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Determination of Cytolytic Malt Modification – Part I: Influence of Variety Characteristics

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ABSTRACT

The malt criteria of viscosity and β-glucan are used as an integral part of routine laboratory control measures to classify the cytolytic malt modification level and to ensure good processability. To optimize separation processes in general, the main focus of barley breeding has been on intensifying the cytolytic modification level and reducing the β-glucan concentration. However, not all new malting barley varieties show good processability despite a low β-glucan content, since the effect of hemicellulose constituents such as arabinoxylan and their enzymatic hydrolysis are largely disregarded. For this reason, the aim of the present work was to investigate the individual cytolytic composition of different malting barley (malt) varieties and to point out differences in cytolytic malt parameters depending on their genetically determined modification level. Appropriate analytical possibilities for arabinoxylan determination and suitable extraction methods for cytolytic evaluation have been further shortcomings in breeding quality control. In addition, the most important cytolytic degrading enzymes have been characterized in this paper. Part one of this two-part study demonstrated that cytolytically highly modified varieties have a lower β-glucan but in consequence a higher arabinoxylan content. With regard to the activity of enzymes, the results suggested small differences in β-glucanase, but not in arabinofuranosidase and xylanase. Finally, a principal component analysis showed that most of the cytolytically highly modified barley varieties (52%) were affected by arabinoxylan. The first part of the paper confirms that while breeding progress has reduced β-glucan, arabinoxylan has become the more dominant impact factor on processability.

KEYWORDS

Arabinoxylan; barley variety; brewhouse; cytolytic modification; filterability; β-glucan

Introduction

Structural substances such as β-glucan and arabinoxylan are frequently found constituents of cell walls of the cereal endosperm and the aleurone cells. In the field of grasses and cereals, these are mainly the non-starch polysaccharides 1,3;1,4-β-glucan and 1,4-β-arabinoxylan, also known as pentosane. This is also the case in barley, where the structural substances constitute about 10% of the barley dry matter.[1] The distribution of polysaccharides varies depending on their origin from aleurone or endosperm (see Table 1).[2,3]

In addition to the distribution of polysaccharides, their final molar mass and concentration in malt may also vary due to variety characteristics and modification processes during malting.[3,4] The degradation mechanisms of specific substance groups during malting are influenced by various enzymes that have been investigated in detail in recent years. These mechanisms are described as cytolysis and include the enzymatic degradation of the substances providing structure and which support the cells that surround the starch in the endosperm.[5] The constituents of endosperm hemicellulose are the substances of β-glucans and arabinoxylan. The enzymatic hydrolysis of the β-glucans in barley is very well studied.[6–8] Three endo-enzymes with different isoforms – 1,3-β-glucanase (EC 3.2.1.39), 1,4-β-glucanase (EC 3.2.1.4) and 1,3;1,4-β-glucanase (EC 3.2.1.73) – can be found in barley. These endo-enzymes degrade high molecular mass β-glucans to β-glucan dextrins of different molar mass (see Figure 1). Further degradation takes place by means of exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.74) resulting in cellobiose, laminaribiose and glucose molecules. The optimal reaction temperature of these degrading enzymes can be found between 40 and 45 °C with a pH optimum between 4.5 and 5.5 (see Table 2). According to the literature one further enzyme occurs in barley, which does not degrade the β-glucans but rather dissolves high molar mass polysaccharides at high temperatures (62–70 °C) and pH (6.3–7.0). This so-called β-glucan-solubilase was first described by Baxter[9] and Bamforth et al.[10] as carboxypeptidase cleaving linkages between β-glucan and firmly-bound peptides in the cell wall. Newer publications state that the solubilase has a similar activity to a 1,4-β-glucanase.[11,12] In this regard, some authors report[11,13] that the enzyme solubilase is not an endogenous barley enzyme but is

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Table 1. Composition (% w/w) of barley cell walls, excerpt from Trafford and Fincher.[2]

Tissue	Arabinoxylan	1,3;1,4-β-glucan	Cellulose	Heteromannan
Aleurone	71	26	2	2
Starchy endosperm	20	70	3	2

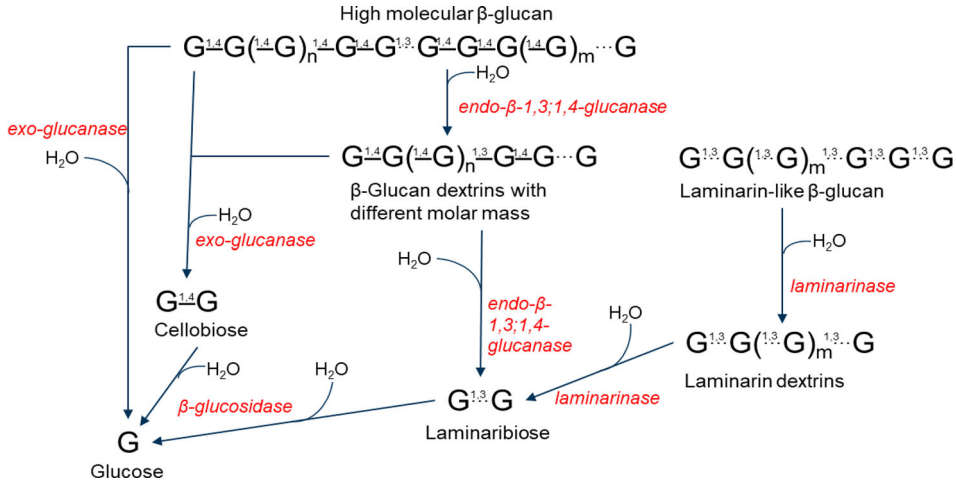

Figure 1. Schematic degradation of β-glucans during the malting and mashing process by endogenous enzymes of the malt; 1,3 and 1,4 are beta-linked glucose units; modified according to [56]; legend: G – glucose molecule.

Table 2. Endogenous enzymes of the malt cytolysis.[3,8,57–61]

	Enzyme	Temperature optimum in mashing [°C]	Inactivation temperature [°C]	pH optimum in mashing
Degradation of β-glucans	β-glucan-solubilase (EC -)	62–70	73	6.3–7.0
	endo-1,3-β-glucanase (EC 3.2.1.39)	<60	70	4.6–5.5
	endo-1,4-β-glucanase (EC 3.2.1.4)	40–45	50–55	4.5–4.8
	endo-1,3;1,4 β-glucanase (EC 3.2.1.73)	40	–	4.8
	laminarinase (EC 3.2.1.6)	–	–	–
	exo-β-glucanase (β-glucan exo-hydrolases) (EC3.2.1.91)	<40	–	4.5
	β-glucosidase (EC 3.2.1.74)	60	–	5.0
Degradation of arabinoxylan	xylan-solubilase (EC -)	–	>65	–
	endo-1,4-β-xylanase (xylan endohydrolases) (EC 3.2.1.8)	45	–	5.0
	exo-xylanase (β-xylosidase) (EC 3.2.1.37)	45	>70	4.5–5.0
	arabinofuranosidase (EC 3.2.1.55)	40–50	60	4.6–4.7
	feruloyl-esterase (EC 3.1.1.73)	30–40	>65	5.9–7.5
	acetylxylan esterase (EC 3.1.1.72)	–	–	–

contributed by fungi associated with the grain husk. However, although the activity of the putative β-glucan solubilase has yet to be fully characterized, this thermostable enzyme plays an important role in the process of the solution of β-glucans during malting and mashing.[8]

