt matrix by hydrolysis with lichenase, an enzyme capable of cleaving β-(1,4)-D-glycosidic linkages, followed by separation with centrifugation. The supernatant is then digested with β-glucosidase to specifically convert the β-glucan to glucose units that are able to undergo a colorimetric reaction with glucose-oxidase peroxidase. The absorbance of the glucose solution is then able to be converted to the concentration of glucose, and thereby glucan mass concentration, by use of a standard curve.

In the colorimetric/fluorometric dye-binding assay, β-glucan forms complexes with fluorescent whitener, Calcofluor (80). These complexes can be measured by fluorescence or spectrophotometry and then related to β-glucan concentration by use of a β-glucan standard of known concentration. In this assay, strong base is used to arrest native enzyme activity. It has been observed that measuring β-glucan content by enzymatic hydrolysis is more accurate than by colorimetric or fluorometric methods (53). Still, the Calcofluor method is an industry standard because the assay has fewer, shorter steps than the enzymatic method and has even been adapted to autoanalyzer apparatuses.

β-glucanase activity is measured less frequently than β-glucan concentration. To measure this activity, McCleary et al. (62) developed a test substrate comprising of a four-unit 1,4-β-glucan oligomer bound to a colorimetric 2-chloro-4-nitrophenyl. This group was found to be bulky enough to resist hydrolysis by β-glucosidase, which would otherwise cause an over-estimation of the endo-β-glucanase activity.

Autoanalyzers perform basic unit operations like pipetting, incubation, and quantifying absorbance of a liquid analyte. This is particularly useful for the routine measurement of properties in beer. However, autoanalyzers introduce some restriction in the parameters available for analysis. For example, the BeerMaster Gallery (Thermo Scientific) used in this study had a set incubator temp of 38°C and measured absorption with lamps of specific wavelengths. Because of these limitations, existing methods need to be adapted to conform to the parameters available from the autoanalyzer. Successful adaptation of the enzymatic β-glucan method to an autoanalyzer may make the method easier to use in malt research and quality control by increasing throughput and reducing lab technician active time. Additionally, an autoanalyzer method for malt β-glucanase could help the assay to be easier to run alongside other malt analyses.

Materials and methods

Malts

Six malts were selected for analysis. Two different brands of Pilsner malt (Admiral Maltings, Alameda, CA, USA; Rahr Malt Co., Shakopee, MN, USA) and one two-row malt (Rahr) were selected because it was estimated that they would have lower β-glucan contents and higher β-glucanase activities compared to other styles of malt. A white wheat malt (Rahr) was tested because it was anticipated to have both low β-glucan and β-glucanase. A Munich malt (Weyermann, Bamberg, Germany) was incorporated into this study to test a malt with higher β-glucan and lower β-glucanase than a typical base malt. Finally, a caramel malt (Simpsons Malt, Berwick-upon-Tweed, UK) was included to observe the effects of malt color on the assays as well as test extremely low β-glucanase and higher β-glucan.

Reagents

The reagents for β-glucan and β-glucanase analysis were obtained from Megazyme (Wicklow, Ireland). The MBG4 kit contained the test substrate 4,6-O-benzylidene-2-chloro-4-nitrophenyl-β-(3)-β-D-cellotriosy-glucoside in 50% DMSO and water (MBG4) and a malt standard of known β-glucanase activity. The KBGLU kit or β-glucan analysis contained a specific lichenase (*endo*-(1-3)(1-4)-β-D-glucan 4-glucanohydrolase), β-glucosidase, GOPOD reagent buffer, and GOPOD reagent enzymes (glucose oxidase, peroxidase, and 4-aminoantipyrine). The KBGLU kit also included a barley standard of know β-glucan content.

Moisture

The moisture content of all malts was measured per ASBC Barley-5A standard method in a drying oven.

β-glucanase

The MBG4 β-glucanase method was selected to analyze the malt samples both manually and with the Gallery (Gallery Plus BeerMaster Discrete Analyzer, Thermo Scientific, Waltham, MA, USA) as illustrated in Figure 2.1. All samples analyzed were milled with a Burr mill with a 0.2 mm gap. Then 0.5 g of each malt was measured into a 15 mL plastic centrifuge tube and combined with 8 mL of 100 mM sodium acetate buffer (pH 6.5). The samples were vortexed and then held for 20 minutes with regular shaking to resuspend the solids in order to extract analytes into the liquid phase. The sample tubes were centrifuged at 3000 x g for 5 minutes. The manual analysis was performed on the supernatant as described in the Megazyme assay protocol. 0.1 mL of MBG4 was combined with 0.5 mL of the enzyme extract and incubated at 30°C for 20 minutes. After the incubation, 0.9mL of Tris buffer was added to stop the reaction. Absorbances at 400 nm were measured at ambient temperature with an Evolution 201 UV-Visible Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

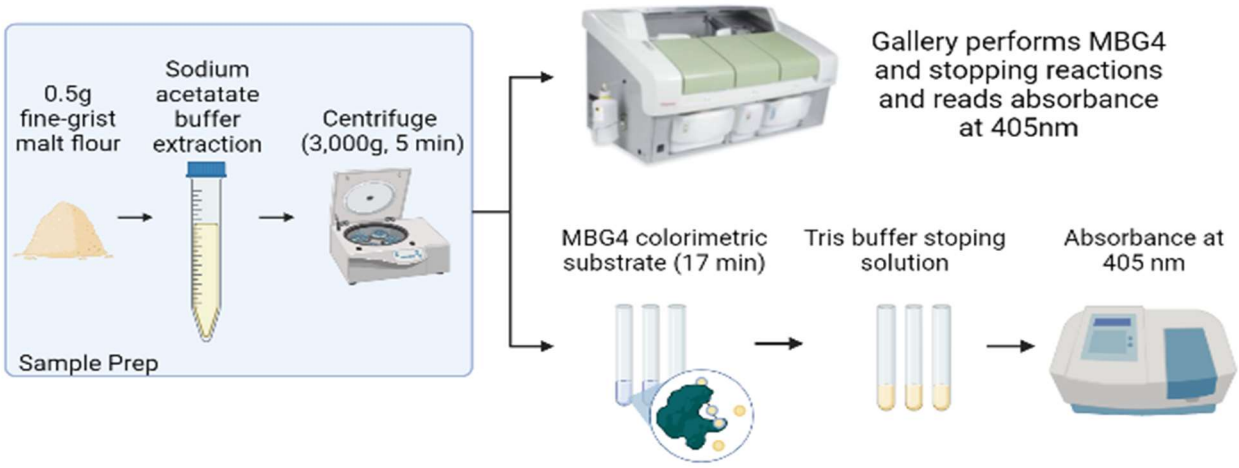

Figure 2.1: A graphic description of the analysis of β-glucanase with the manual method and with the Gallery autoanalyzer.

Approximately 1 mL of the same supernatant was added to Gallery sample cups. Incubation of 0.5 µL of sample with 0.1 µL of the MBG4 reagent was extended to 22 minutes from the original 20 minutes to compensate for the higher temperature in the Gallery incubator (38°C instead of 30°C). The increase in incubation time resulted in improved correlation between the standard bench method and the Gallery adapted method. A final absorbance measurement was taken at 405 nm. A reagent blank was measured by replacing 50 µL of the sample with sodium acetate extraction buffer.

A malt control standard provided by Megazyme was used to calibrate both the spectrophotometer and the Gallery. A unit of activity is defined as the amount of enzyme needed to release one micromole of 2-chloro-4-nitrophenol from the MBG4 substrate in one minute.

β-glucan

A Megazyme kit for (1-3),(1-4)-β-glucan analysis (K-BGLU, Wicklow, Ireland) was selected to assess the β-glucan content of the malts manually and by Gallery measurement as seen in Figure 2.2. Sample preparation was performed in duplicate. Samples were ground at a fine grist setting (< 2 mm) using a MIAG sample mill. 1.0 grams of malt samples were vortexed and boiled with 5mL aqueous ethanol (50% v/v) and 5mL of sodium phosphate buffer (20 mM, pH 6.0) for 5 minute. The samples were

centrifuged (1,000 x g, 10 minutes) and decanted to separate simple sugars in the supernatant from the polysaccharides in the malt. The ethanol wash was only performed once since comparisons with double-washed samples showed no significant difference in β-glucan content. Malt solids were resuspended in 5 mL sodium phosphate buffer (20 mM, pH 6.0) and were digested with lichenase for one hour at 40°C. Samples were diluted to 15 mL with distilled water and centrifuged (1,000 x g, 10 minutes). The supernatant was analyzed in duplicate (for a total of 4 data points per malt) according to the method detailed in the Megazyme protocol, section D. 0.1mL of the supernatant was combined with 0.1 mL of β-glucosidase. After a 15-minute incubation at 40°C, 3 mL of the GOPOD reagent was added and allowed to react for 20 minutes at 40°C. Absorbances were measured at 510 nm (A510) with the visible spectrophotometer.

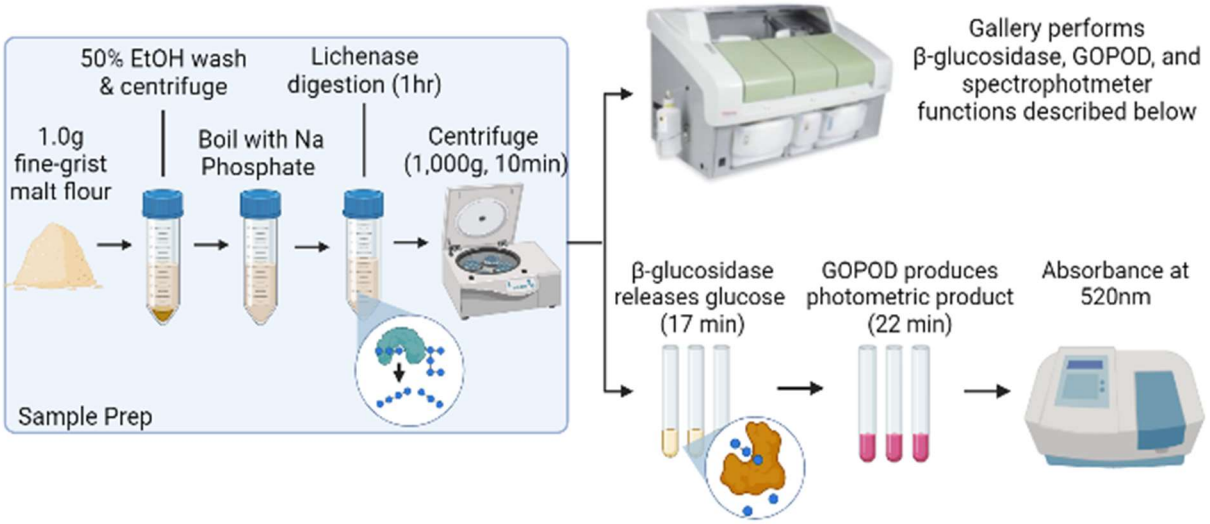

Figure 2.2: A graphical description of the manual enzymatic β-glucan method compared with the Gallery adapted method.

Approximately 1 mL of the same supernatant containing hydrolyzed β-glucan was added to Gallery sample cups. The Gallery was programmed to combine 8 µL of sample with 8 µL of β-glucosidase. Since the incubation temperature was changed from 40°C to 38°C to fit Gallery settings, the first incubation was extended from 15 minutes to 20 minutes. At the end of the first incubation, 240 µL

of glucose oxidase peroxidase (GOPOD) reagent was added. After a second incubation of 22 minutes (increased from the original 20 minutes), the absorbance was measured at 520 nm (A520). Samples were measured against a blank using 8 µL sodium acetate buffer instead of β-glucanase. Each supernatant sample was analyzed in duplicate for a total of 4 data points per malt. Absorbance was converted to concentration using a Gallery calibration with 1 mg/mL D-glucose.

The assays were calibrated with glucose standards, with a separate standard curve generated on both the spectrophotometer and the Gallery. The glucose standard curve was measured at A520 and A510 to investigate the effect of the absorbance wavelength on the results.

Statistical analysis

Data was plotted in Excel. A Student's paired t-test was used to analyze the significance of the difference in results between the Gallery and standard analysis. Correlation values between the two versions of each method were determined.

Results

Moisture contents varied from 5.7% in white wheat malt to 9.2% in Munich I (wet basis). These values were used to calculate percentages by dry weight for the β-glucan and β-glucanase analyses.

When analyzing β-glucanase results, the malt control was reliably measured at 0.1 U/g, dry basis. As anticipated, the pilsner and 2-row malts had fair amounts of β-glucanase activity (Figure 2.3A). Munich I and white wheat had minor amounts of activity, while caramel malt had essentially none (Figure 2.3A). There were no significant differences between the Gallery and manual β-glucanase measurements within a malt sample as determined by a Student's paired t-test (α=0.1).

When plotted against each other, the two methods had a slope of 1.1 (σ = 0.030), a y-intercept of -0.009 (σ = 0.002), and a coefficient of determination of 0.996 (Figure 2.3B). The Gallery β-glucanase method saved approximately 27 minutes of researcher active time to measure six malt samples as recorded by a stopwatch.

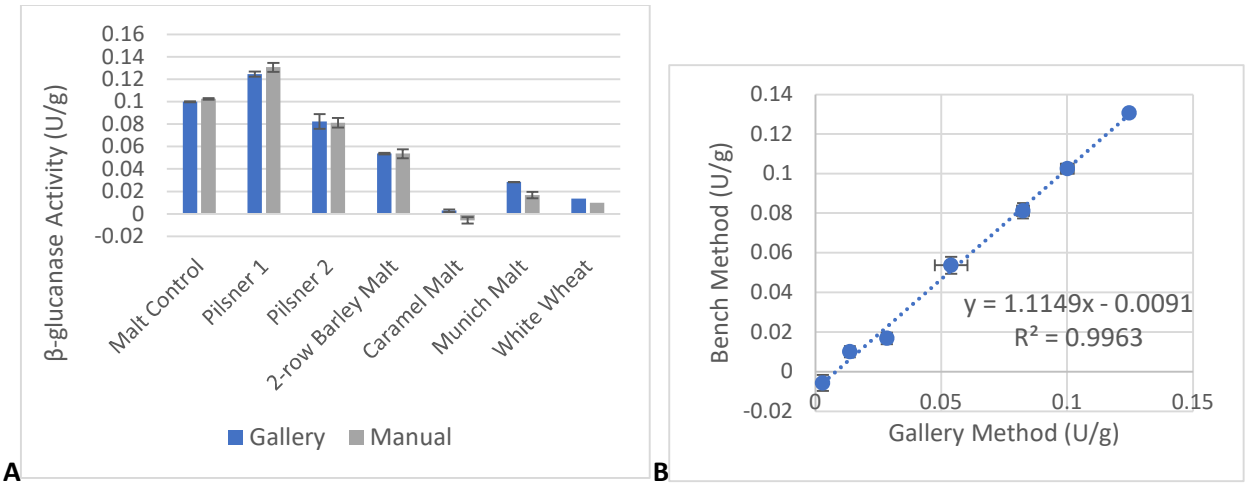

Figure 2.3: β-glucanase results for the Gallery and manual methods. (a) Measurement of β-glucan in each malt sample analyzed. (b) Correlation of the Gallery method vs the manual method. The equation of best fit and the correlation value are displayed on the chart. Error bars represent one standard deviation from the mean with n=4.