Besides β-glucans, arabinoxylans are contained in the cell wall of the barley (constituents of hemicelluloses in the endosperm). Depending on their location in spelt or endosperm, two types of pentosan are described in the literature, whereby especially the type and number of substituents on the xylose backbone differ.[3,14] The enzymatic hydrolysis of arabinoxylan has not been studied in detail compared to the β-glucans. According to the literature, the endo-xylanase (EC 3.2.1.8) degrades high molecular mass arabinoxylan into arabinoxylan dextrins of different molecular masses (see Figure 2). Other enzymes such as exo-xylanase (EC 3.2.1.37), arabinofuranosidase (EC 3.2.1.55), β-xylosidase (EC 3.2.1.37) and feruloyl-esterase (EC 3.1.1.73) depolymerize the cell wall arabinoxylan, resulting in xylose and arabinose mono- and oligosaccharides as well as ferulic acid (see Figure 2).[15]

The optimal reaction temperature and pH for the arabinoxylan degrading enzymes can be found in Table 2. In comparison to the degradation of β-glucans, much less information has been reported in the literature. As with the β-glucans, the literature also states an equivalent enzyme, which can dissolve high molecular mass arabinoxylan at higher temperatures [3, 16]. Detailed information on the

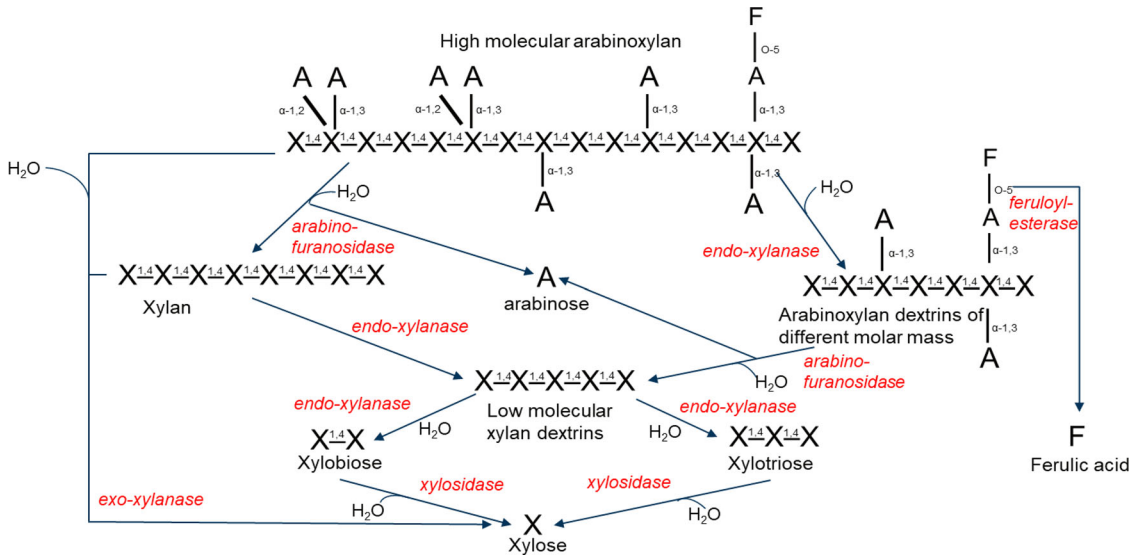

Figure 2. Schematic degradation of arabinoxylan during the malting and mashing process by endogenous enzymes of malt; 1,4 are beta-linked xylose units; modified according to [14, 56]; legend: X – xylose molecule, A – arabinose molecule, F – ferulic acid.

type of enzyme or process technological impact for the brewing process (see Table 2) can be found in the current literature.[3,17]

Evaluation of cytolytic malt parameter

Plant breeding is the most important tool for developing new varieties and adapting them to the changing environment, the changing needs of consumers and the growing demands of the brewing industry. In addition to improving agronomic properties such as resistance, yield and quality properties, a priority objective of malting barley breeding is to guarantee good processability or to focus on modern breeding goals (e.g. no-LOX, thermostable enzymes). Good processability includes the right balance and homogeneity in malt modification (cytolytic, proteolytic and amylolytic criteria) of a variety in the malting process. In addition, the intensity of malt modification is driven by economic aspects such as water and time savings. In the brewing sector, haze formation in wort or beer must additionally be avoided while ensuring trouble-free progress during lautering and beer filtration. In conclusion, malt quality has a decisive influence on the beer production process and quality of the final product.

There has been considerable success in terms of breeding in recent years. Besides increased yields, the breeding progress of the required malt quality parameters (specifications – especially of spring barley varieties) has been enormous in the last 50 years. The purpose of required quality specifications is to guarantee good processability in the brewing process. A barley malt analysis typically describes the three primary modification processes by analytical criteria (specifications) that have occurred in the kernel during the malting process: cytolysis, proteolysis and amylolysis.[5,18,19] If modern brewhouse procedures often entail mashing-in at temperatures above 60 °C, it is a task for breeders and maltsters to effect a homogenous and complete degradation of the cell walls and to attain a suitable level of protein modification. In modern breweries modifying the temperatures and rests in the mash program is not practicable if brewing operations are to remain on schedule. Thus, mashing is largely limited to amylolysis.

For malt quality evaluation, the amylolytic criteria are extract, final attenuation and the enzyme activity of α- and β-amylase; proteolytic parameters are crude protein content, soluble nitrogen, the calculated Kolbach index (ratio of soluble nitrogen to crude protein) and free amino nitrogen (FAN); cytolytic parameters are friability, viscosity and β-glucan content. Parameters such as crude protein and friability are measured directly from the kernel, while the other parameters require a prior extraction method (specific laboratory Congress mashing procedure) to bring the substance in solution as close as possible to applications in the brewery. Common laboratory extraction procedures are EBC-hot water extract, EBC-Congress mashing procedure and isothermal 65 °C mashing procedure according to MEBAK.[20] Owing to the 45 °C rest, the Congress mash method promotes cytolytic degradation. However, with this method, once the temperature of the mash reaches 45 °C, it is immediately heated up to 70 °C. The isothermal 65 °C mash (grist:water ratio 1:6; 0.2 mm; 65 °C for 1 h, add 50 ml water after 30 min) promotes with its high mashing-in temperature and the intensive β-glucan-solubilase rest, high β-glucan contents and distinguishes variations more clearly.[5,21] In consequence, for cytolytic quality assessment both procedures had been applied. Malting barley evaluation and selection for breeding programs in Germany, with aim of malting barley approval, were only based on the Congress mash procedure up to 2012. Required specifications for the cytolytic quality criteria to guarantee good processability depending on the laboratory extraction method are shown in Table 3.

Looking back on the last 50 years of malting barley breeding, for a long time the cytolytic modification of the barley was the limiting factor for optimizing the malting process in the malthouse (e.g. shorter germination time) to achieve the

Table 3. Cytolytic malt analysis quality attributes with different mashing-in temperatures according to MEBAK [5, 20].