When the β-glucan concentration was measured by dry weight, caramel malt and Munich I malt had higher β-glucan contents as expected. The base malt barley had mean β-glucan under 0.2% w/w, dry basis (Figure 2.4A). Wheat malt had the lowest β-glucan content, with a mean value of less than 0.1% by both methods (Figure 2.4A). The barley control was found to have 2.74±0.044% β-glucan by Gallery analysis, and 3.18±0.115% β-glucan by manual analysis. There were no significant differences between the Gallery and manual β-glucan measurements within a malt sample as determined by a Student's paired t-test (α=0.1).

When the two β-glucan methods were plotted against each other, the slope of the line of best fit was 1.18 (σ = 0.0095) with a y-intercept of –0.043 (σ = 0.010) and a coefficient of determination of 0.988 (Figure 2.4B). The adaptation of the β-glucan method to the Gallery saved approximately 48 minutes of researcher active time as recorded by stopwatch when measuring 6 malt samples.

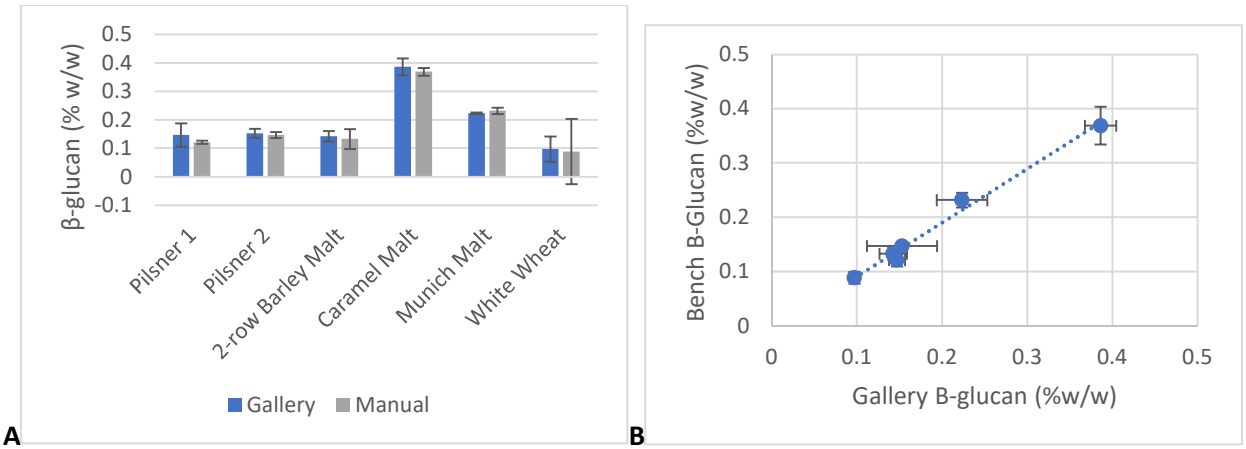

Figure 2.4: β-glucan results for the Gallery and manual methods. (a) Measurement of β-glucan in each malt sample analyzed. (b) Correlation of β-glucan values for the Gallery method vs the manual method. Equation of best fit and the correlation value are displayed on the chart. Error bars represent one standard deviation from the mean with n=4.

When the glucose standard curve was measured at A510 and A520, the coefficients of determination were 0.9955 and 0.9956 respectively. The slope of A510 was slightly steeper (0.35 mL/mg) compared to the slope of A520 (0.34 mL/mg).

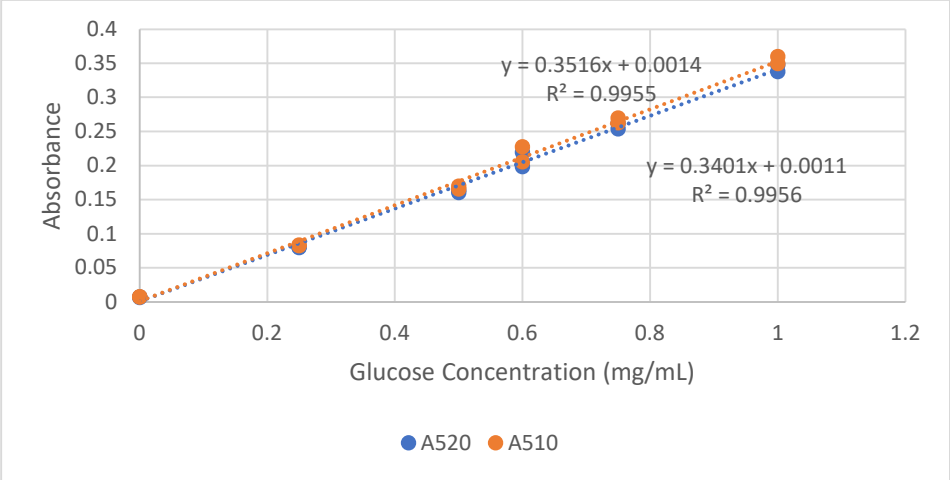

Figure 2.5: Glucose standard curves measured by the spectrophotometer at absorption wavelengths of 520 nm (A520) and 510 nm (A510). Correlation constants and equations for the lines of best fit are displayed on the chart.

Discussion

When the manual method and Gallery methods were plotted against each other, slopes that approached one and intercepts that approached zero indicate that the methods can be directly

compared. When adapting the methods from manual analysis to Gallery analysis, it was important to consider the incubation temperature and the absorbances available with the Gallery's hardware. With adjustments in absorbance wavelengths and incubation times, the Gallery was able to produce statistically consistent results compared to the standard manual analysis. This level of reproducibility is common for the enzymatic assay of β-glucan (53).

Since the β-glucanase extraction buffer is not specific for the enzyme, but the MBG4 substrate is, it is reasonable to expect that this Gallery method could be used to analyze β-glucanase activity in wort during the early stages of mashing (62).The standards of deviation were somewhat smaller in the manual method than the Gallery method, but it is possible that the small amounts of reagent in the Gallery method were more prone to uneven mixing and therefore a less consistent result. The lack of activity measured in the caramel malt provides an indication that with proper blank preparation, color from the malt should not yield an inflated measure of β-glucanase activity. While brewers do not expect any β-glucanase activity from specialty malts, it may be useful to understand how incorporating specialty malts may dilute the enzyme strength of base malts.

One limitation of this β-glucan method is that it does not measure polymer length. This could be amended by fractioning the β-glucan in the sample prior to digestion by lichenase (61). Another drawback is that the assay cannot be run in parallel with other assays on the same sample. This is because the GOPOD reaction would pick up reducing sugar from other hydrolysis products and the extraction process destroys other analytes. Lastly, for both methods, there is still some sample preparation that has to occur before the Gallery can perform its analysis. In the case of β-glucanase, sample prep is limited to a short extraction at room temperature. However, to measure malt β-glucan, the samples need to be boiled for 5 minutes, centrifuged for 10 minutes, and then digested for 1 hour with lichenase before an analyzable supernatant is obtained.

Conclusions

The McCleary methods (62,63) for 1,3-1,4-β-glucan and MBG4 β-glucanase were successfully adapted for the Gallery autoanalyzer. The analysis was robust to colored specialty malts. The adaptation of the β-glucanase and β-glucan methods will help to increase throughput with reduced researcher active time, making both methods more feasible to perform in a research and/or quality lab.

Chapter 3

Simultaneous evaluation of β-glucan and native β-glucanase during different mash temperature profiles

Abstract

High wort β-glucan may contribute to brewery processing problems such as poor run-off, slow filtration, and unwanted haze. To investigate how β-glucanase impacts wort β-glucan throughout mashing, 10 different mashes were considered with varied temperature profiles, malt bills, and levels of malt modification. Mashes were sampled periodically and evaluated for β-glucan concentration and β-glucanase activity using methods adapted to the BeerMaster Gallery autoanalyzer (Gallery). Enzyme activity quickly decayed in modified IoB mashes (average half-life 12.4 minutes) accompanied by logarithmic accumulation of wort β-glucan. IoB β-glucan percent extraction ranged from 30.3% to 99.5%. In EBC mashes, a slow decay in enzyme activity was followed by an increased rate of activity decay after 30 minutes. The β-glucan concentration in well-modified samples remained steady while enzyme activity was appreciable, though concentration increased after 40 minutes as β-glucanase activity waned. As a result, percent β-glucan extraction remained relatively low, ranging from 11.9% to 34.3 %. β-glucanase activity at lower temperatures compensates for high malt β-glucan. Measuring wort β-glucan in an EBC mash is insufficient in predicting malt performance in other mash styles. Methods for β-glucan and β-glucanase analysis adapted for the Gallery autoanalyzer increased throughput, enabling analysis of the enzyme and substrate throughout mashing.

Introduction

Barley and other grains used for brewing contain a starchy endosperm packaged in layers of protein and cell wall polysaccharides (8). For barley, and in some other adjunct grains, β-glucan makes

up a large part of the fibrous cell walls—impeding the extraction of fermentable sugars from the grain (39,56). According to the American Malting Barley Association (4), malting must reduce the β-glucan content of barley from around 2-6% by weight to less than 100 ppm, on the order of a hundred-fold reduction (47). Not only is the β-glucan content reduced, but polymer length shortens and becomes more uniform during malting (61).

Even after considerable reduction during malting, barley malt β-glucan can cause problems in the brewing process, such as poor run-off, slow filtration, and unwanted haze (12). It has been observed that wort viscosity increases linearly with increasing β-glucan concentration from 50 to 1000 mg/L, and thus wort within the typical range of β-glucan concentration from 100-300 mg/L can be impacted by viscosity related issues (46,47). Polymers of higher molecular weight (MW) have a greater impact on viscosity when added to a model beer solution at the same concentration as lower MW β-glucan (73). Incomplete hydrolysis of β-glucan in endosperm cell walls can also lead to reduced extraction of fermentable sugar by impeding diastase enzymes access to starch (12). It has been suggested that the complete hydrolysis of β-glucan to D-glucose could even contribute additional fermentable sugars (56). In addition, flavor volatiles are known to associate with β-glucan polymers in a 1 g/L model beer solution, leading to enhanced agglomeration of the polymers associated, unwanted haze or precipitates, and potential loss of beer aroma (54).

To prevent problems associated with β-glucans, maltsters work to produce well modified malts with low EBC wort β-glucan levels. Modification refers to the degree of endosperm transformation during germination. Modification is associated with a softening of the endosperm due in large part to the digestion of the protein matrix surrounding the starch granules. Brewers may lower mash-in temperatures, search for more homogenously well-modified malts, or choose to add exogenous enzymes when β-glucan problems arise in their breweries (12,13). Brewers operating a soft mash starting below saccharification temperatures can use slightly under-modified malts without too much

concern for problems with β-glucans. However, for brewers mashing in at temperatures at or above 65°C, the EBC wort β-glucan value included in a certificate of analysis likely does not accurately reflect the β-glucans extracted in a hot mash.

Quality assurance and research malt analysis is also often done in mashes with 100% base malt. Base malt is the majority constituent of a brewer's grain mixture (grist), and it is typically well-modified and kilned at lower temperatures to produce a malt with a high amount of starch and starch degrading enzymes. Brewers use different base malts in different brews with the inclusion of specialty malts and barley. Specialty malts usually contribute additional carbohydrates and proteins that need to be hydrolyzed without providing any additional enzymes. To observe β-glucanase and β-glucan through a range of mashes, this study used a pale malt, Pilsner malt, caramel malt, and barley. We hypothesize that the EBC mash will be useful in describing some malt behavior, but it may have some limitations in predicting the behavior of poorly modified grists, grists with adjuncts, and grists with specialty malts. Additionally, the authors wanted to investigate how colorimetric measurements adapted for the Gallery would perform on worts with higher color values on the Standard Reference Method chart (SRM).

Barley breeders have also sought to reduce β-glucan through genetics. However, research has shown that while cultivar is important, barley β-glucan is also influenced heavily by the environment (14,68). Additionally, barley β-glucan is a poor predictor for wort β-glucan (27). Therefore, breeding barley for low β-glucan is not enough to prevent problems associated with β-glucan. Furthermore, increasingly hot and dry conditions due to climate change impacts grain filling and maturation (14,75). Researchers have observed that hot and dry conditions can cause increased barley β-glucan percentages, although this is not always the case (70,75). There have been suggestions to incorporate more thermostable β-glucanase into the genome of barley to increase the enzyme's survival after kilning and impact during mashing (45,57). A more in-depth understanding of the relationship between β-glucan and β-glucanase during mashing may suggest new ways to adapt to changing barley quality.

When considering wort β-glucan, it is important to also consider malt β-glucanase which begins to digest β-glucan during malting when (1-3,1-4)-endo-β-glucanase is generated in the aleurone and scutellum of the grain (81). The enzymatic degradation of cell walls contributes to the overall modification of the grain. Furthermore, since an estimated 37% of isoenzyme EII's activity is retained after kilning, β-glucanase is relevant during mashing (60). However, the impact of barley β-glucanase is limited by its relatively low thermostability; its activity rapidly decays at temperatures above 45°C (60).

Previously, enzyme activity in the mashing process has been measured by the change in length (i.e., molecular weight) of β-glucan polymers over time and by change in wort viscosity (11,60). By measuring both enzyme and substrate concurrently throughout mashing, this study seeks to provide insight towards the importance of β-glucanase in two different mash conditions. To investigate how malt β-glucanase and mash profile influence the final β-glucan content of wort, an IoB mash and an EBC mash were conducted with multiple grists, including with simulated under-modified malt. Samples of wort taken periodically throughout the mashes were analyzed for wort β-glucan concentration and β-glucanase activity simultaneously using high-throughput methods adapted for the Gallery Autoanalyzer.

Materials and methods

Materials

Malt was sourced from Admiral Maltings in Alameda, California. A pale two-row malt (Gallagher's Best, lots 21-045 and 21-123) and a Pilsner malt (Admiral Pils, lots 21-042 and 21-125) were used as base malts in this study. Both malts were well modified with EBC wort β-glucan concentrations of 79 mg/L and 88 mg/L, respectively, as reported by the manufacturer. A caramel malt (Kilnsmith, lot 21-010) was used in 90:10 and 80:20 ratio additions with the base malts to create darker worts. Un-malted whole barley (UC Tahoe, harvested in Davis, CA, 2017) was added in 10 and 20 percent additions to the base malts to simulate under-modified malt. Combinations of base malt, kilned malt, and un-malted barley resulted in a total of ten samples as seen in Figure 1.