Cytolytic analysis parameter depending on extraction method	Recommended value
Friability	>85%
Whole glassy kernels	<2%
Viscosity (adj. to 8.6%), Congress mash	<1.56 mPa × s
Viscosity (adj. to 8.6%), isothermal 65 °C mash	<1.6 mPa × s
β-glucans (adj. to 8.6%), Congress mash	<200 mg/L
β-glucans (adj. to 8.6%), isothermal 65 °C mash	<350 mg/L
Homogeneity	>75%

specifications required by the brewers.[22] A brief summary is possible based on standardized barley evaluation results of different European malting barley varieties. While the average friability value was about 80% in the early eighties, it increased by an average of about 10% in twenty years. Accordingly, the viscosity has decreased from about 1.50 mPa × s to 1.45 mPa × s. Equally, the proteolytic parameters have risen, for example the Kolbach index from 41% to 47%. A significant increase was also achieved in terms of amylolytic characteristics (extract level and amylolytic enzyme capacity).

As mentioned before, previously mainly cytolysis was the limiting factor and required an intensive malting and brewing procedure. This is also reflected by the fact that the malting regime and the laboratory mashing procedure for malting barley selection (breeding programs) were adapted several times over the past decades to achieve higher discrimination between the varieties. For example, the MEBAK standard malting procedure was adjusted in 2005/2006 (45% steeping degree, 15 °C germination temperature) by shortening the germination time by one day down to six days. In 2003 the first "highly modified" barley variety Marnie was approved. Since about 2007, the cytolytic solution properties of new barley variety registrations have more and more been divided into two classifications based on their modification characteristics: moderately modified and very highly modified varieties.

The focus of barley breeders' efforts on improving malt cytolytic modification has resulted in the reduction of viscosity and the related soluble β-glucan. High molecular weight β-glucan fractions have been related to viscosity increases and could lead to processing problems in the brewery, such as less brewhouse yield, slow lautering performance and filtration, as well as haze formation in beer.[5,7,23–25] However, some other sources attest to the favorable influence of high molecular weight β-glucans on foam and mouthfeel.[26,27] However, as long as the β-glucans are not in a gel form, caused by shear forces, quantities of up to approximately 300 mg/l are not critical (recommended specification according to MEBAK).[20] But β-glucan gel can lead to filtration issues even at concentrations as low as 10–15 mg/L.[23] Since β-glucan also provides positive effects, for example on sensory perception like palate fullness and mouthfeel or foam, the tendency to reduce the substance to the detection limit should be reconsidered. Even the stability of the molecular structure of the cell wall is influenced by the molecular size and fine structure of β-glucans.[7]

Finally, new malting barley varieties enable an economical malting procedure by saving water (degree of steeping/proteolysis) and time (cytolysis) as well as a reduction in malting losses and energy and related production costs. In consequence, these modern varieties with high malting flexibility and modification characteristics are extremely interesting for the brewing value chain. However, despite a low β-glucan content and quality criteria at the required level, problems during lautering and filtration still occur from time to time in the brewing industry. Clarifying the cytolytic degradation in more detail and considering disregarded substance groups (e.g. arabinoxylan) and related enzymes remain challenging areas for research.

For breeding progress based on the Congress mash, the discrimination of the common cytolytic parameters of new barley varieties offers limited information. As a consequence, the German Brewing Barley Association (Braugersten-Gemeinschaft e.V.), organizers of the German breeding program Berliner Program, as well as the Bundessortenamt (responsible for admission and registration in the National Variety List) decided in 2012 to switch to the isothermal 65 °C mashing procedure according to MEBAK (similar to the hot water extract – EBC method).[21] Thus, the isothermal 65 °C method has become more and more established as the common laboratory mashing procedure instead of the traditional Congress mash in response to the changing barley characteristics and the economic efforts in the brewing industry.[21,28] This confirms that the influence of the chosen extraction method is fundamental for the information value of results. For the evaluation of cytolytic characteristics in barley and malt, especially in terms of the targeted breeding of new barley cultivations, only standard parameters such as viscosity, homogeneity (friability), modification and possibly also β-glucan content in the laboratory wort were analyzed in practice. Even in this broad range of cytolytic parameters, a lack of knowledge in the assessment of processability prediction with regard to the changing composition of cytolytic cell wall polysaccharides and corresponding enzymes because of breeding progress exists. Even if the standard methods used for cytolytic evaluation are in a proposed specification (see Table 3), not all effects on processing performance (lautering and filtration process) are detectable with these common methods.

In summary, various enzymes have been identified that can degrade arabinoxylan and β-glucan (see Table 2) resulting in mono-, oligo- and polysaccharides of different molar masses. Furthermore, the applied mashing procedure (temperature and mashing pH) has an impact on the extraction and degradation of these polysaccharides during malting and mashing. The multiplicity interaction of these malt cytolytic hydrolyzing factors ultimately affects wort non-starch polysaccharide composition and concomitant separation processes during the brewing process such as lautering and beer filtration.

Thus, a defined extraction method for the barley analysis with respect to more detailed information on cytolytic parameters such as arabinoxylan and β-glucan is very important. Furthermore, there is a lack of measurement

methods for the determination of arabinoxylan and its molar mass distribution in malt and wort. Given the good availability of cytolytically highly modified malts with a low β-glucan content, performance problems should occur less in terms of separation processes. Nevertheless, there are processing problems, especially during the lautering, which cannot be explained by the common malt analysis methods. Thus the aim of this paper is the investigation of a standardized extraction method for arabinoxylan and β-glucan from malt and the investigation of the impact of these polysaccharides on the lautering process. Furthermore, the impact of the related cytolytic enzymes on processing should be considered in order to obtain detailed information about the complex cytolytic system.

Experimental

Selection of barley samples and micromalting

In total, nine summer and three winter malting barley samples as well as 49 selected malting barley malt samples from the 2016 harvest (summer barley, different proveniences in Germany, Switzerland, Sweden, Denmark and Austria) were analyzed with regard to their cytolytic composition. Afterwards, a classification according to cytolytic modification characteristics of the varieties was made based on expert knowledge and with the help of the breeders' expertise by means of their cytolytic quality parameter results (friability, β-glucan and viscosity) of recent years. The distribution based on the cytolytic modification level of the investigated barley varieties resulted in 21 highly modified, eight minimally modified and 20 medium modified samples. All malt samples were malted as standard according to MEBAK R-110.00.008 [2016-03] [20] and standard malt parameters were analyzed based on the isothermal 65 °C laboratory mashing regime R-207.00.002 [2016-03] analogous to common variety evaluation in barley breeding programs. The cytolytic malt quality parameters (friability R-200.14.011 [2016-03], viscosity R-205.10.282 [2016-03], β-glucan-content R-200.26.174 [2016-03]) were measured according to MEBAK procedures. For the production of laboratory mash the malt samples were dry-milled to 0.2 mm in a laboratory mill (DLFU mill, Bühler company, Braunschweig, Germany) and ground malts were isothermally mashed (ratio 1:6) at 65 °C for 1 h according to the MEBAK procedure.[20]

Extraction method for determination of cytolytic compounds

For the extraction of cell wall polysaccharides in barley and malt, three different extraction methods were investigated. For the hot water extraction, 25 mL boiling water was mixed with 125 mg malt flour and shaken for 30 min. The samples were then centrifuged at 3500 g for 10 min and the supernatant was used for further analysis. Besides this hot water extraction, a Congress mash (fine grist 0.2 mm, ratio 1:4) as well as an isothermal 65 °C mash (fine grist 0.2 mm, grist:-water ratio 1:6, 1 h at 65 °C, addition of 50 ml of water after 30 min, weight up to 450 g) according to MEBAK R-207.00.002 [2016-03] were tested.[20]

Original gravity, real extract (EBC 9.4) and viscosity (EBC 9.38) were measured using Anton Paar DMA 4500, Alcolzyer 2000ME and Lovis 2000ME (Anton Paar, Graz, Austria) using a glass capillary viscosimeter.

Polysaccharide concentration

β-Glucan content

β-Glucan measurement was performed according to Kupetz et al.[29] using a high-throughput 96-well microplate assay in order to ensure greater sample throughput and simultaneous high measurement accuracy. Thus, fluorometric and colorimetric methods were transferred to a BioTek synergy H4 multimode plate reader (BioTek, Bad Friedrichshall, Germany). Initially, 15 µL of calibration standard (β-glucan calibration standard – DHB13, Eurofins, Vejen, Denmark) was transferred to a 96-well plate using a pipetting robot (BioTek Precision XS, BioTek Instruments, Inc., Winooski, United States) to create a seven-point calibration.

For the fluorometric assay, 300 μL of the dye solution, made from the dilution of 5 ml of Calcofluor standard solution (Sigma) with 495 mL of degassed Tris-HCl buffer (0.1 mol/L pH 8.0), was pipetted into each well of the 96-well plate.[30] Fluorescence intensity was recorded at an excitation wavelength of 360 nm and an emission wavelength of 445 nm using the BioTek synergy H4 microplate reader (BioTek Instruments, Inc., Winooski, United States). For the calculation of β-glucan content of beer samples, a second-order nonlinear regression curve was plotted between the fluorescence intensity and the β-glucan concentration. A seven-point calibration curve was generated. All measurements were performed in quadruplicate.

For the colorimetric assay, 15 μL of sample was mixed with 300 μL of Congo red solution consisting of 100 mg/L Congo red dye (C6767 Sigma, Sigma-Aldrich, Germany) dissolved in Tris-HCl buffer, pH 8.0. The Congo red dye was filtered using filter paper circles (black ribbon, Whatman Schleicher & Schuell, Dassel, Germany). Following incubation at 25 °C for 20 min, the absorbance at 550 nm was measured using the BioTek synergy H4 plate reader. The concentration was calculated using second-order nonlinear regression. All measurements were performed in quadruplicate.

Arabinoxylan content

Before the measurement of arabinoxylan in malt extracts could be performed, hexose sugars had to be separated from the wort, because of a reaction with phloroglucinol resulting in a negative absorbance difference if high contents of starch degradation products are present.[31] Thus, removal of starch was performed by means of fermentation using *Saccharomyces diastaticus*.[32]

Arabinoxylan content in malt extracts was also determined using acid hydrolysis and staining of resultant furfural residues with phloroglucinol according to the Douglas

method.[31,33] A calibration curve using xylose standard (100 mg D(+)-xylose in 100 mL distilled water) with a concentration between 0 mg/L and 0.3 mg/L in distilled water was used for quantification. Reaction reagent for acid hydrolysis consisted of 110 mL glacial acetic acid and 2 mL hydrochloric acid (fuming, 37%) as well as the dye phloroglucinol (2 g, 79330 Aldrich, Sigma-Aldrich, Germany) dissolved in 10 mL of pure ethanol. Malt extracts were diluted 1:8 and pipetted into brown test tubes. Two mL of sample or calibration standard were mixed with 10 mL reaction reagent. Samples were boiled for 25 min and immediately cooled in ice water. Measurement of absorbance was performed at 550 nm and 505 nm. Concentration was determined using two-degree polynomial regression of delta absorbance and concentration of calibration standards. These results were multiplied with dilution factor and factor 0.88 (pentose sugar) to correct the incorporation of water during hydrolysis.[34] All measurements were performed in quadruplicate. All hydrolysis and measurements were checked using an arabinoxylan standard (wheat AX medium viscosity Megazyme, Dublin, Ireland) in a defined initial weight, in two different weights.

Enzyme activity

β-Glucanase

The β-glucanase activity was determined using an MBG4 assay kit from Megazyme (Dublin, Ireland). Mixed linkage-β-glucanase cleaves 2-chloro-4-nitrophenol (CNP) from the substrate 4,6-O-benzylidene-2-chloro-4-nitrophenyl-β-(3^1-β-D-cellotriosyl-glucoside) (BCNPBG4). The rate of release of CNP relates directly to the activity of malt β-glucanase.

For the determination of malt β-glucanase, 0.5 g malt grist was weighted into centrifuge tubes. Eight mL of extraction buffer (100 mM sodium acetate, pH 4.5) was added and the samples were incubated for 15 min at room temperature (less than 30 °C). After centrifugation at 3,000 g for 5 min, 100 µL sample was pipetted into a PCR plate, where 20 µL of MBG4 substrate had already been placed. After the required incubation time of 20 min at 30 °C in a PCR cycler, the reaction was stopped with 180 mL stop reagent (Tris buffer pH 10.0). Then 200 µL aliquots of these samples were transferred to a 96-well microtiter plate and absorbance at 400 nm was measured against a blank sample.

The activity was calculated according to the formula provided by Megazyme using ε_{mM} for 2-chloro-4-nitrophenol of 12.456. All measurements were performed in quadruplicate. To test the method, a malt sample (provided by Megazyme, Dublin, Ireland) with known β-glucanase activity was included in each measurement.

Endo-xylanase

The xylanase activity was determined using an XylX6 assay kit of Megazyme (Dublin, Ireland) with a few changes. This method allows the determination of endo-xylanase activity in malt using the substrate 4,6-O-(3-ketobutylidene)-4-nitrophenyl-β-D-45-glucosyl-xylopentaoside (XylX6) in combination with a β-xylosidase. Activity of an endo-xylanase generates a non-blocked colorimetric oligosaccharide that is rapidly hydrolyzed by the ancillary β-xylosidase.[35] The rate of formation of 4-nitrophenol is therefore directly related to xylanase activity.

For the determination of malt β-xylanase, 0.5 g malt grist was weighed into centrifuge tubes and 8 mL extraction buffer (100 mM sodium acetate, pH 4.5) was added to each sample. The samples were incubated for 15 min at room temperature, with the samples vortexed every 5 min. After centrifugation at 3,000 g for 5 min, 100 µL malt extract was pipetted into a PCR plate, where 20 µL of XylX6 substrate had already been placed. After incubation for 30 min at 30 °C, 180 µL stop reagent solution (Tris buffer pH 10.0) was added into each cavity of the PCR plate. Then 200 µL aliquots of these samples were transferred to a 96-well microtiter plate and absorbance at 400 nm was measured against a blank sample.

The activity was calculated according to the formula provided by Megazyme using the ε_{mM} for 4-nitrophenol of 18.1. All measurements were performed in quadruplicate. To test the method, a malt sample (not provided) as well as a xylanase standard enzyme solution (provided by Megazyme, Dublin, Ireland) with known xylanase activity was included in each measurement.