Figure 3.1: Description of the 10 different malt bills used in both the EBC and modified IoB mashes for a total of 20 different mashes.

Methods

Initial malt β-glucan and β-glucanase

To measure β-glucanase activity, the McCleary method (62) was adapted for the Gallery using Megazyme reagent kits (MBG4, Wicklow, Ireland) described in Chapter 2. Similarly, the McCleary mixed linkage β-glucan method (63) was performed with a KBGLU reagent kit (Megazyme). Initial data was used to calculate an estimated total β-glucan content contributed by each of the grists described in Figure 3.1.

Mash Protocol

Mashes were performed using and IEC Mash Bath (Thornberry, Australia) according to the EBC method (ASBC Malt-4) and a modified IoB method (28). Both mashes were performed with each of the ten different malt bills are listed in Figure 3.1. Samples were ground with a Buhler mill (Braunschweig, Germany) at the setting prescribed by each method. During the water addition at the beginning of each mash, 0.9 mL of 1M $CaCl_2$ solution was added, resulting in a final Ca^{++} ion concentration of 0.3 M in the wort. Calcium ions were added to both mashes to avoid a potential source of variability as some β-glucanases are inhibited by Ca^{2+} ions (67). Wort samples of 1-2 mL were drawn periodically from each mash and passed through a type 40 Whatman filter paper placed in the bottom of a 20 mL Monoject

syringe (Covidien, Mansfield, MA, USA) into a gallery sample cup to clarify samples before spectrophotometric evaluation in the Gallery. Wort samples were drawn at 2, 5, 10, 15, 20, 30, 35, 40, 45, 50, 55, 60, 65 minutes during the EBC mashes. During the modified IoB mash, wort samples were drawn at 2, 4, 6, 8, 10, 15, 20, 50, and 60 minutes. At the end of each mash, wort was filtered through a type 40 filter paper and collected for further analyses.

Mash analysis

For each sample, β-glucanase activity and wort β-glucan content were measured in the Gallery. The β-glucanase assay was performed as described in Chapter 2, but instead the activity of the wort was measured rather than a room temperature extract. Final wort β-glucan activity from each mash was used as a sample blank to account for the color of the wort.. The wort β-glucan assay was performed with a high molecular weight β-glucan kit supplied by Thermo Scientific. In this method a strong base reagent to first denature any β-glucanase activity and a Calcofluor fluorescent dye solution that binds to β-glucan polymers producing a color that absorbs light at 405nm. This Calcofluor method was used instead of the previously described β-glucan method because the latter would measure the hydrolysis products of starch in addition to β-glucans.

Viscosity and filtration

To evaluate mash separation, finished worts were cooled to 25°C and filtered through Cytivia filter paper (32). The filtered volume of wort collected after 25 minutes (FV25) was measured using a graduated cylinder.

Filtered wort viscosity was measured by a MCR 92 rheometer with plate and plate geometry (Anton Paar, Graz Austria). A shear rate sweep from 1-100 s^{-1} was conducted. Viscosity was measured in duplicate and reported at 100 s^{-1}.

Statistical analysis

GraphPad Prism was used to plot and curve-fit β-glucanase activity and β-glucan concentration data over time (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA). The software was also used to calculate the integral of the β-glucanase activity over time. Significant differences between final β-glucan concentration results were calculated using Student's paired T-Test in Excel with level of significance $P<0.05$.

Results and discussion

For all mashes, β-glucan concentration was influenced by the decay of β-glucanase activity over time at high temperature. The Gallery adapted colorimetric β-glucanase method was able to produce consistent β-glucanase activity values for room temperature malt extract and for wort, including those with caramel malt (degree SRM > 18). The ability to run this assay on the autoanalyzer makes analyzing malt and wort β-glucanase more feasible in concert with other spectrophotometric analyses.

EBC mash

In the EBC mash, β-glucanase activity decayed slowly over the first 30 minutes of the mash at 45°C from a maximum activity at 282 U/L to a minimum activity at 57 U/L (Figure 3.2). Because the temperature changed over the course of the EBC mash, the rate of decay of β-glucanase activity changed as well. To represent this, two curves were fit over two different time phases: phase one from 0–35 minutes and phase 2 from 40–65 minutes. Both decay functions operated by the following equation where "A" is β-glucanase activity at time "t," with rate constant "K." The plateau is given by the estimation of "A" as $t \rightarrow \infty$.

Equation 3.1 $$A = (A_0 - Plateau * e^{-K*t}) + Plateau$$

It is possible that the slow decay in enzyme activity from 2 to 35 minutes was the result of somewhat higher-than-optimal temperatures that cause some denaturation in agreement with the trend observed in isothermal mashes (41,60). As temperature increased, the rate of decay changed as

seen with differences in K and half-life in Table 3.1. Half-life was determined by the following equation where K is the rate constant.

Equation 3.2 $$half - life = \frac{ln(2)}{K}$$

Table 3.1: Key values and correlation constants (R^2) from the curve fit of β-glucan concentration and β-glucanase activity in the EBC mash as calculated by Equations 3.1, 2, and 3 in GraphPad software. The β-glucan data was fit with a third-degree polynomial function, and the two phases of β-glucanase decay were calculated with an exponential decay function.

Sample	β-glucan	β-glucanase Phase 1			β-glucanase Phase 2		
	R^2	K (min^{-1})	Half-Life (min)	R^2	K (min^{-1})	Half-Life (min)	R^2
Pilsner	0.96	0.03	22.67	0.68	0.05	13.70	0.91
90:10 PC	0.97	0.07	9.86	0.94	0.02	33.41	0.89
80:20 PC	0.93	0.03	21.93	0.92	0.10	7.26	0.92
90:10 PB	0.82	0.01	111.30	0.43	0.09	7.96	0.98
80:20 PB	0.65	0.07	10.21	0.10	0.09	7.52	0.80
Pale Two-Row	0.96	0.07	10.32	0.96	0.23	3.02	0.88
90:10 2RC	0.78	0.07	10.38	0.87	0.10	6.75	0.84
80:20 2RC	0.95	0.07	10.33	0.95	0.14	4.92	0.95
90:10 2RB	0.78	0.05	14.67	0.70	0.05	13.28	0.98
80:20 2RB	0.87	0.11	6.37	0.22	0.06	12.33	0.93

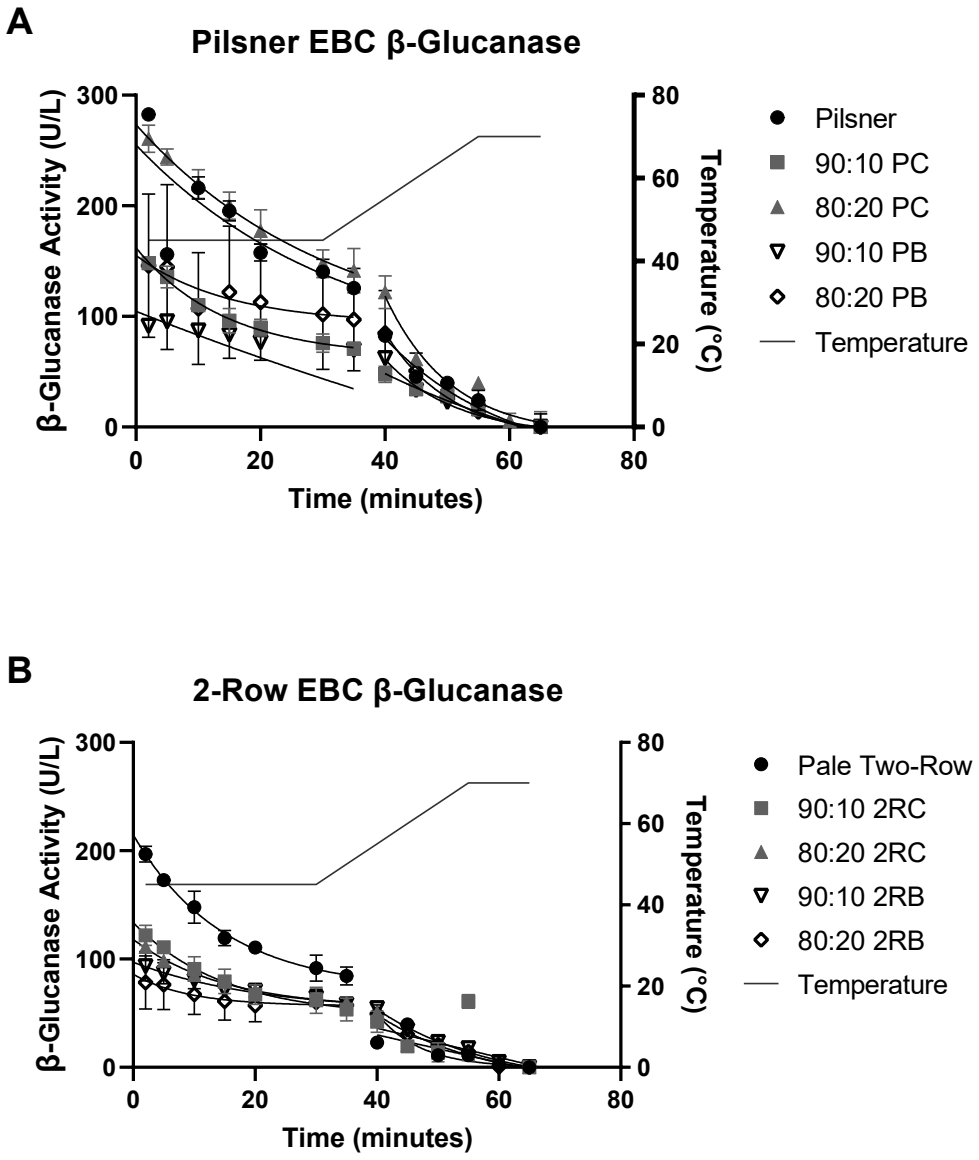

Figure 3.2: β-glucanase activity throughout the temperature progression of the EBC mash for (A) Pilsner malt and (B) two-row pale malt. Two curves were fit sequentially to each sample using Graph Pad software to indicate the change in rate of decay with change in temperature. Error bars represent one standard deviation. PC is Pilsner malt and caramel malt blend, PB is Pilsner malt and barley blend, 2RC is two-row pale malt and caramel malt blend, and 2RB is two-row pale malt and barley blend.

Because there was still appreciable β-glucanase activity, the β-glucan content of the wort for grists without barley samples subtly increased then decreased over the first 30 minutes of the mash at an average of 9 mg/L in well-modified mashes (Figure 3.3A). This relationship suggests that in well-modified malts the β-glucanase digests β-glucan about as quickly as β-glucan is solubilized into the wort

(59). In contrast, for “under-modified” malt samples containing un-malted barley, the amount of β-glucan in solution initially increased to become quite concentrated with barley-containing mashes between 62 mg/L and 336 mg/L during the first twenty minutes (Figure 3.3B). Concentration then decreased over time until 35 minutes into the EBC mash as β-glucanase digested the polymers. Oher researchers have observed that the activity of β-glucanase from malt is enough to neutralize the contribution of barley β-glucan after 45-60 minutes at 48°C (41). In the EBC mash used in this study, one sample (80:20 two-row and barley) reached comparable levels to all-malt mashes by the end of the 30-minute rest at 45°C.

β-glucan concentration then increased for all samples during the temperature ramp as β-glucanase activity exponentially decayed from a maximum of 141 U/L to zero U/L for all samples (Figure 3.2). Overall, this gives the curve the appearance of a third-degree polynomial function given by the following equation where “B” is the β-glucan concentration at time “t” and constants “r” representing some combination of factors influencing the shape of the curve.

Equation 3.3 $$B = r_0 + (r_1 * t) + (r_2 * t^2) + (r_3 * t^3)$$

As the temperature ramped past 55°C, the β-glucanase rate of decay increased and half-life decreased (Figure 3.2, Table 3.1). As a result of the loss in β-glucanase activity, the β-glucan concentration in the wort increased with time after the 40-minute mark to final values between 80 mg/L and 388 mg/L. It also should be noted that some glucanase activity may have been recovered when samples were placed in the ice bath. Because all samples were cooled in the ice bath, this is likely not a factor in variation between samples, but rather an inflation of the measured activity until enzyme denaturation was complete at around 60 minutes.

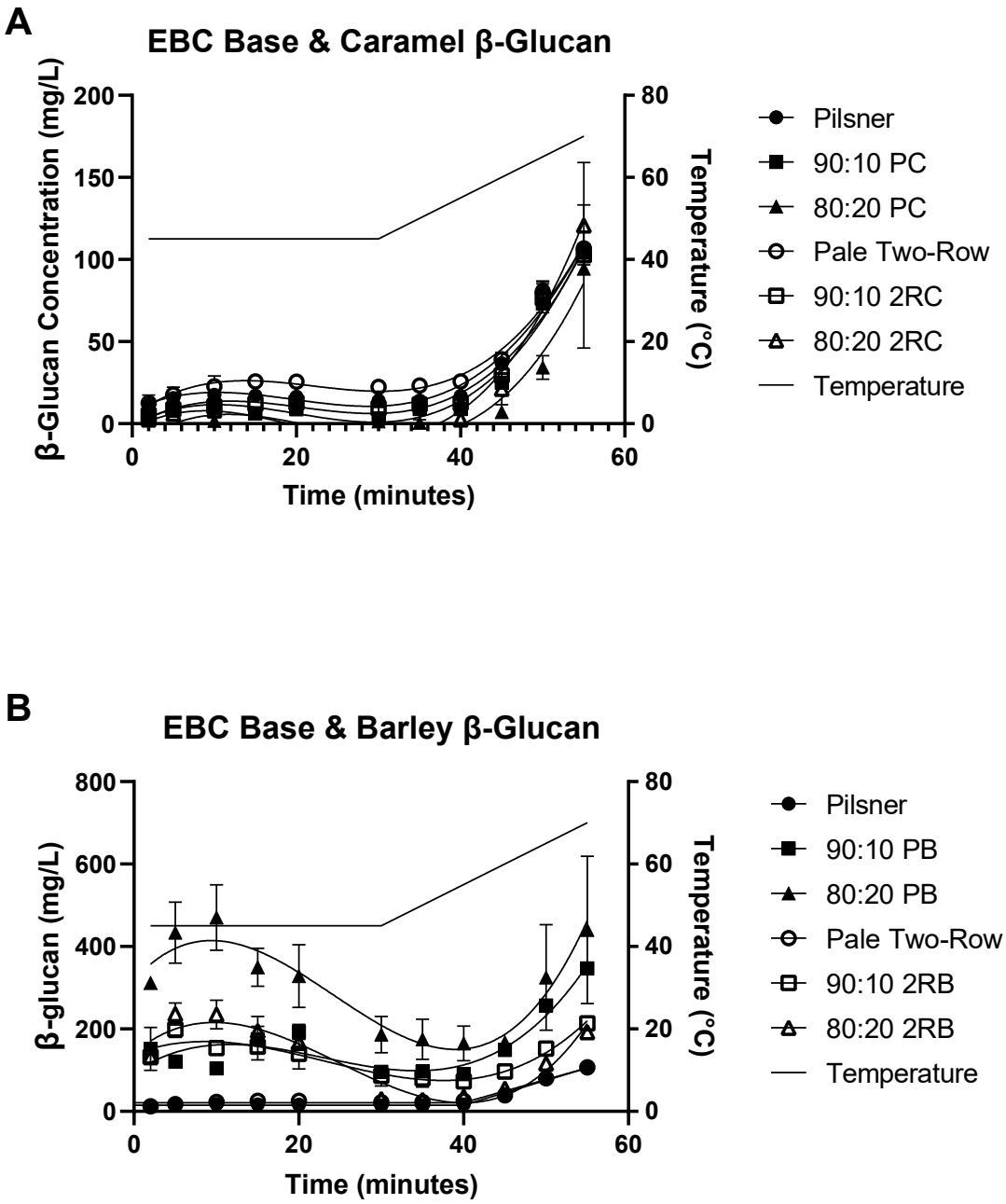

Figure 3.3: β-glucan concentration over the temperature progression of the EBC mash for base malt in comparison and combined with (A) caramel malt and (B) barley. Error bars represent one standard deviation. PC is Pilsner malt and caramel malt blend, PB is Pilsner malt and barley blend, 2RC is two-row pale malt and caramel malt blend, and 2RB is two-row pale malt and barley blend.