Arabinofuranosidase

This method allows the determination of arabinofuranosidase activity in malt using the substrate p-nitrophenyl-a-L-arabinofuranosidase (pnp-Ara, Megazyme, Ireland, Lot 150301).

For the determination of malt arabinofuranosidase activity, 0.5 g malt grist was weighed into centrifuge tubes and 8 mL extraction buffer (100 mM sodium acetate, pH 5.0) was added to each sample. The samples were incubated for 15 min at room temperature. After centrifugation at 3,000 g for 10 min, 10 µL malt extract was pipetted into a PCR plate, where 100 µL of substrate (100 mM sodium acetate, 10 mM pnp-Ara, pH 5,0) had already been placed. After the required incubation time of 240 min at 40 °C in the PCR cycler, the reaction was stopped by pipetting 20 µL sample and substrate mixture into 190 µL stop buffer reagent (Tris buffer pH 10.0). Absorbance at 400 nm was measured against a blank sample.

The activity was calculated according to the formula provided by Megazyme using the ε_{mM} for 4-nitrophenol of 18.1. All measurements were performed in quadruplicate. To test the method, a malt sample (not provided) as well as an arabinofuranosidase standard enzyme solution (purchased by Megazyme, Dublin, Ireland) with known arabinofuranosidase activity was included in each measurement.

Statistics

Statistical analyses, to determine Pearson correlation coefficients, averages and standard deviations as well as principal component analysis, were carried out using OriginPro 2018 G (OriginLab Cooperation, Northampton, USA).

Table 4. Impact of extraction method on the content of β-glucan and arabinoxylan; legend: n.d. – not detectable, AX – arabinoxylan content (n = 4), β-Glc – β-glucan content, fluorimetric (n = 4).

Sample ID	Hot water extraction (barley)		Hot water extraction (malt)		Congress mash (malt)		Isothermal 65 °C mash (malt)	
	β-Glc [mg/L]	AX [mg/L]	β-Glc [mg/L]	AX [mg/L]	β-Glc [mg/L]	AX [mg/L]	β-Glc [mg/L]	AX [mg/L]
1	n.d.	239	n.d.	823	95	918	278	922
2	231	299	n.d.	610	n.d.	690	n.d.	1104
3	287	322	n.d.	637	n.d.	815	16	1485
4	449	281	n.d.	645	n.d.	1180	23	1375
5	n.d.	322	n.d.	771	160	1297	548	1078
6	246	197	n.d.	654	111	584	462	821
7	376	226	n.d.	725	106	581	307	1291
8	658	331	n.d.	990	80	1135	501	798
9	401	386	n.d.	1000	147	1842	210	1375
10	849	286	n.d.	940	85	1381	240	1319
11	n.d.	217	n.d.	797	54	1167	97	1265
12	47	224	n.d.	775	197	848	282	1397

Results

Since barley has a complex cytolytic composition, and β-glucan and arabinoxylans can impact the beer production process, a detailed study of these components, not only in the context of breeders' programs, is of particular interest. Especially the compound β-glucan is of high interest, encompassing the complete barley supply chain. However, compared to β-glucan, arabinoxylan has largely been ignored. This was not the least because of insufficient measurement methods for the quantification of arabinoxylan and a minor influence reported in the literature.[36] After some process performance problems in recent years (e.g. lautering and filtration difficulties) of modern highly modified malting barley varieties, even with low β-glucan content and good cytolytic quality parameters (e.g. viscosity, friability), arabinoxylan is again coming into the focus of research. Even the use of exogenous enzyme preparations (technical β-glucanases) in the processing of these barley malt varieties outside the German Purity Law resulted in no performance increase.

For this reason, the cytolytic composition of different common malting barley malt samples should be characterized more significantly and informatively through the application of various extraction methods for the examination of specific cell wall polysaccharides. Subsequently, the evaluation of the modification level of the investigated samples depends on the extraction method and variety characteristics. The intention is to define an analytical application for water-soluble cell wall polysaccharide analysis with high information value for the cytolytic discrimination of different malting barley varieties. Afterwards, in the second part of this study, the influence of the specific cell wall polysaccharides on the processability, especially the lautering process, is examined in more detail.

Besides a measurement directly in malt grist (total amount), an extraction could be performed in order to analyze the water-soluble fraction of the polysaccharides. Depending on the used extraction method and tested barley varieties some authors defined different fractions of these cell wall polysaccharides, in particular arabinoxylan and β-glucans.[37,38]

Thus, different extraction procedures (including established laboratory mashing schemes) were tested to investigate the impact of the method on the water-soluble content of arabinoxylan and β-glucan of the 12 barley samples and corresponding malt samples. Besides a hot water extraction, a standardized Congress and isothermal 65 °C mash were applied. These three methods have the advantage that they work with different temperatures and thus the specific cytolytic enzymes are active or bypassed (see Table 4). The hot water extraction of the barley samples resulted in an average β-glucan content of 295 mg/L ($n = 12$) and an average arabinoxylan content of 278 mg/L ($n = 12$). In the corresponding malt samples no β-glucan could be determined, but total arabinoxylan ranged from 610–1000 mg/L (average: 781 mg/L, $n = 12$). In contrast, Congress mash resulted in an average β-glucan content of 86 mg/L and arabinoxylan content of 1037 mg/L ($n = 12$). The isothermal 65 °C mash had the highest concentrations in β-glucan 247 mg/L ($n = 12$) and arabinoxylan 1,186 mg/L ($n = 12$). Since β-glucan content was not measurable in hot water extract and arabinoxylan content was low, this method was not further considered, even if an advantage of fast screening with this method is possible due to shorter extraction times.

Finally, the results were evaluated statistically to find out whether β-glucan and arabinoxylan content were related to each other depending on the extraction method (Table 5). Since the degrading cytolytic enzymes are not taken into account in the isothermal 65 °C mash (see Table 5), these results indicate a connection between the composition of β-glucan and arabinoxylan in barley malt depending on the cytolytic structure of the barley variety. This could indicate a different composition of cytolytic substances depending on the barley variety based on the genetically determined modification level (combination of cell wall structure and enzymatic activity/potential).

In order to investigate the impact of endogenous cytolytic enzymes on the degradation products and to derive variety differences, 49 malt samples were mashed with the Congress as well as isothermal laboratory mashing procedure and the content of water-soluble polysaccharides was investigated. Arabinoxylan (see Figure 3a) ranged between 798 and 1690 mg/L in the isothermal mash with an average of 1182 mg/L ($n = 46$) and between 451 and 1842 mg/L ($n = 46$) in the Congress mash (average: 1081 mg/L). The broader range of arabinoxylan concentrations shows the significant influence of arabinoxylan-degrading enzymes during the Congress mash due to a 45 °C rest. A comparable result was found for the β-glucan concentrations. Using the

Table 5. Pearson correlation coefficients determined using the data of Table 4 (n = 12) to determine the best extraction procedure for cytolytic malt characteristics.