IoB mash

In the modified IoB mash, β-glucanase activity exponentially decayed through the entire mash (Figure 3.4). This decay can be represented by the same function as Equation 3.1. The maximum activity recorded (62 U/L) was much less than the activities observed in the EBC mash. The half-life of the β-glucanase enzyme was on average 13.9 minutes (Table 3.2). The half-life was calculated as seen in equation 3.2. Since β-glucanase activity was lost relatively rapidly, β-glucan extracted from the grist into the wort was allowed to accumulate with negligible hydrolysis (Figure 3.5). This was modeled with an exponential growth function (Equation 3.4) where "B" is the concentration of β-glucan at time "t," with rate constant k. The plateau is given by the estimate of β-glucan at t→∞.

Equation 3.4 $$B = B_0 + (Plateau - B_0) * (1 - e^{-k*t})$$

At the higher temperature of the IoB mash compared to the EBC mash, more β-glucan was solubilized into the liquor fraction. This was compounded by higher temperatures that decreased β-glucanase activity. Therefore, the β-glucan extracted from the malt accumulated in the wort, as there was little to no enzyme activity breaking it down.

Table 3.2: Key values and correlation constants (R^2) from the curve fit of β-glucan and β-glucanase in the IoB mash as calculated by equations 3.1, 2, and 4 in GraphPad software. The β-glucan data was fit with an exponential growth function (Equation 3.4), and the β-glucanase data was fit with an exponential decay function (Equation 3.1).

Sample	β-glucan		β-glucanase		
	k ($minutes^{-1}$)	R^2	K ($minutes^{-1}$)	Half-Life (minutes)	R^2
Pilsner	0.10	0.94	0.08	8.39	0.81
90:10 PC	0.11	0.96	0.05	14.75	0.75
80:20 PC	0.10	0.98	0.09	7.63	0.66
90:10 PB	0.16	0.92	0.26	2.71	0.74
80:20 PB	0.46	0.85	0.19	3.68	0.82
Pale Two-Row	0.10	0.95	0.07	10.15	0.69
90:10 2RC	0.17	0.93	0.01	59.87	0.66
80:20 2RC	0.22	0.85	0.06	11.48	0.44
90:10 2RB	0.14	0.80	0.06	10.76	0.77
80:20 2RB	0.13	0.94	0.07	9.77	0.71

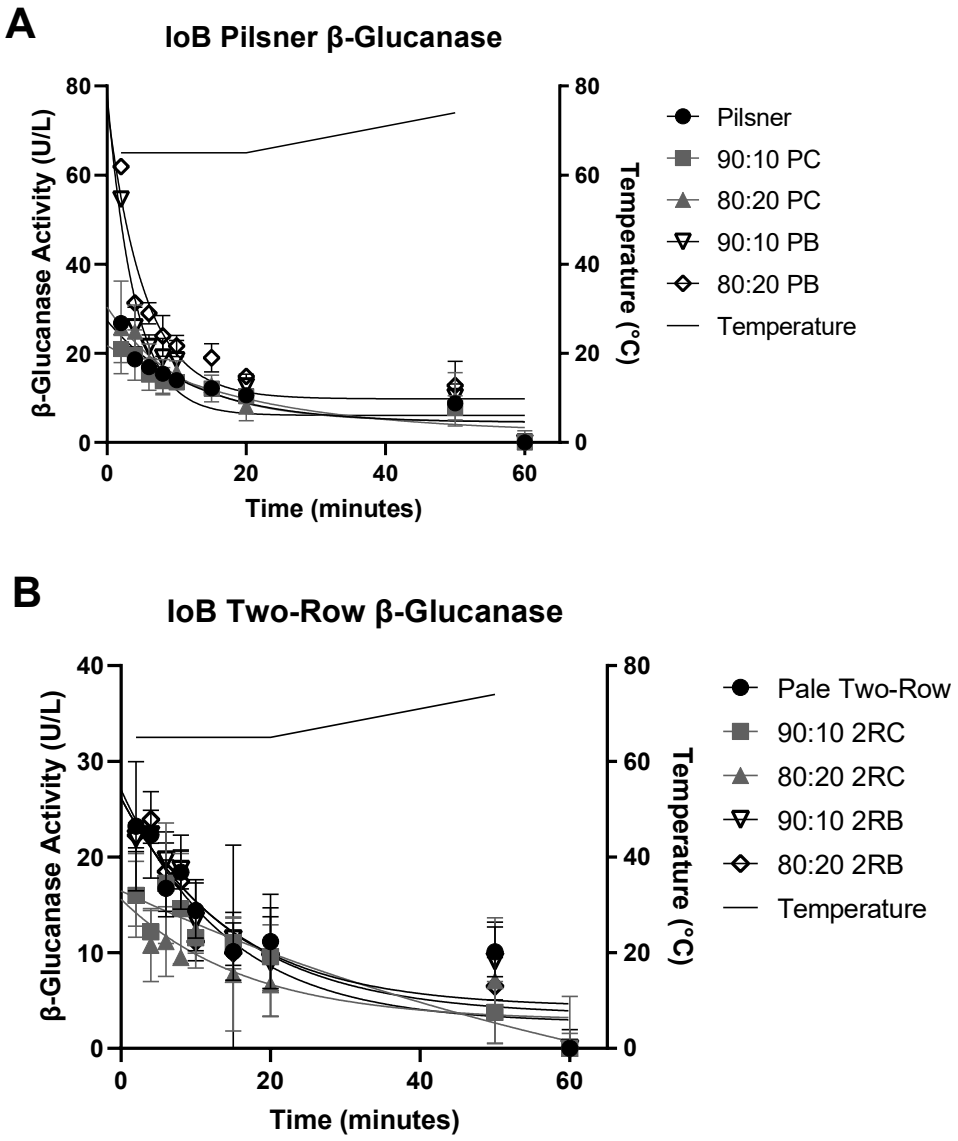

Figure 3.4: β-glucanase throughout the temperature progression of the IoB mash for (A) Pilsner malt and (B) two-row pale malt. Error bars represent one standard deviation. PC is Pilsner malt and caramel malt blend, PB is Pilsner malt and barley blend, 2RC is two-row pale malt and caramel malt blend, and 2RB is two-row pale malt and barley blend.

The concentration of β-glucan in the wort appeared to have an exponential increase over the course of the IoB mash with rate constants as displayed in Table 3.2. It should be noted that enzyme activity, while significantly less than what was measured in the EBC mash, still was measurable at 65°C, in contrast with findings by Home et al. (41) but in agreement with other authors (11,60). This was perhaps due to the higher grist to liquor ratio in the IoB mash compared to the 1:4 used by Home et al.

(11) which offers some protection to enzymes. The initial period of enzyme activity followed by rapid accumulation of β-glucan could cause problems for some brewers since it has been observed that partial hydrolysis of β-glucans may cause more unwanted haze (93). Final β-glucan ranged from 176 mg/L in 80:20 PC malt to 1810 mg/L in 80:20 2RB. With final glucan contents in the range of 232-1810 mg/L it was possible that extraction kinetics were improved with warmer temperatures in addition to reduced β-glucanase activity (10,69).

The final β-glucan concentrations were much higher in mashes containing barley than the mashes with fully modified malts. Mashes with 10% barley had about three times the β-glucan concentration of fully modified mashes. Mashes with 20% barley had 130% more β-glucan concentration compared to the 10% barley mashes (Figure 3.5B). When comparing a well modified malt germinated for 90 hours to a poorly modified malt only germinated for 60 hours, EBC wort was observed to have an eight-fold increase in β-glucan content as a result of poor modification (59). The highest EBC value of 388 mg/L β-glucan from the 80:20 Pilsner and barley mash was on the same order, although less than the 583.8 mg/L concentration observed by Lee and Bamforth (59). The difference may come from a different enzyme profile in fully modified malt diluted with barley compared to an arrested germination malt. In the IoB mashes, final wort β-glucan values in this study were 2.4-3.3 times larger than the wort β-glucan levels observed by Lee and Bamforth (59) in both well and poorly modified malts at 65 and 75°C mash temperatures. The comparison of IoB and EBC data across these studies indicates that both the presence of enzymes and their thermostability influence the build-up of wort β-glucan.

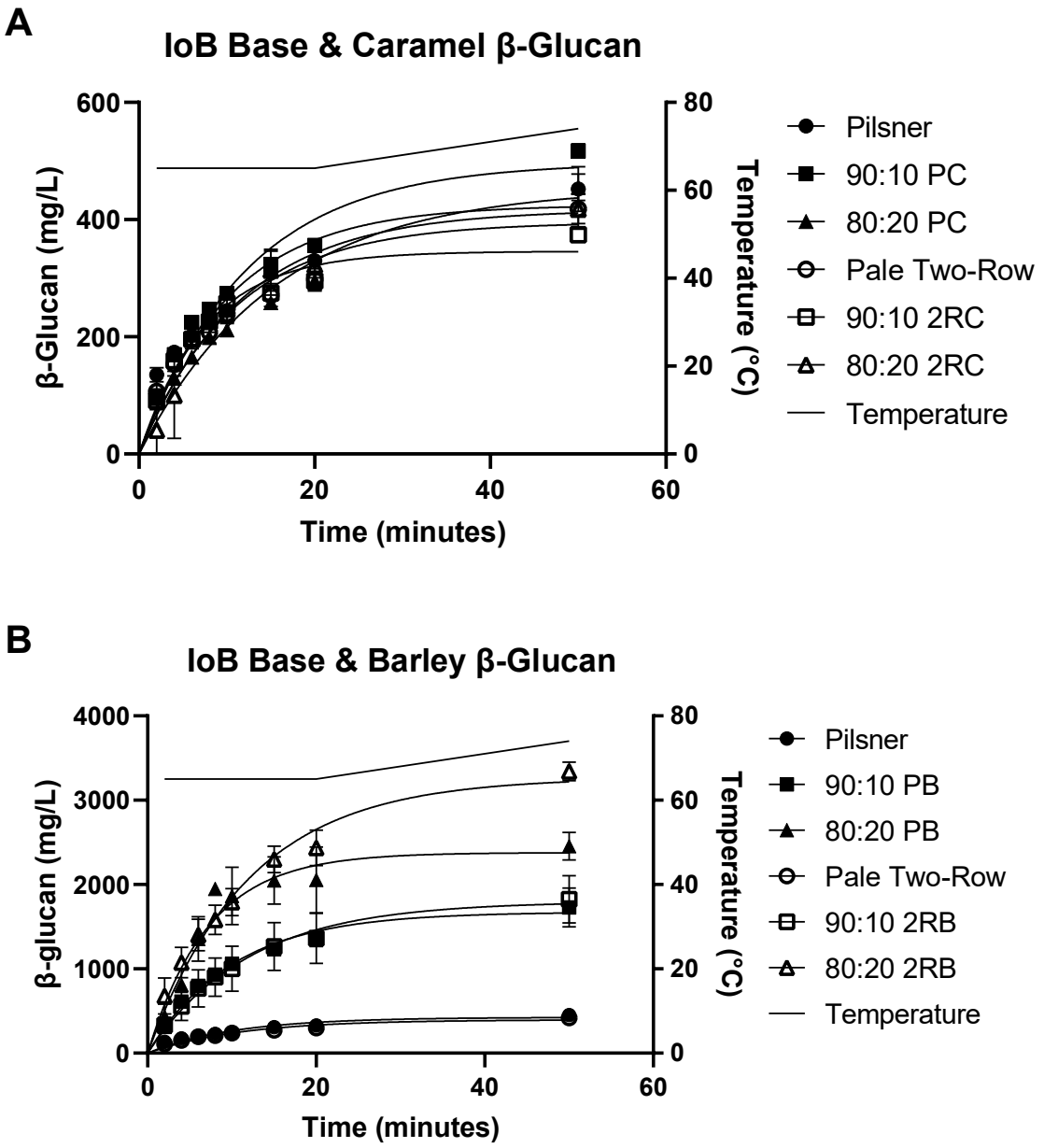

Figure 3.5: β-glucan concentration over the temperature progression of the IoB mash for base malt in comparison and combined with (A) caramel malt and (B) barley. Error bars represent one standard deviation. PC is Pilsner malt and caramel malt blend, PB is Pilsner malt and barley blend, 2RC is two-row pale malt and caramel malt blend, and 2RB is two-row pale malt and barley blend.

Caramel malt

In the EBC mash, caramel malts contributed significantly more β-glucan compared to the Pilsner base malt (Figure 3.3A, Table 3.3). Similarly, the EBC two-row pale mash had significantly less wort β-

glucan than the 80:20 two-row and caramel EBC mash, although there was no significant difference with the 90:10 two-row and caramel mash. This trend was similar in the IoB mash where the Pilsner and Pilsner-caramel mashes were distinct from one another, but the two-row had some overlap in the 90:10 two-row and caramel condition (Figure 3.5A, Table 3.3). It was possible that the structure of the β-glucan differed between the two base malts influencing how quickly it was degraded to a small enough molecular size to escape detection by the calcofluor method (53). While these differences are statistically significant, all base and caramel malt blends produced wort within a reasonable range for brewing (47). IoB mashes extracted only 30-43% of total glucan available and EBC glucan extracts varied between 12-16%.

Table 3.3: Average final wort β-glucan concentration values for base malts and caramel malt blends in IoB and EBC mashes.

Malt	EBC Final Glucan (mg/L)	IoB Final Glucan (mg/L)
Pilsner	81 ± 3.6^{f}	260 ± 9.6^{c}
90:10 PC	85 ± 7.8^{d}	309 ± 8.6^{a}
80:20 PC	108 ± 3.7^{b}	275 ± 11^{b}
Two-Row Pale	86 ± 9.0^{cd}	232 ± 11^{e}
90:10 2RC	122 ± 19^{abc}	235 ± 1.6^{cde}
80:20 2RC	131 ± 5.0^{a}	249 ± 12^{d}

Letters indicated significant difference determined by Student's paired t-test with $P<0.05$.

Comparison: EBC and IoB

In both mashes, a combination of warm water extraction and some hydrolysis of β-glucan resulted an increase of β-glucans measurable in the liquor fraction of the mash. In the EBC mash, lower temperatures allowed for continued hydrolysis of the polymers to lower molecular weight oligomers not detected by the calcofluor method (53). However, in the IoB mash, lack of β-glucanase activity after 12 minutes allowed many β-glucan polymers to remain in solution without further hydrolysis. Additionally, continued stirring at warm temperatures extracted more of the water-soluble fiber over time.

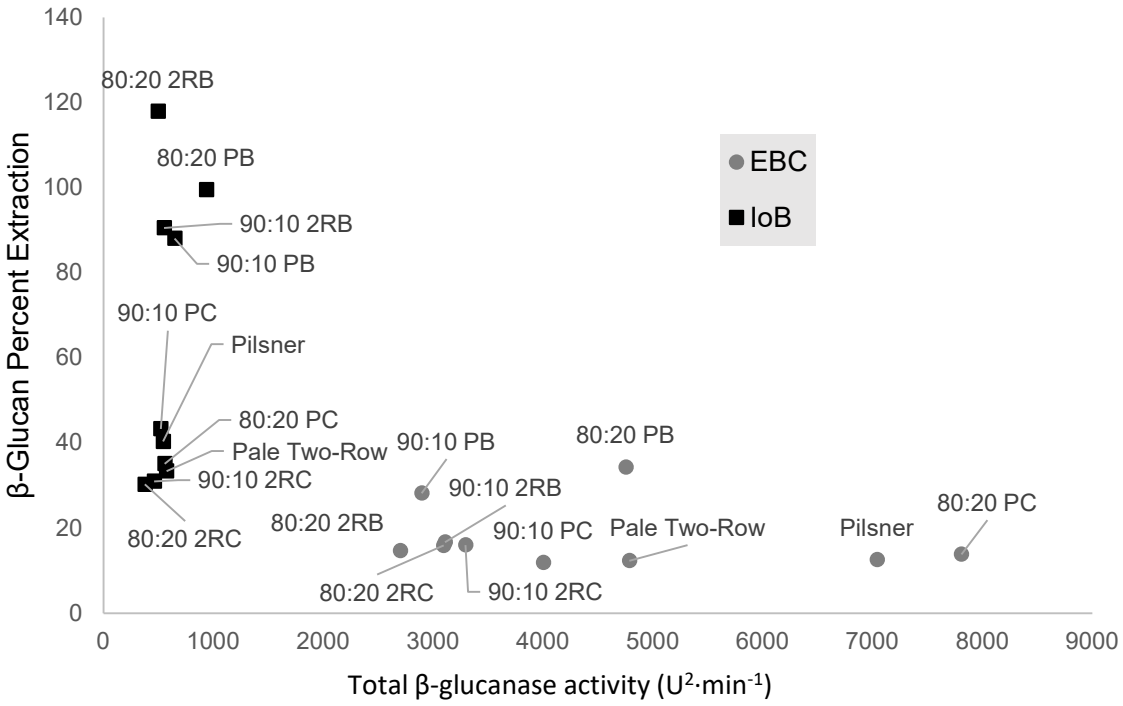

Figure 3.6: Plot of the final β-glucan percent extraction and total β-glucanase activity for each mash. The total β-glucanase was integrated in GraphPad to estimate the total β-glucanase activity over the duration of each mash. Percent extract was determined through dividing the final wort β-glucan content by the baseline β-glucan content of each grist and multiplying by 100% to calculate the total amount of β-glucan contributed to each mash. Total β-glucanase activity was computed using GraphPad Prism's area under curve function. PC is Pilsner malt and caramel malt blend, PB is Pilsner malt and barley blend, 2RC is two-row pale malt and caramel malt blend, and 2RB is two-row pale malt and barley blend.

Despite the dramatically higher β-glucan content of the IoB mashes (especially those with 10-20% barley), the wort viscosity was not affected by increased in β-glucan (Table 3.4). However, β-glucan content did affect wort filtration. For all mash samples with a wort β-glucan below 200 mg/L there was a significant positive correlation between β-glucan concentration and FV25. There was also significant but weaker correlation between β-glucan and FV25 for samples above 200 mg/L β-glucan. There was no correlation between FV25 and β-glucan in the EBC mashes, but there was a significant correlation of FV25 with β-glucan with worts made from IoB mashes. Based on these correlations, differences in wort β-glucan from mash temperature profile and malt bill have some effect of filtration but essentially no effect on wort viscosity in this study. It is also possible that differences in starch hydrolysis or other cell-

wall polymers, like arabinoxylan, as influenced by mash parameters could have an effect on the mash filtration rate.

Table 4: Correlation values (R^2) of wort β-glucan content with final wort viscosity and filtrate collected after 25 minutes (FV25). Standard error of each correlation is listed in column σ.*Indicates statistically significant at a 95% confidence interval.

Condition	**Viscosity (mPa·s)**		**FV25 (mL)**	
	R^2	**σ**	**R^2**	**σ**
EBC	0.0010	129.4	0.0075	13.9
IoB	0.030	584.2	0.66*	16.3
β-glucan > 200 mg/L	0.070	505.4	0.36*	24.3
β-glucan < 200 mg/L	0.0034	0.12	0.83*	14.7

To compare the modified IoB and EBC mashes, the β-glucan percent extraction was calculated and plotted against the integral of the activity decay curve. To find the total β-glucanase activity over time, GraphPad Prism software was used to calculate the integral of β-glucanase activity from the curve of best fit from time zero to the end of each mash. This was then plotted against the percent extraction given by dividing the final wort β-glucan by the β-glucan measured in the grist by enzymic extraction under ideal conditions and detection by the GOPOD method.

The β-glucan percent extractions for all the IoB mashes varied much more than the percent extraction in EBC mashes (Figure 3.6). Since β-glucanase activity decayed quickly for all IoB samples, original malt β-glucan content had a greater influence on β-glucan extraction; malts with higher amounts of β-glucan experienced higher percent extractions. These percent extractions were higher than what has been reported in literature, possibly due to continuous stirring in these small-scale mashes (41,47). Conversely, EBC mashes had more variability in integrated β-glucanase activity (Figure 3.6). The temperature ramp likely introduced additional variability in the rate of decay compared to when starting at a constant 65°C in the modified IoB mash. Both trends indicate that β-glucans in worts

produced by well modified malts are less impacted by different mash temperature profiles as has been previously observed (30,41).

The percent extract above 100% in some IoB mashes with barley could be explained by the higher standard deviations observed with high concentration samples that required dilution to be within the standard curve. Additionally, the method for measuring total β-glucan relied on extraction at lower temperatures (40°C) compared to the modified IoB mash (65°C), and hence temperature difference could impact β-glucan solubility. It is possible that using both the reducing sugar method and Calcofluor method resulted in an under-estimate of total glucan or an overestimate of final wort β-glucan. However, percent extraction is still a useful metric when comparing the EBC and IoB mashes over a range of malt β-glucan contents.

While the incorporation of caramel malt or un-malted barley reduced the total concentration of β-glucanase, the total activity (integral of β-glucanase decay during the mash) was not correlated with final β-glucan extraction (Figure 3.6). Since temperature profile (IoB vs EBC) and initial β-glucan content are better predictors of final β-glucan content, brewers should look towards selecting homogenous low-β-glucan malts and mashing at lower temperatures to avoid problems with β-glucan. However, as many barley growing climates change, it may be more difficult to control β-glucan content in raw materials. Mash schedules that allow for some β-glucanase activity can be more robust to high β-glucan malts. But considering that many breweries cannot perform temperature ramps, adding exogenous β-glucanase or the incorporation of thermostable β-glucanase enzymes into the malting barley genome may offer a solution for high β-glucan malt (57).

Conclusion

This work shows that β-glucanase affected by mash temperature can dramatically skew final wort β-glucan content. The EBC mashes had more cumulative β-glucanase activity and lower final β-glucan concentration compared to IoB mashes. Since the temperature profile in the modified IoB mash

resulted in a β-glucanase half-life of approximately 12 minutes, malt β-glucan content had a greater influence on final wort β-glucan concentration. Low wort β-glucan concentration has little effect on wort viscosity but does impact the rate of mash filtration. Malt β-glucanase can help remedy high malt β-glucan if the temperature profile allows, as in the EBC mash. In contrast, low malt β-glucan is needed for IoB mash conditions to produce a low β-glucan wort. Listing malt β-glucanase activity on a routine malt analysis would do little to contextualize the role of heat in the determination of wort β-glucan. Additionally, β-glucanase activity in an EBC mash could mask high malt β-glucan levels that would be more apparent in an IoB mash, especially when under-modified malt, adjunct grain, or specialty malt is incorporated.

Chapter 4

Comparing the effects of thermostable β-glucanases from *Trichoderma* and wild barley in a modified IoB mash

Abstract

In an effort to reduce wort β-glucan in the IoB mash, the use of two thermostable exogenous β-glucanases was investigated. A thermostable β-glucanase allozyme from wild barley was produced by recombinant *E. coli* and added to IoB mashes with base malt and 10-20% unmalted barley. The wild barley enzyme's effect on wort β-glucan, viscosity, and filtration was evaluated in comparison to a more thermostable β-glucanase from the filamentous fungus *Trichoderma*. Due to the higher level of thermostability in the *Trichoderma* β-glucanase, mashes with this enzyme produced worts with negligible β-glucan that filtered well. The wild barley enzyme was able to reduce wort β-glucan compared to previous mashes with no added enzyme, but not to the same extent as with the *Trichoderma* enzyme. While there was a weak correlation between wort β-glucan and viscosity, all enzyme-added mashes had viscosities within the normal range for wort. Thermostable β-glucanase from wild barley could be a tool to reduce wort β-glucan, but it would likely have less impact on β-glucan content and wort quality compared with existing commercial enzymes.

Introduction

Barley endosperm cell walls are approximately 60-70% β-glucan for a total of 2-6% of the grain's weight (47). Barley β-glucan is a soluble fiber made up of D-glucose units joined by β-1,3 or β-1,4 bonds to form a polymer 1,200-1,850 units long. During the malting process β-1,3-1,4-endoglucanase (β-glucanase) cleaves the polymer, resulting in two smaller β-glucan polymers. The reduced molecular size dramatically reduces the polysaccharide's ability to affect viscosity, wort separation, or beer filtration

(61,73). Excess undegraded β-glucan in malt or wort is undesired when making high quality beer. Therefore, brewing scientists have explored different methods to reduce β-glucan during mashing in addition to the hundred-fold reduction that occurs during malting (4).

β-glucanase is a potential solution for brewers seeking to reduce β-glucan levels during beer processing. There are at least three distinct avenues to how this could be achieved. First, exogenous β-glucanase from a fungal or bacterial source could be added. Commercial enzyme blends have been shown in studies to reduce β-glucan content in wort and beer made with under-modified malts or unmalted barley (20,83). Second, β-glucanase expression could be upregulated during malting. There are concerns, however, that over-expression of β-glucanase during germination could create problems with early plant development for farmers (35). The use of gibberellic acid and other plant hormones to accelerate the malting process and specifically β-glucan hydrolysis has been well documented (82). Third, the thermostability of β-glucanase could be increased so that enzyme activity during the mash could "scrub" β-glucans that are extracted into the wort (67). Increased thermostability could come from site-specific mutagenesis or by selectively breeding thermostable β-glucanase alleles from wild barley into the conventional barley genome (58,85).

Previous experiments have shown that native β-glucanase activity is sufficient to break down the β-glucan contributed by a 20% unmalted barley addition (Chapter 3). Therefore, this study focuses on using exogenous enzymes from *Trichoderma* (+T) and wild barley (+W) in a modified IoB mash.

Materials and methods

Malt

Two-row and Pilsner malts were sourced from Admiral Maltings (Alameda, CA). UC Tahoe barley was grown at UC Davis in 2017. Mashes were conducted with 100% base malt or a blend with ratios of 90:10 or 80:20 base malt to unmalted barley.

Enzymes

Exogenous β-glucanase from *Trichoderma longibrachiatum* was sourced from Millipore-Sigma (Burlington, MA). 375mg of enzyme powder was dissolved in 100mL water to make a solution of 37.5 U/mL. This solution was prepared fresh daily.

A thermostable β-glucanase from wild barley was engineered from the sequence published by Lauer et al. (57) and expressed in BL21(DE3) *Escherichia coli* as described by Caster (19). Heat shock at 42°C was used to transform the plasmids containing the desired genetic sequences into the *E. coli* culture. The transformed cells were plated on Lysogeny broth agarose selection plates with kanamycin. The inoculated plates were incubated for 18 hours at 37°C. A single colony was used to inoculate 2 mL of the bacterial growth medium (Terrific broth with kanamycin), and the broth was incubated with shaking overnight at 37°C. The 2 mL culture was dosed into 500 mL of growth medium and allowed to shake overnight at 37°C. Once the cells had multiplied to a healthy population, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to encourage the expression of the desired β-glucanase protein and held for 18 hours at 18°C with shaking. The culture was centrifuged at 4700 rpm to form a pellet of cells. Media was decanted, and the pellet was frozen at –20°C until ready for use.

To extract the protein, the cells were thawed and resuspended in phosphate buffered saline (PBS) containing 30 mM imidazole, pH 7.5. The cells were sonicated at an amplitude of 30 µm for 2 minutes to lyse. The lysed cell solution was centrifuged at 4700 rpm for 45 minutes to separate the protein from cell debris. The lysate supernatant was filtered through an immobilized metal ion chromatography column containing 1mL of 50% Ni-NTA resin slurry that would bind the protein of interest. After a six-fold wash with PBS, the protein was eluted with 5mL of PBS buffer containing 200mM imidazole, pH 7.5. The eluent was collected in a 6mL, 10 kDa molecular weight limit spin concentrator.