Wort characteristic	Compared extraction procedure (raw material)	Correlation coefficient, P-value
Arabinoxylan	Hot water extraction (malt)/ Congress mash (malt)	r = 0.719, P < 0.05
	Hot water extraction (barley)/ Congress mash (malt)	r = 0.670, P < 0.05
β-Glucan	Isothermal 65 °C mash (malt)/ Congress mash (malt)	r = 0.701, P < 0.05
Compared wort characteristic	**Mashing procedure (raw material)**	**Correlation coefficient, P-value**
Arabinoxylan/ β-glucan	Isothermal 65 °C mash (malt)	r=-0.640, P < 0.05

fluorimetric method, β-glucan content (see Figure 3a) in the isothermal mash ranged between <5 and 710 mg/L (average: 240 mg/L, $n = 46$) and in the Congress wort between <5 and 293 mg/L with an average of 89 mg/L ($n = 46$). The colorimetric β-glucan method resulted in much lower contents of <5–349 mg/L with an average of 59 mg/L in the isothermal mash and <5–171 mg/L (average: 22 mg/L, $n = 46$) in the Congress mash. Corresponding to the cell wall polysaccharide concentration, higher viscosities (see Figure 3b) were observed in the isothermal mashes (1.367–1.585 mPa × s, average: 1.464 mPa × s, $n = 46$) than in the Congress mashes (1.366–1.478 mPa × s, average: 1.421 mPa × s, $n = 46$).

The statistical evaluation is shown in Table 6. It was striking that the arabinoxylan and β-glucan (fluorimetric) content in the isothermal mash again correlated negatively. The viscosity and β-glucan content correlated with both the Congress mash and the isothermal mash. This shows that just the water-soluble β-glucan fraction has an impact on the malt quality parameter viscosity. In contrast to the correlations often described in the literature,[34, 39, 40] no correlation between water-soluble arabinoxylan content and viscosity could be determined. This is consistent with the findings of Cach and Annemüller[14] as well as Burberg et al.[41]

In addition, the data were evaluated on the basis of the classification in modification level of the respective varieties. In the isothermal mash, the highly modified barley malt samples had an average β-glucan content of 176 mg/L (range: 0–710 mg/L), which was the lowest value within the three groups and was expected due to the classification type. In contrast, arabinoxylan was with 1254 mg/L (range: 798–1690 mg/L) the highest average in the groups. Independently, the viscosity reached a mean value of 1.445 mPa × s, which was the lowest value and confirms the correlation to water-soluble β-glucan content. In comparison, medium modified barley malt samples had an average β-glucan of 268 mg/L (range: 49–546 mg/L) and arabinoxylan content of 1141 mg/L (range: 810–1682 mg/L). Despite the lowest average concentration in arabinoxylan, the highest viscosity was determined in these malt samples (1.478 mPa × s with a range between 1.404 and 1.585 mPa × s). The minimally modified barley malt samples contained the highest average β-glucan content of 280 mg/L (range: 94–548 mg/L). Arabinoxylan (1202 mg/L, range: 964–1498 mg/L) and viscosity (1.462 mPa × s, range: 1.414–1.542 mPa × s) showed values between the highly and medium modified barley varieties.

The results of the extraction methods were comparable, although lower levels were generally found in Congress wort. Likewise, the lowest mean β-glucan content (60 mg/L, range: 0–294 mg/L) and highest arabinoxylan content (1,081 mg/L, range: 660–1431 mg/L) in the cytolytically highly modified barley varieties could be measured. This resulted in the lowest average viscosity of 1.415 mPa × s. The medium modified samples had the lowest arabinoxylan content (942 mg/L, range: 451–1555 mg/L), but the highest viscosity (1.423 mPa × s, range: 1.377–1.469 mPa × s). The β-glucan content ranged from <5–236 mg/L with an average of 94 mg/L. Minimally modified barley malt samples had the highest average β-glucan value (127 mg/L, range: 0–291 mg/L), but a medium arabinoxylan content (1,240 mg/L, range: 848–1842 mg/L) as well as viscosity (1.427 mPa × s, range: 1.366–1.478 mPa × s).

Thus, these results confirm the classification of the varieties according to their modification characteristics, since β-glucan and viscosity function as cytolytic quality criteria. Nevertheless, the findings highlight that highly modified malt samples had the highest water-soluble arabinoxylan content, which certainly may affect the brewing process. In addition, it could be shown that the solubility of the cell wall polysaccharides (concentration of water-soluble fraction) depends strongly on the extraction temperature during the mashing procedure. As different cytolytic enzyme activities exist in the selected extraction methods, they strongly influence the concentration of the resulting cell wall polysaccharide. Moreover, Mangan et al.[35] described a correlation between cytolytic enzyme activity and viscosity of the wort. For this reason, the specific cytolytic enzyme activity of the barley malts was the focus of this work. Figure 4 shows the distribution of β-glucanase, endo-xylanase and arabinofuranosidase activity in the investigated malt samples.

Average β-glucanase activity was detected with 790 U/kg in a range between 233 and 1,243 U/kg. Arabinoxylan degrading enzymes endo-xylanase (range: 21–64 U/kg) and arabinofuranosidase activity (range: 62–274 U/kg) had an average of 38 U/kg and 165 U/kg, respectively. These activities are consistent with the values reported in the literature.[35,42,43] Comparing these results to the malt characteristics achieved a low significant correlation ($r = -0.324$, $P < 0.05$) between total water-soluble arabinoxylan content in isothermal mash and xylanase activity. The viscosity in the isothermal mash correlated only with the arabinofuranosidase activity ($r = 0.307$, $P < 0.05$). This shows that the viscosity increases with a higher arabinofuranosidase activity and the resulting debranching due to a lower number of arabinose side chains on the arabinoxylan molecule. However, high arabinofuranosidase activity also makes the xylan backbone more susceptible to depolymerization by xylanase.[44,45] Thus, the low residual activity of xylanase (see Table 2) due to the high mashing temperature could cause an increase in water-soluble arabinoxylan content. This may also contribute to the xylan-solubilase and its

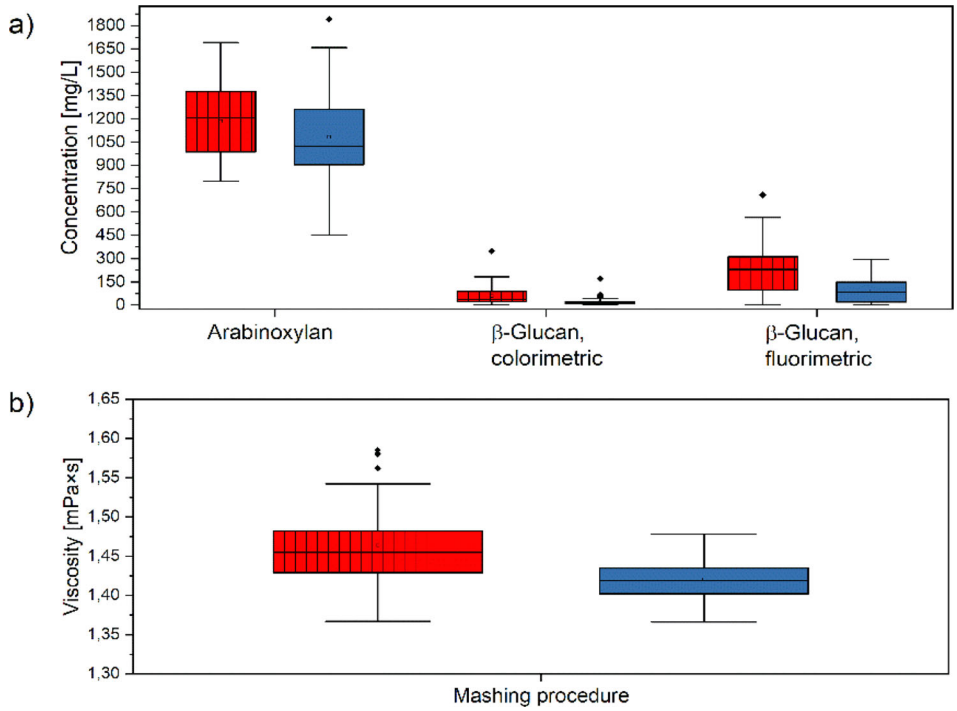

Figure 3. Arabinoxylan (n = 4), colorimetric and fluorimetric β-glucan content (n = 4) in isothermal 65 °C mash and Congress mash (a) as well as corresponding viscosity (b) (n = 49); legend: red box with stripes – isothermal 65 °C mashing procedure, blue box – Congress mashing procedure.