The protein solution was purified batchwise using the same vessel. The PBS buffer was exchanged for a 100 mM sodium acetate buffer at pH 4.5, via centrifugation, to match the buffer used in previous β-glucanase tests. This protein solution had an enzyme activity of 364 U/L.

Mash

A modified IoB mash (28) was conducted as described in the previous chapter. Base malts were combined 90:10 and 80:20 with barley to test a wide range of β-glucan contents. Exogenous β-glucanase (1mL of bacterial enzyme solution and 0.2 mL of wild barley enzyme solution) was added at the beginning of mashes. The amount added was selected to be within the range of initial mash β-glucan activities of the EBC mashes conducted in Chapter 3. However, the volume addition of wild barley enzyme was reduced to conserve the enzyme solution, and therefore to be able to use it in more conditions. The 100% Pilsner malt +W condition was excluded to prioritize other assays. The β-glucan content and β-glucanase activity was measured intermittently throughout the mash using the Gallery methods described in Chapters 2 and 3.

Viscosity and Filtrate Volume

To evaluate mash separation, finished worts were cooled to 25°C and filtered through Cytivia filter paper (32). The wort filtrate volume collected after 25 minutes (FV25) was measured using a graduated cylinder. Filtered wort viscosity was measured by MCR 92 rheometer with plate and plate geometry (Anton Paar). A shear rate sweep from 1-100 s^{-1} was conducted, measuring the resulting torque. Viscosity was measured in duplicate for each sample and reported at 100 s^{-1}.

Statistical Analysis

Both β-glucan and β-glucanase data measured over the course of the mash were fit to exponential curves using GraphPad Prism 9.4.0 (673) software. Excel was used to find correlation between β-glucan content, wort viscosity, and FV25.

Results

Table 4.1: Correlation constants and key parameters from the exponential decay functions (Equation 4.1) of β-glucanase activity and β-glucan content in IoB mashes with thermostable β-glucanase from *Trichoderma* added (+T). Mashes were made with grists of 100% two-row malt (2-Row), or 90% two-row malt with 10% barley (90102RB), or 80% two-row malt with 20% barley (80202RB). This was repeated with 100% Pilsner malt, 90% Pilsner with 10% barley (9010PB), or 80% Pilsner with 20% barley (8020PB).

	β-Glucanase Decay			**β-Glucan Decay**		
Sample	**K (min^{-1})**	**Half-Life (minutes)**	**R^2**	**K (min^{-1})**	**Half-Life (minutes)**	**R^2**
2-Row +T	0.04	15.79	0.94	0.01	56.94	0.92
90102RB +T	0.07	10.18	0.96	0.02	43.17	0.98
80202RB +T	0.07	10.37	0.97	0.05	14.45	0.94
Pilsner +T	0.09	7.49	0.95	0.03	26.39	0.91
9010PB +T	0.07	10.47	0.98	0.06	11.19	0.98
8020PB +T	0.11	6.10	0.96	0.06	12.52	0.92

Exogenous thermostable β-glucanase activity from *Trichoderma* decayed slowly throughout the mash with an average half-life of 10.1 minutes, although activity was measurable throughout (Figure 4.1A, Table 4.1). The decay function can be described by the following equation where "A" is β-glucanase activity at time "t," with rate constant K. The plateau is given by the estimation of "A" as t→∞.

Equation 4.1 $$A = (A_0 - Plateau * e^{-K*t}) + Plateau$$

Half-life was then calculated as follows.

Equation 4.2 $$half - life = \frac{ln(2)}{K}$$

Addition of thermostable β-glucanase from *Trichoderma* dramatically reduced β-glucan content compared to levels in +W mashes or in the mashes in Chapter 2 (Figure 4.1B). These data were described by a similar exponential decay function where "B" is the concentration of β-glucan at time "t," with rate of decay "K." The plateau is given by the estimation of "B" as t→∞.

Equation 4.3 $$B = (B_0 - Plateau * e^{-K*t}) + Plateau$$

This resulted in a wort with a final β-glucan content ranging from 7.2±0.001 mg/L in 100% two-row base malt to 50.2±3.2 mg/L in 20% barley addition to two-row malt.

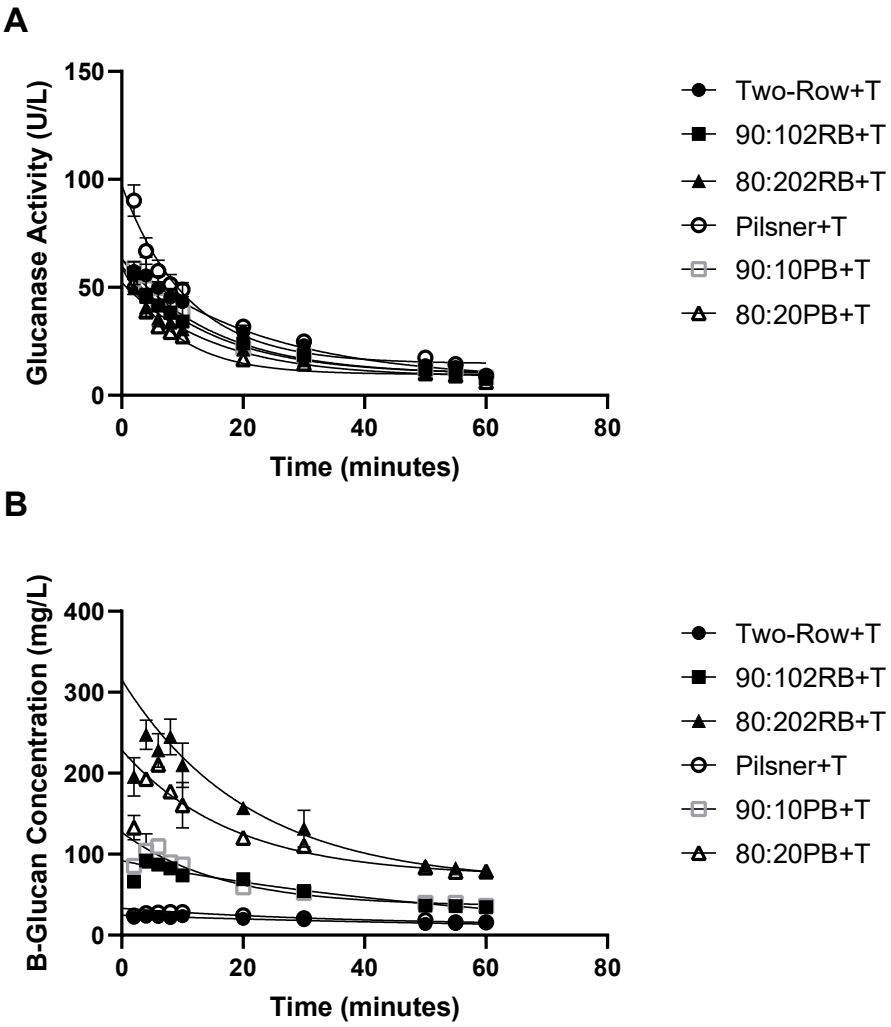

Figure 4.1: β-glucanase activity (A) and β-glucan content (B) over time during IoB mash with thermostable β-glucanase from *Trichoderma* added (+T). Mashes were made with grists of 100% two-row malt (2-Row), or 90% two-row malt with 10% barley (90102RB), or 80% two-row malt with 20% barley (80202RB). This was repeated with 100% Pilsner malt, 90% Pilsner with 10% barley (9010PB), or 80% Pilsner with 20% barley (8020PB).Error bars represent one standard deviation from the mean.

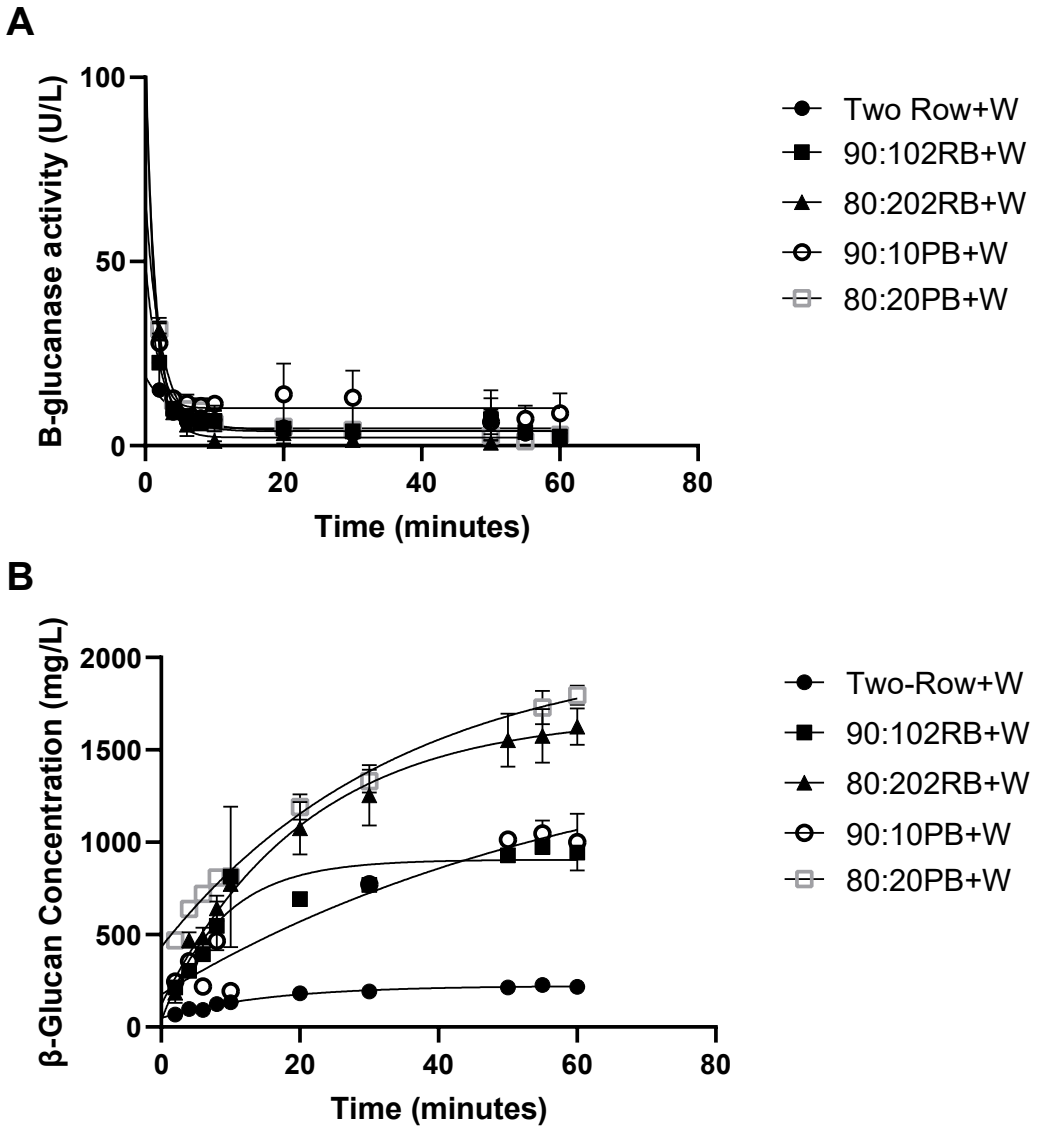

Figure 4.2: β-glucanase activity (A) and β-glucan content (B) over time during IoB mash with thermostable β-glucanase from wild barley added (+W). Results displayed are for Pilsner and pale two-row malts in combination with 10% and 20% barley. Error bars represent one standard deviation from the mean.

Addition of the mutant wild barley enzyme (+W) contributed some additional enzyme activity, and the half-life averaged to 1.6 minutes (Equation 4.2, Table 4.2). Over the course of the mash, β-glucanase activity decayed exponentially (Equation 4.1) and β-glucan accumulated in the wort by a function of exponential growth over time (Figure 4.2). The accumulation of β-glucan over time was fit

using Equation 4.4 where "B" is the concentration of β-glucan at time "t," and with rate constant "k." The plateau estimates the concentration of β-glucan as t→∞.

$$B = B_0 + (Plateau - B_0) * (1 - e^{-k*t}) \quad \textit{Equation 4.4}$$

The accumulation of β-glucan over the course of +W mashes resulted in a range of final β-glucan contents from 151.2±3.9 in 100% two-row base malt to 991.4±28.1 mg/L in 20% barley addition to Pilsner base malt.

Table 4.2: Constants from the non-linear curve fit of exponential growth of wort β-glucan and exponential decay of β-glucanase in IoB mashes with added β-glucanase from wild barley (+W). Curves were fir using Graph Pad Prism software and equations 4.1, 2, and 3.

	β-glucan association		β-glucanase decay		
Samples	K	R-squared	K	half-life	R-squared
Two-Row + W	0.06549	0.8598	0.2222	3.12	0.4102
90:10 2RB + W	0.1163	0.7247	0.4691	1.478	0.7265
80:20 2RB + W	0.04814	0.959	0.6324	1.096	0.9544
90:10 PB + W	0.01582	0.9085	0.8824	0.7855	0.5632
80:20 PB + W	0.02656	0.7965	0.4306	1.61	0.9059

Wort β-glucan had an insignificant correlation with wort viscosity in +T mashes (r^2 = 0.24) and +W mashes (r^2 = 0.32) at a 95% confidence interval. (Figure 4.3A). Similarly, wort β-glucan was insignificantly correlated with FV25 in +T mashes (r^2 = 0.36) and +W mashes (r^2 = 0.407) (Figure 4.3B). Significance was determined at a 95% confidence interval.

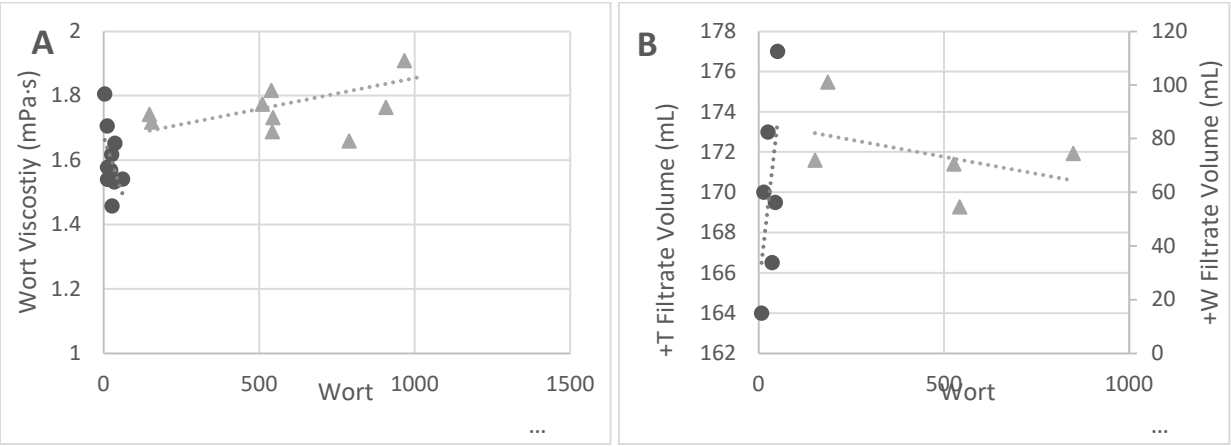

Figure 4.3: (A) Wort viscosity plotted against wort β-glucan content. (B) Wort filtrate volume after 25 minutes plotted against wort β-glucan content for mashes with β-glucanase from *Trichoderma* (●) plotted on the primary y-axis and wild barley (▲) plotted on the secondary y-axis.

Discussion

The thermostable β-glucanase from wild barley was not as effective at reducing the β-glucan content of +W mashes when compared with +T mashes. The lower thermostability of the wild barley enzyme resulted in a more rapid decay as evidenced by the shorter half-life and larger K values in the +W mashes compared to the +T mashes (Tables 4.1 and 4.2). Exacerbating this effect, the *Trichoderma* β-glucanase was added at a higher activity per volume than the wild barley, and a lower addition rate would enable a clearer comparison of each enzyme's efficacy. Nonetheless, the lower thermostability of the wild enzyme would result in a less dramatic reduction of β-glucan at an equal initial activity.

As a result of the highly persistent *Trichoderma* enzyme activity, there was little to no accumulation of β-glucan in the wort throughout the mash. This trend resembles the β-glucan accumulation in a 40°C or 45°C isothermal mash performed by Home et al. (41). In contrast, the quick decay in activity in the +W mash resulted in a first order increase of β-glucan into the wort. If minimal β-glucan extraction is desired in a hot mash, adding a commercial enzyme with high thermostability at a high dosage is a viable way to achieve this goal. However, addition of wild barley thermostable β-glucanase at approximately the level existing in malt was able to reduce, but not eliminate, wort β-glucan. Therefore, incorporating this wild barley enzyme into the domesticated barley genome has the potential to reduce wort β-glucan levels in IoB style mashes, but is not as effective as existing commercial enzymes (20,31).

When compared to mashes without enzyme addition, the wild barley enzyme does confer some benefit. In Figure 4.4 the β-glucan accumulation over the course of a modified IoB mash with pale two-row base malt is shown. For mashes with wild barley enzyme added, there was approximately 65% less final wort β-glucan with 100% two-row malt compared to the no-enzyme condition from Chapter 3, Figure 3.5. For mashes with 10% barley and wild enzyme there was a 52% reduction in β-glucan content. For mashes with 20% barley and wild enzyme there was a 47% reduction in β-glucan compared to the

no-enzyme condition. A previous study has shown that the wild barley allozyme had 8% higher activity than the domesticated barley β-glucanase at 55°C (57). At the higher temperature in the modified IoB mash, this small boost in thermostability was enough to confer some benefit, but further increase in thermostability or addition rate would be needed to reduce wort β-glucan to levels comparable with bacterial enzymes (20).

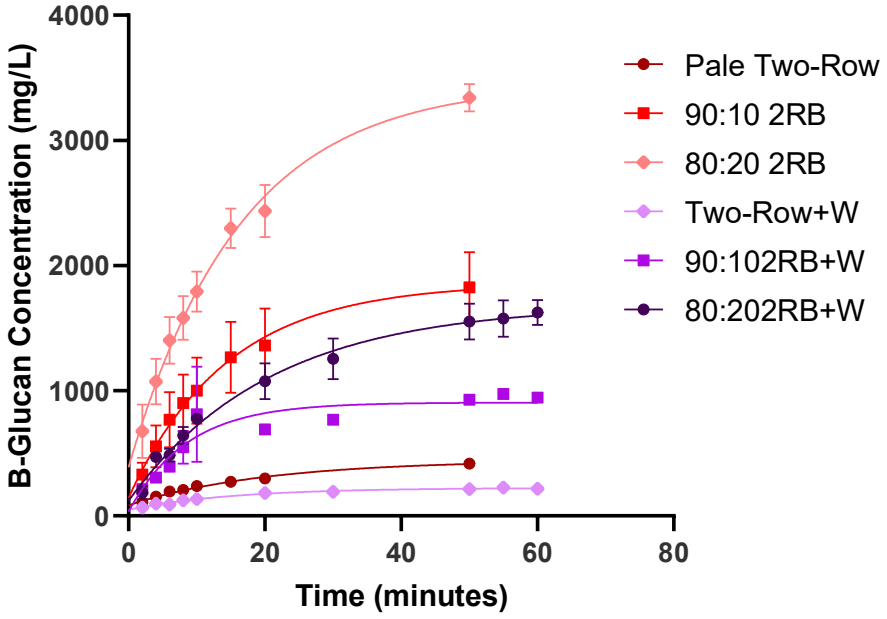

Figure 4.4: β-glucan accumulation over time in IoB mashes comparing two-row and barley mashes with the addition of wild enzyme and without. 2RB is two-row and barley.

Enzyme addition had limited correlation with wort FV25 viscosity. Other studies have found that while β-glucanase addition can have an impact on wort filtration, α-amylase addition seems to have a larger impact (36). The rate of β-glucanase addition likely affects FV25 since the wild enzyme condition had little change in wort filtration, but the +T condition resulted in much more FV25. Previous studies have observed a correlation between viscosity and β-glucan content (23,32). Wort viscosities in this study ranged from 1.46-2.01 mPa·s, which is within a common and acceptable range for wort (46,73).

The higher concentration of β-glucan in the +W worts resulted in lower FV25 compared to +T worts, but the low correlations between β-glucan content and FV25 within each data set indicate that

other malt polymers may be involved. For instance, the highly efficient breakdown of β-glucan in +T mashes could lead to better starch hydrolysis without β-glucan structures' interference. Unhydrolyzed starch in spent grains has been shown to reduce mash filtration efficacy (36). However, analyzing the spent grain's residual starch content would be necessary to confirm this hypothesis.

Conclusion

Addition of exogenous enzymes resulted in a decrease of wort β-glucan in modified IoB mash conditions. Due to the increased thermostability and higher dose rate of the β-glucanase from *Trichoderma*, +T mashes ended with negligible β-glucanase while the +W mashes finished at relatively higher β-glucan concentrations. Wort FV25 was significantly higher in +T mashes compared to +W mashes. Wort viscosity for both exogenous enzyme mashes was within a normal range. Addition of wild barley thermostable β-glucanase is an effective strategy to reduce wort β-glucan, but it has limited impact on wort viscosity or FV25 and is not as effective as more thermostable bacterial β-glucanase.

Bibliography

1. Aastrup, S., and Erdal, K. Quantitative determination of endosperm modification and its relationship to the content of 1,3:1,4-β-glucans during malting of barley. *Carlsberg Res. Commun.* (online). 10.1007/BF02906161, 1980.

2. Aastrup, S. The effect of rain on β-glucan content in barley grains. *Carlsberg Res. Commun.* (online). 10.1007/BF02906187, 1979.

3. Aastrup, S. Selection and characterization of low β-glucan mutants from barley. *Carlsberg Res. Commun.* (online). 10.1007/BF02911913, 1983.

4. American Malting Barley Association. MALTING BARLEY BREEDING GUIDELINES IDEAL COMMERCIAL MALT CRITERIA. 2021.

5. Asare, E. K., Jaiswal, S., Maley, J., Båga, M., Sammynaiken, R., Rossnagel, B. G., et al. Barley grain constituents, starch composition, and structure affect starch in vitro enzymatic hydrolysis. *J. Agric. Food Chem.* (online). 10.1021/jf200054e, 2011.

6. ASBC. Malt-4. 2011.

7. Ballance, G. Structural analysis and enzymic solubilization of barley endosperm cell-walls. *Carbohydr. Res.* (online). 10.1016/S0008-6215(00)83575-2, 1978.

8. Bamforth, C., and Kanauchi, M. A Simple Model for the Cell Wall of the Starchy. 2001.

9. Bamforth, C. W., Martin, H. L., and Wainwright, T. A role for carboxypeptidase in the solubilization of barly β-glucan. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1979.tb03937.x, 1979.

10. Bamforth, C. W., and Martin, H. L. β-glucan and β-glucan solubilase in malting and mashing. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1981.tb04052.x, 1981.

11. Bamforth, C. W., and Martin, H. L. The degradation of β-glucan during malting and mashing: the role of β-glucanase. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1983.tb04190.x, 1983.

12. Bamforth, C. W. B-glucan and b-glucanase in malting and brewing: practical aspects. 1994.

13. Bamforth, C. W. Progress in brewing science and beer production. *Annu. Rev. Chem. Biomol. Eng.* (online). 10.1146/annurev-chembioeng-060816-101450, 2017.

14. Barmeier, G., Hu, Y., and Schmidhalter, U. Partitioning and translocation of dry matter and nitrogen during grain filling in spring barley varieties and their roles in determining malting quality. *Front. Plant Sci.* (online). 10.3389/fpls.2021.722871, 2021.

15. Bathgate, G. N., Palmer, G. H., and Wilson, G. THE ACTION OF ENDO-β-1,3-GLUCANASES ON BARLEY AND MALT β-GLUCANS. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1974.tb03617.x, 1974.

16. Brazil, C., Oliveira, D. F. de, Duarte, R. A., Galo, J. M., Lucchetta, L., Santos, E. da C. dos, et al. β-Glucanase Addition in Brewing Malt Produced by Reduced Time of Germination. *Braz. arch. biol. technol.* (online). 10.1590/1678-4324-2019180315, 2019.

17. Cammarano, D., Ceccarelli, S., Grando, S., Romagosa, I., Benbelkacem, A., Akar, T., et al. The impact of climate change on barley yield in the Mediterranean basin. *European Journal of Agronomy* (online). 10.1016/j.eja.2019.03.002, 2019.

18. Carbonell, J. V., Sendra, J. M., and Todo, V. KINETICS OF β-GLUCAN DEGRADATION IN BEER BY EXOGENOUS β-GLUCANASE TREATMENT. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1990.tb01018.x, 1990.

19. Caster, R. W. Utilizing Crowd Sourced Engineering to Design a Lactone Ring Hydrolyzing Enzyme for Aflatoxin B1 Degradation - Dissertations & Theses. 2018.

20. Cimini, A., De Francesco, G., and Perretti, G. Effect of crossflow microfiltration on the clarification and stability of beer from 100% low-β-glucan barley or malt. *LWT* (online). 10.1016/j.lwt.2017.07.033, 2017.

21. Colleoni-Sirghie, M., Kovalenko, I. V., Briggs, J. L., Fulton, B., and White, P. J. Rheological and molecular properties of water soluble (1,3) (1,4)-β-d-glucans from high-β-glucan and traditional oat lines. *Carbohydr. Polym.* (online). 10.1016/S0144-8617(03)00003-1, 2003.

22. Cooper, C. M., Evans, D. E., Yousif, A., Metz, N., and Koutoulis, A. Comparison of the impact on the performance of small-scale mashing with different proportions of unmalted barley, Ondea Pro®, malt and rice. *J. Inst. Brew.* (online). 10.1002/jib.325, 2016.

23. Cornaggia, C., Evans, D. E., Draga, A., Mangan, D., and McCleary, B. V. Prediction of potential malt extract and beer filterability using conventional and novel malt assays. *J. Inst. Brew.* (online). 10.1002/jib.567, 2019.

24. De Arcangelis, E., Djurle, S., Andersson, A. A. M., Marconi, E., Messia, M. C., and Andersson, R. Structure analysis of β-glucan in barley and effects of wheat β-glucanase. *J. Cereal Sci.* (online). 10.1016/j.jcs.2018.12.002, 2019.

25. Demeester, A., Laureys, D., Baillière, J., Huys, J., Vermeir, P., De Leyn, I., et al. Comparison of Congress Mash with Final 65 °C Mash for Wort Production with Unmalted Barley, Tritordeum, and Quinoa, with or without Pregelatinization and/or Enzyme Addition. *ASBCJ* (online). 10.1080/03610470.2021.1989571, 2021.

26. Dugulin, C. A., De Rouck, G., and Cook, D. J. Green malt for a green future – feasibility and challenges of brewing using freshly germinated (unkilned) malt: A review. *ASBCJ* (online). 10.1080/03610470.2021.1902710, 2021.

27. Edney, M. J., LaBerge, D. E., and Langrell, D. E. Relationships Among the β-Glucan Contents of Barley, Malt, Malt Congress Extract, and Beer. *ASBCJ* (online). 10.1094/ASBCJ-56-0164, 1998.

28. Evans, D. E., and Fox, G. P. Comparison of Diastatic Power Enzyme Release and Persistence during Modified Institute of Brewing 65°C and Congress Programmed Mashes. *ASBCJ* (online). 10.1094/ASBCJ-2017-4707-01, 2017.

29. Evans, D. E., Goldsmith, M., Dambergs, R., and Nischwitz, R. A Comprehensive Revaluation of Small-Scale Congress Mash Protocol Parameters for Determining Extract and Fermentability. *ASBCJ* (online). 10.1094/ASBCJ-2011-0111-01, 2011.

30. Evans, D. E., Goldsmith, M., Redd, K. S., Nischwitz, R., and Lentini, A. Impact of mashing conditions on extract, its fermentability, and the levels of wort free amino nitrogen (FAN), β-glucan, and lipids. *ASBCJ* (online). 10.1094/ASBCJ-2012-0103-01, 2012.

31. Evans, D. E., Redd, K., Haraysmow, S. E., Elvig, N., Metz, N., and Koutoulis, A. The Influence of Malt Quality on Malt Brewing and Barley Quality on Barley Brewing with Ondea Pro, Compared by Small-Scale Analysis. *ASBCJ* (online). 10.1094/ASBCJ-2014-0630-01, 2014.

32. Evans, D. E., Stewart, S., Stewart, D., Han, Z., Han, Y., and Able, J. A. Profiling malt enzymes related to impact on malt fermentability, lautering and beer filtration performance of 94 commercially produced malt batches. *ASBCJ* (online). 10.1080/03610470.2021.1979891, 2021.

33. Furtado, G. P., Carli, S., Meleiro, L. P., Salgado, J. C. S., and Ward, R. J. Enhanced hydrolytic efficiency of an engineered CBM11-glucanase enzyme chimera against barley β-d-glucan extracts. *Food Chem.* (online). 10.1016/j.foodchem.2021.130460, 2021.