Table 6. Pearson correlation coefficients determined using the data of Figure 3 and Figure 4 (n = 49) to determine the best extraction procedure for cytolytic malt characteristics.

Wort characteristic	**Compared mashing procedure**	**Correlation coefficient, P-value**
β-glucan, colorimetric	Isothermal 65 °C mash/ Congress mash	r = 0.797, P < 0.05
β-glucan, fluorimetric	Isothermal 65 °C mash/ Congress mash	r = 0.572, P < 0.05
Viscosity	Isothermal 65 °C mash/ Congress mash	r = 0.588, P < 0.05
Compared wort/ malt characteristics	**Mashing procedure**	**Correlation coefficient, P-value**
Arabinoxylan/ β-glucan, fluorimetric	Isothermal 65 °C mash	r=-0.413, P < 0.05
Arabinoxylan/ xylanase activity	Isothermal 65 °C mash	r=-0.324, P < 0.05
Viscosity/ β-glucan, fluorimetric	Isothermal 65 °C mash	r = 0.712, P < 0.05
Viscosity/ β-glucan, fluorimetric	Congress mash	r = 0.636, P < 0.05
Viscosity/ β-glucan, colorimetric	Isothermal 65 °C mash	r = 0.632, P < 0.05
Viscosity/ β-glucan, colorimetric	Congress mash	r = 0.530, P < 0.05
Viscosity/ arabinofuranosidase activity	Isothermal 65 °C mash	r = 0.307, P < 0.05

higher temperature optimum, similar to the β-glucan-solubilase. However, there is no profound information about this enzyme in the literature.

Furthermore, viscosity in the Congress mash and β-glucanase activity correlated negatively, with $r = -0.316$, $P < 0.05$. During the Congress mashing process, a large amount of β-glucan is dissolved and hydrolyzed to low molecular weight β-glucan during the 45 °C rest, due to the optimal temperature of the β-glucanase. However, the water-soluble β-glucan has a greater influence on the total viscosity of the wort than arabinoxylan.

The evaluation according to the specific barley modification characteristics based on the classification only resulted in slight differences in average enzyme activity. Highly modified malt samples had an average β-glucanase activity of 746 U/kg, medium modified samples 810 U/kg and minimally modified samples 842 U/kg (xylanase activity: 38 U/kg, 38 U/kg and 40 U/kg, arabinofuranosidase activity: 164 U/kg, 167 U/kg and 168 U/kg, respectively). The low β-glucanase activity of the cytolytically highly modified varieties is consistent with the lower measurable β-glucans in the wort. Furthermore, the results may indicate that cytolytic enzyme activity is less influenced by variety characteristics but by growth conditions and the resulting cell wall structure of the substrate.[46]

In order to elaborate the correlation between barley malt variety characteristics (classification) and specific cytolytic malt quality parameters (analytical specifications such as viscosity, arabinoxylan, β-glucan) analyzed in the Congress wort and isothermal 65 °C wort, a principal component analysis was carried out (not taking into account the enzyme activities, since these showed only slight differences between the varieties). The intention was to find a maximum differentiation in terms of cytolytic characteristics of barley varieties based on an extraction procedure with highly informative value. It was found that the Congress wort was unsuitable for further differentiation of the cytolytic characteristics, since the cytolytic enzymes strongly hydrolyzed the cell wall polysaccharides during the 45 °C rest and thus no differences in the composition were visible for

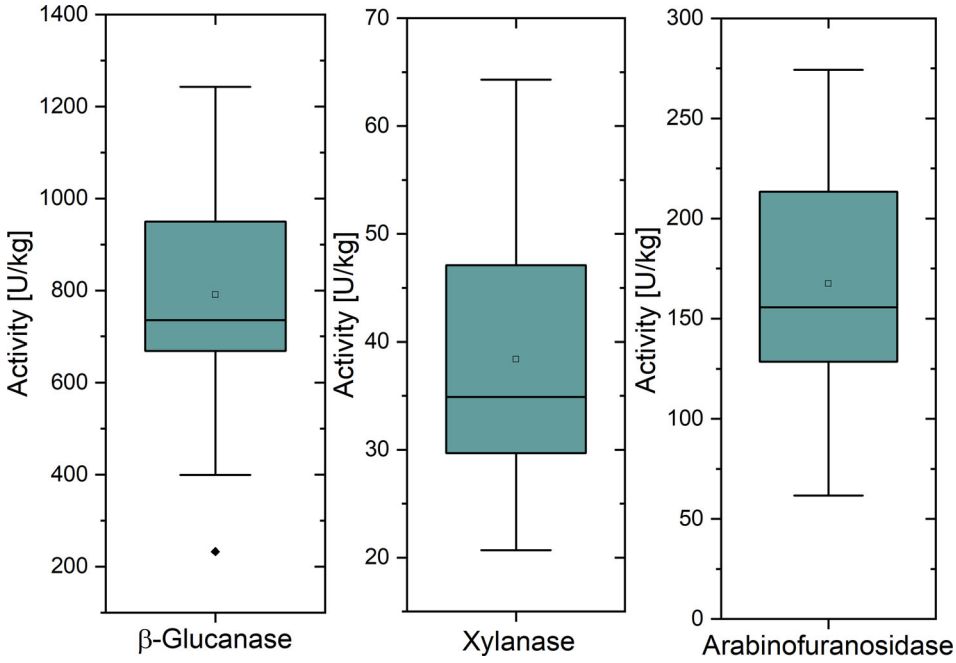

Figure 4. β-Glucanase (n = 4), endo-xylanase (n = 4) and arabinofuranosidase activity (n = 4) of the investigated malt samples (n = 49).