34. Garcia-Gimenez, G., Barakate, A., Smith, P., Stephens, J., Khor, S. F., Doblin, M. S., et al. Targeted mutation of barley (1,3;1,4)-β-glucan synthases reveals complex relationships between the storage and cell wall polysaccharide content. *Plant J.* (online). 10.1111/tpj.14977, 2020.

35. Gibson, C. E. The expression of hydrolytic enzymes in germinating barley grain. 2017.

36. Goode, D. L., Wijngaard, H. H., and Arendt, E. K. Mashing with unmalted barley--impact of malted barley and commercial enzyme (Bacillus spp.) additions. 2005.

37. Grujic, O., and Pejin, J. The application of triticale malt as the substitute for barley malt in wort production. *Acta per. tech.* (online). 10.2298/APT0738117G, 2007.

38. Hennemann, M., Gastl, M., and Becker, T. Inhomogeneity in the lauter tun: a chromatographic view. *Eur. Food Res. Technol.* (online). 10.1007/s00217-018-03226-4, 2019.

39. Henry, R. J. A comparison of the non-starch carbohydrates in cereal grains. *J. Sci. Food Agric.* (online). 10.1002/jsfa.2740361207, 1985.

40. Henry, R. J. Changes in β-glucan and other carbohydrate components of barley during malting. *J. Sci. Food Agric.* (online). 10.1002/jsfa.2740420407, 1988.

41. Home, S., Pietilä, K., and Sjoholm, K. Control of glucanolysis in mashing. *ASBCJ* (online). 10.1094/ASBCJ-51-0108, 1993.

42. Hrmova, M., Harvey, A. J., Wang, J., Shirley, N. J., Jones, G. P., Stone, B. A., et al. Barley beta-D-glucan exohydrolases with beta-D-glucosidase activity. Purification, characterization, and determination of primary structure from a cDNA clone. *J. Biol. Chem.* (online). 10.1074/jbc.271.9.5277, 1996.

43. Huismann, M., Gormley, F., Dzait, D., Speers, R. A., and L. Maskell, D. Unfilterable beer haze part I: the investigation of an india pale ale haze. *ASBCJ* (online). 10.1080/03610470.2021.1937460, 2022.

44. Igarashi, H., and Amaha, M. STUDIES ON FROZEN BEER PRECIPITATES II. CHEMICAL STRUCTURE OF THE β-GLUCAN ISOLATED FROM THE PRECIPITATES. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1969.tb03214.x, 1969.

45. Jensen, L. G., Olsen, O., Kops, O., Wolf, N., Thomsen, K. K., and von Wettstein, D. Transgenic barley expressing a protein-engineered, thermostable (1,3-1,4)-beta-glucanase during germination. *Proc Natl Acad Sci USA* (online). 10.1073/pnas.93.8.3487, 1996.

46. Jin, Y.-L., Speers, A., Paulson, A. T., and Stewart, R. J. Effects of β-Glucans and Environmental Factors on the Viscosities of Wort and Beer. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.2004.tb00189.x, 2004.

47. Jin, Y. L., Speers, R. A., Paulson, A. T., and Stewart, R. J. Barley b-Glucans and Their Degradation During Malting and Brewing. 41 (3) :231–240 2004.

48. Kahle, E.-M., Zarnkow, M., and Jacob, F. Identification and differentiation of haze substances using Raman microspectroscopy. 2020.

49. Kanauchi, M., and Bamforth, C. W. Release of β-Glucan from Cell Walls of Starchy Endosperm of Barley. *Cereal Chemistry Journal* (online). 10.1094/CCHEM.2001.78.2.121, 2001.

50. Kanauchi, M., and Bamforth, C. W. The Relevance of Different Enzymes for the Hydrolysis of β-glucans in Malting and Mashing. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.2008.tb00332.x, 2008.

51. Kuntz, R. J., and Bamforth, C. W. Time course for the development of enzymes in barley1. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.2007.tb00276.x, 2007.

52. Kupetz, M., Aumer, J., Harms, D., Zarnkow, M., Sacher, B., and Becker, T. High-throughput β-glucan analyses and their relationship with beer filterability. *Eur. Food Res. Technol.* (online). 10.1007/s00217-016-2748-3, 2017.

53. Kupetz, M., Procopio, S., Sacher, B., and Becker, T. Critical review of the methods of β-glucan analysis and its significance in the beer filtration process. *Eur. Food Res. Technol.* (online). 10.1007/s00217-015-2498-7, 2015.

54. Kupetz, M., Sacher, B., and Becker, T. Impact of flavouring substances on the aggregation behaviour of dissolved barley β-glucans in a model beer. *Carbohydr. Polym.* (online). 10.1016/j.carbpol.2016.01.070, 2016.

55. Laitila, A., Manninen, J., Priha, O., Smart, K., Tsitko, I., and James, S. Characterisation of barley-associated bacteria and their impact on wort separation performance. *J. Inst. Brew.* (online). 10.1002/jib.509, 2018.

56. Langenaeken, N. A., De Schepper, C. F., De Schutter, D. P., and Courtin, C. M. Carbohydrate content and structure during malting and brewing: a mass balance study. *J. Inst. Brew.* (online). 10.1002/jib.619, 2020.

57. Lauer, J. C., Cu, S., Burton, R. A., and Eglinton, J. K. Variation in barley (1 → 3, 1 → 4)-β-glucan endohydrolases reveals novel allozymes with increased thermostability. *Theor. Appl. Genet.* (online). 10.1007/s00122-017-2870-z, 2017.

58. Lauer, J. C., Yap, K., Cu, S., Burton, R. A., and Eglinton, J. K. Novel Barley (1→3,1→4)-β-Glucan Endohydrolase Alleles Confer Increased Enzyme Thermostability. *J. Agric. Food Chem.* (online). 10.1021/acs.jafc.6b04287, 2017.

59. Lee, Y.-T., and Bamforth, C. W. Variations in Solubility of Barley β-Glucan during Malting and Impact on Levels of β-Glucan in Wort and Beer. *ASBCJ* (online). 10.1094/ASBCJ-2009-0226-01, 2009.

60. Loi, L., Barton, P. A., and Fincher, G. B. Survival of barley (1→3, 1→w4)-β-glucanase isoenzymes during kilning and mashing. *J. Cereal Sci.* (online). 10.1016/S0733-5210(87)80008-5, 1987.

61. Marconi, O., Tomasi, I., Dionisio, L., Perretti, G., and Fantozzi, P. Effects of malting on molecular weight distribution and content of water-extractable β-glucans in barley. *Food Res. Int.* (online). 10.1016/j.foodres.2014.07.035, 2014.

62. McCleary, B. V., Mangan, D., Daly, R., Fort, S., Ivory, R., and McCormack, N. Novel substrates for the measurement of endo-1,4-β-glucanase (endo-cellulase). *Carbohydr. Res.* (online). 10.1016/j.carres.2013.12.001, 2014.

63. McCleary, B. V., and Nurthen, E. MEASUREMENT OF (1 → 3)(1 → 4)-β-d -GLUCAN IN MALT, WORT AND BEER. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1986.tb04392.x, 1986.

64. Megazyme. B-glucan MW Standards. 2022.

65. Müller, J. J., Thomsen, K. K., and Heinemann, U. Crystal Structure of Barley 1,3–1,4-β-Glucanase at 2.0-Å Resolution and Comparison with*Bacillus* 1,3–1,4-β-Glucanase. *Journal of Biological Chemistry* (online). 10.1074/jbc.273.6.3438, 1998.

66. Muller, R. The influence of grain components on infusion mash performance. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1991.tb01056.x, 1991.

67. Niu, C., Zhu, L., Hill, A., Alex Speers, R., and Li, Q. Construction of a highly thermostable 1,3-1,4-β-glucanase by combinational mutagenesis and its potential application in the brewing industry. *Biotechnol. Lett.* (online). 10.1007/s10529-016-2212-2, 2017.

68. Oscarsson, M., Andersson, R., Åman, P., Olofsson, S., and Jonsson, A. Effects of cultivar, nitrogen fertilization rate and environment on yield and grain quality of barley. *J. Sci. Food Agric.* (online). 10.1002/(SICI)1097-0010(199811)78:3<359::AID-JSFA126>3.0.CO;2-R, 1998.

69. Palmer, G. H., and Agu, R. C. Effect of Mashing Temperatures and Endo-β-Glucanase on β-Glucan Content of Malt Worts. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1999.tb00024.x, 1999.

70. Passarella, V. S., Savin, R., and Slafer, G. A. Are temperature effects on weight and quality of barley grains modified by resource availability? *Aust. J. Agric. Res.* (online). 10.1071/AR06325, 2008.

71. Pérez-Vendrell, A. M., Guasch, J., Francesch, M., Molina-Cano, J. L., and Brufau, J. Determination of β-(1–3), (1–4)-D-glucans in barley by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* (online). 10.1016/0021-9673(95)00694-X, 1995.

72. Sá, R. M., and Palmer, G. H. Assessment of enzymatic endosperm modification of malting barley using individual grain analyses. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.2004.tb00179.x, 2004.

73. Sadosky, P., Schwarz, P. B., and Horsley, R. D. Effect of Arabinoxylans, β-Glucans, and Dextrins on the Viscosity and Membrane Filterability of a Beer Model Solution. *ASBCJ* (online). 10.1094/ASBCJ-60-0153, 2002.

74. Savin, R., and Nicolas, M. E. Effects of short periods of drought and high temperature on grain growth and starch accumulation of two malting barley cultivars. *Functional Plant Biol.* (online). 10.1071/PP9960201, 1996.

75. Savin, R., Stone, P. J., and Nicolas, M. E. Responses of grain growth and malting quality of barley to short periods of high temperature in field studies using portable chambers. *Aust. J. Agric. Res.* (online). 10.1071/AR9960465, 1996.

76. Scheffler, A., and Bamforth, C. W. Exogenous β-glucanases and pentosanases and their impact on mashing. *Enzyme Microb. Technol.* (online). 10.1016/j.enzmictec.2005.01.009, 2005.

77. Schnitzenbaumer, B., and Arendt, E. K. Effect of unmalted oats (Avena sativa L.) on the quality of high-gravity mashes and worts without or with exogenous enzyme addition. *Eur. Food Res. Technol.* (online). 10.1007/s00217-013-2105-8, 2014.

78. Scott, R. W. The viscosity of worts in relation to their content of β-glucan. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1972.tb06401.x, 1972.

79. Scott, R. W. SOLUBILIZATION OF β-GLUCAN DURING MASHING. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1972.tb03472.x, 1972.

80. Sendra, J. M., Carbonell, J. V., Gosalbes, M. J., and Todo, V. DETERMINATION OF β-GLUCAN IN WORT AND BEER BY ITS BINDING WITH CALCOFLUOR, USING A FLUORIMETRIC FLOW-INJECTION-ANALYSIS (FIA) METHOD. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1989.tb04639.x, 1989.

81. Slakeski, N., and Fincher, G. B. Developmental Regulation of (1-->3, 1-->4)-beta-Glucanase Gene Expression in Barley : Tissue-Specific Expression of Individual Isoenzymes. *Plant Physiol.* (online). 10.1104/pp.99.3.1226, 1992.

82. Slakeski, N., and Fincher, G. B. Barley (1----3,1----4)-beta-glucanase isoenzyme EI gene expression is mediated by auxin and gibberellic acid. *FEBS Lett.* (online). 10.1016/0014-5793(92)80977-o, 1992.

83. Steiner, E., Auer, A., Becker, T., and Gastl, M. Comparison of beer quality attributes between beers brewed with 100% barley malt and 100% barley raw material. *J. Sci. Food Agric.* (online). 10.1002/jsfa.4651, 2012.

84. Stewart, D., Freeman, G., and Evans, E. Development and Assessment of a Small-Scale Wort Filtration Test for the Prediction of Beer Filtration Efficiency. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.2000.tb00526.x, 2000.

85. Stewart, R. J., Varghese, J. N., Garrett, T. P., Høj, P. B., and Fincher, G. B. Mutant barley (1-->3,1-->4)-beta-glucan endohydrolases with enhanced thermostability. *Protein Eng.* (online). 10.1093/protein/14.4.245, 2001.

86. Takayanagi, S., Amaha, M., Satake, K., Kuroiwa, Y., Igarashi, H., and Murata, A. Studies on frozen beer precipitates i. formation and general characters. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1969.tb03213.x, 1969.

87. Thomsen, K. K., Jackson, E. A., and Brenner, K. Genetic Engineering of Yeast: Construction of Strains That Degrade β-Glucans with the Aid of a Barley Gene. *ASBCJ* (online). 10.1094/ASBCJ-46-0031, 1988.

88. Tsitko, I., Manninen, J., Smart, K., James, S., and Laitila, A. Management of barley-associated bacterial biofilms: a key to improving wort separation. *J. Inst. Brew.* (online). 10.1002/jib.506, 2018.

89. Walters, M. T., Seefeld, R., Hawthorne, D. B., and Kavanagh, T. E. Composition and kinetics of particle formation in beer post packaging. *ASBCJ* (online). 10.1094/ASBCJ-54-0057, 1996.

90. Wilhelmi, C., and Morgan, K. The hydrolysis of barley beta-glucan by the cellulase EC 3.2.1.4 under dilute conditions is identical to that of barley solubilase. *Carbohydr. Res.* (online). 10.1016/s0008-6215(00)00298-6, 2001.

91. Woonton, B., Jacobsen, J., Sherkat, F., and Stuart, M. Effect of post-harvest storage period on barley germination and malt quality. 2001.

92. Wu, J., Zhao, L., Li, J., Jin, S., and Wu, S. Aggregation and gelation of oat β-glucan in aqueous solution probed by NMR relaxometry. *Carbohydr. Polym.* (online). 10.1016/j.carbpol.2017.01.070, 2017.

93. Yamashita, H., Hayase, F., and Kato, H. .BETA.-Glucanases in malt that form insoluble materials. *Agric. Biol. Chem.* (online). 10.1271/bbb1961.49.1313, 1985.

94. Ying, R., Saulnier, L., and Rondeau-Mouro, C. Films of arabinoxylans and β-glucans extracted from cereal grains: Molecular motions by TD-NMR. *Carbohydr. Polym.* (online). 10.1016/j.carbpol.2011.05.033, 2011.

95. Yin, X. S., MacGregor, A. W., and Clear, R. M. FIELD FUNGI AND β-GLUCAN SOLUBILASE IN BARLEY KERNELS*. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1989.tb04626.x, 1989.

96. Yin, X. S., and MacGregor, A. W. AN APPROACH TO THE IDENTIFICATION OF A ß-GLUCAN SOLUBILASE FROM BARLEY[1]. *J. Inst. Brew.* (online). 10.1002/j.2050-0416.1988.tb04591.x, 1988.

97. Yousif, A. M., and Evans, D. E. Changes in malt quality during production in two commercial malt houses. *J. Inst. Brew.* (online). 10.1002/jib.609, 2020.