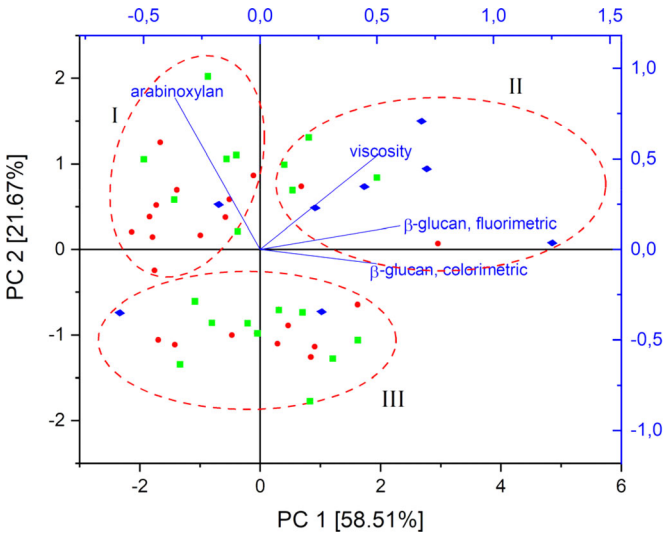

Figure 5. Principal component analysis: specific cytolytic malt parameters and their impact on barley malt modification characteristics (n = 49). Classification: I: mostly highly modified barley varieties, II: mostly minimally modified barley varieties, III: mostly medium modified barley varieties; legend: ● highly modified malt samples, ■ medium modified malt samples, ◆ minimally modified malt samples.

discrimination. This was also confirmed by the results of the PCA (data not shown). In contrast, the results of the isothermal mash cytolytic parameters, shown in the PCA biplot in Figure 5, indicate distinct variety differences depending on the cytolytic modification. While the first component is mainly defined by β-glucan and viscosity (almost 59%), the second component (almost 22%) is dominated by arabinoxylan. Furthermore, the figure displays the distribution of the samples versus the cytolytic characteristics (classifications). Three clusters of samples could be found in the biplot. The first cluster is dominated by highly modified malt samples, with 52% of all highly modified samples inside this cluster. Sixty-three percent of minimally modified samples can be found in cluster II and 50% of medium modified samples in cluster III. Since cluster I is mainly defined by the second component, the cytolytic composition of new highly modified malting barley varieties is mainly impacted by arabinoxylan, which is consistent with the high concentrations found in comparison to the other barley varieties. The second cluster was mostly defined by β-glucan content (fluorimetric) and viscosity, which agrees with the high β-glucan content of the minimally modified barley samples. Cluster III was affected by high molar mass β-glucan, determined with colorimetric assay.[29,47]

In conclusion, these results verify that barley breeding programs with a focus on cytolytic modification significantly affect structural changes of the barley cell wall (hemicellulose of endosperm) and in consequence influence the resulting malt quality parameters, especially based on the non-starch polysaccharides (degradation products). Since highly modified samples had a very low β-glucan content, the cell wall seems to consist mainly of arabinoxylan. In addition, slightly lower β-glucanase activity could be detected in these varieties. Medium modified samples achieved the highest viscosity, but the lowest arabinoxylan contents. Minimally modified samples were affected by both β-glucan and arabinoxylan, whereas in the isothermal 65 °C mash the viscosity was lower than in the medium modified wort samples.

Discussion

Due to progresses in breeding in recent years, not only large increases in yield but also an improvement in the brewing quality criteria (e.g. extract) of barley have been achieved.[48] In addition to these required quality specifications, the processability of barley and barley malt in the malting and brewing process has become increasingly important. That means

mainly the reduction of critical parameters to guarantee good processability, whereby particularly the cytolytic modification was a limiting factor. The main focus has been on the decrease of the water-soluble β-glucan in wort, since these polysaccharides are known to have a negative impact on beer viscosity and filtration performance.[49] However, in practice, some of these new malting barley varieties of high quality did not show improvements in lautering performance, with increased wort turbidity and lower flux. For this reason, the differences between the barley varieties with various cytolytic modification characteristics were examined in this work.

The extraction methods used showed significant differences in the total concentration of the endosperm cell wall polysaccharides. Especially the β-glucan concentrations were low when using a Congress mashing procedure that included a rest at 45 °C. As a consequence, the differences between the barley varieties applying this extraction method were very low.

Various authors have described mashing processes at high temperatures to extract β-glucan.[28,50–53] Evans et al.[50] noted that β-glucan content was relatively stable when mashing above 62.5 °C. However, these results are not comparable, since different grist:water ratios and slightly different extraction procedures were used, despite the same mashing temperature.[17,54] This is probably influenced by the inactivation of β-glucan-degrading enzymes and an optimum temperature of β-glucan-solubilase (see Table 2) as well as an accessibility and solubility improvement of β-glucan due to the changes in malt caused by starch gelatinization.[7] The activity of β-glucan-solubilases at higher mashing temperatures (above 62 °C) results in high concentrations of β-glucan, but due to an inactivation of β-glucanases at these temperatures, no further degradation in molar mass can occur.[51] Thus, it is not only possible to analyze the proportion of soluble β-glucan (ratio of two effects: degradation to low molecular β-glucan and solubility of high molecular β-glucan) using an extraction method with higher mashing-in temperature, but also the activity of solubilase can be estimated. Furthermore, to obtain reliable information about malt quality, malt composition and expected processability, mashing procedures close to the technological processes of modern brewhouses in practice (modern infusion processes) are more suitable. In addition, the high temperature promotes the solubility of the polysaccharide as an important cause of the gelatinization of starch (amylolytic enzyme rest). Considering detailed information on cytolytic composition, the results of the isothermal 65 °C mash achieved significant differences between the varieties with different modification levels. Although the highly modified varieties had a low β-glucan content and viscosity, these varieties had a higher water-soluble arabinoxylan content. Li et al.[55] achieved the highest arabinoxylan content using an isothermal mash at 80 °C, whereas mashing at 40 °C resulted in the degradation of arabinoxylan. To what extent the xylan solubilase participates in the extraction at these temperature ranges cannot be determined from the literature. However, an influence is also suspected here.

Enzyme activity observed only slight variations between the varieties with different cytolytic modification levels. Only the β-glucanase activity showed a greater difference. Lower activity is associated with lower β-glucan concentrations in the wort in highly modified barley varieties. Various authors show different measuring ranges for the enzyme activities, although these are difficult to compare due to other substrates or raw material-based influencing factors (variety, provenance).[17,54]

In summary it could be shown that newly bred cytolytically highly modified varieties have a lower β-glucan content but, compared to other varieties, a significant increase in arabinoxylan content, which is the dominant factor in processing. These differences can be represented by the selected practical isothermal 65 °C laboratory mashing procedure. Due to the small differences between the cytolytic enzymes, no information on variety characteristics could be gained. To what extent these variations in the cytolytic composition affect separation processes (especially lautering) will be clarified in part two of this paper.

Conclusion and outlook

The results show a great impact of the extraction procedure on the concentration of the cytolytic polysaccharides. Depending on the respective hydrolyzing enzymes, different information is obtained for the evaluation of the malting barley. The isothermal 65 °C mashing process is particularly suitable because of its practical approach to modern brewing processes. In addition, it was found that due to the high mashing temperature, variety characteristics can be clearly identified. Hot water extraction or the Congress mashing procedure, however, did not provide sufficient information to distinguish these characteristics. The Congress mash does not allow enough time for an adequate β-glucan solubilase rest to be able to show specific variety characteristics. Thus, variation in cytolytic modification among different lots of malt cannot be sufficiently characterized by means of the Congress mash method.

By using the isothermal 65 °C mashing process, it has been shown that cytolytically highly modified malting barley varieties have low β-glucan concentrations but a high arabinoxylan concentration. Since the breeding of these varieties aimed at a low β-glucan content, this variety property could be proven. The aim was trouble-free processing in the brewhouse and during beer filtration. However, some of these varieties tend to have low lautering performance. To what extent this depends on the particular cytolytic composition will be shown in the second part of this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

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