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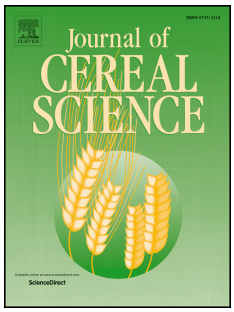

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Barley genotypic β-glucan variation combined with enzymatic modifications direct its potential as a natural ingredient in a high fiber extract

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ABSTRACT

β-Glucan was extracted from eight different barley genotypes varying in β-glucan content and molecular structure using Termamyl® SC (T), Attenuzyme® (A) and Attenuzyme® Flex (AF) amylolytic enzymes in combinations. Extracts from barley lines Lys5f, KVL408, KVL1104 and CDC Fibar exceeded 4 g β-glucan/l, providing European Food Safety Authority (EFSA) and U.S. Food and Drug Administration (FDA) recommended amounts (3 g β-glucan/day) from three portions. TAF extracts of Lys5f and KVL408 grains reached extraordinary high concentrations of 8-9 g β-glucan/l. The β-glucan molecular mass decreased with enzyme treatment T < TA < TAF due to minor lichenase side activity. Extractability was generally higher and molecular mass lower for barley lines low in triosyl/tetraosyl (DP3/DP4) ratios than for those high in DP3/DP4 ratios (Lys5f, KVL408 and KVL1104). Overall, the higher β-glucan content and structural robustness in Lys5f and KVL408 raw materials favor these in a β-glucan rich extract with potential for EFSA and FDA health and nutrition claims.

Keywords

Barley beta-glucans; Extractability; Molecular structure; Enzymatic hydrolysis

1. Introduction

The human population at large fails to consume adequate amounts of dietary fiber. Recently, renewed interest in barley as a food grain has evolved due to its high content of physiologically active soluble fibers, especially mixed linkage (1→3, 1→4) β-glucan (BG).

BG dietary fibers have a remarkable range of health benefits including the promotion of heart health, stabilization of blood glucose levels, stimulation of immune responses, satiety increase and maintenance of body weight (El et al., 2012; Mikkelsen et al., 2014; Wood, 2010). Scientific evidence has led to the approval of barley BG with health claims by the United States Food and Drug Administration (FDA, 2005) and the European Food Safety Authority (EFSA). EFSA has authorized health claims for the ability of barley BG to maintain or reduce blood cholesterol levels and reduce post-prandial glycemic responses (Harland, 2014). Furthermore, barley grain fibers are approved by EFSA for contributing to an increase in fecal bulk, which relates to gut health. The recommended intake of BG is minimum 3 g per day and the health claims allow food producers to label products containing at least 1 g BG per serving. In addition to the content of BG in the products, also the physico-chemical properties of BG, which may be dependent on combinations of molecular mass, solubility and viscosity, are essential for providing the health effects (Mikkelsen et al., 2014; Wood, 2010). BG depolymerization, as might be imparted by food processing, typically leads to reduction in BG viscosity. However, low molecular mass BGs have shown to form gel like structures more readily than high molecular mass BGs (Wood et al., 2010), and low molecular mass BGs might be physiologically as effective as those with high molecular mass provided that the concentration or active dose estimated by increased releasability is sufficiently high (Frank et al., 2004; Naumann et al., 2006). Kerckhoffs et al. (2003) found that beneficial physiological effects of BGs can be decreased when incorporated into solid foods such as bread and cookies. Liquid matrices, such as beverages and soups, may therefore be preferable for inducing health effects from BG meals as the liquid state allows hydration and release of the BG before ingestion. Liquids

containing high levels of BGs are typically very viscous, which renders them less attractive as beverages. Hence, there is an unmet need for methods and materials for preparing appealing and functional extracts with high content of natural BG and there is sparse evidence of how combined processing and genotypic variation in barley BG structure affect its major physical and potential health beneficial properties. In the present study, we evaluate the extractability and quality of BG liquid formulations from eight different barley lines using mashing protocols and selected combinations of amylolytic enzymes.

In barley grains, BG is located in pericarp, scutellum, aleurone layer and starchy endosperm as a cell wall component (Dornez et al., 2011). BG is a structural polysaccharide, but it also provides glucose during grain germination (Burton et al., 2010). Barley is a genetically diverse cereal crop and it is classified as spring or winter type, two- or six-rowed, hulled or hull-less, and malting or feed by the end-use type. Based on grain composition, barley can be further classified as normal, waxy or high amylose starch types, high lysine and high BG. De-hulling and pearling of barley grains reduces the contents of insoluble fiber, protein, ash and free lipids from the outer layers including the hull (palea and lemma), bran (pericarp, testa, aleurone) and germ (embryo), and increases the content of starch and soluble BG fiber originating from the endosperm (Baik and Ullrich, 2008). BG content in barley grains typically ranges from 2.5% to 12% by weight (Izydorczyk and Dexter, 2008), but extreme levels of 15-20% have been reported for the high BG/low starch mutant line Lys5f (Munck et al., 2004). This mutant line along with its barley mother line, Bomi, was included in the present study.

BGs are comprised of glucose units connected by β (1→4)-linkages (~70%) and β (1→3)-linkages (~30%) in a linear manner (Mikkelsen et al., 2010). Blocks of β (1→4)-linked sequences, with cellotriose and cellotetraose units constituting ~90% of the molecule, are separated by single β (1→3)-linkages (Burton et al., 2010). The molar ratio of the cellotriosyl to cellotetraosyl units with degree of polymerization (DP) of 3 to 4, respectively, is referred to as the DP3/DP4 ratio and is

considered as a fingerprint of the individual BGs from various barley lines and tissues. Typically, small amounts (~10%) of cellulosic oligosaccharides with DP5-15 are also present (Woodward et al., 1988). DP3/DP4 ratios of barley BGs have generally been reported in the range of 1.8-3.5. Lower ratios are typically found in the endosperm (2.7-3.2) tissue compared to pericarp (3.4-4.2) and aleurone (3.8-4.1) outer layers of the barley grain (Izydorczyk and Dexter, 2008). Both, linear regions of repeated units of cellotriosyl or cellotetraosyl, as well as the long cellulosic oligomer blocks, have been suggested to decrease BG solubility due to chain alignment and aggregation (Burton et al., 2010; Woodward et al., 1988). Thus, the DP3/DP4 ratio provides an indication of solubility and BGs with ratios close to 1:1 are found to be more soluble than BGs with either very high or very low DP3/DP4 ratios having longer stretches with repetitive structures (Burton et al., 2010). Barley BGs typically comprise more than 1000 glucosyl residues and reported molecular mass values range 130 to 2,500 kDa. The large variations reflect the diversity of genotypical botanic origin, but also result from the methodology of extraction and molecular mass determination (Irakli et al., 2004).

A wide range of laboratory and pilot scale BG extraction protocols have been reported (Benito-Roman et al., 2011; Benito-Roman et al., 2014; Limberger-Bayer et al., 2014; Mikkelsen et al., 2013; Wood, 2010). In brief, they involve milling, inactivation of endogenous hydrolytic enzymes, extraction with hot water or alkaline solutions, removal of starch and protein using hydrolytic enzymes and/or centrifugation, recovery of BG from the extract by ethanol precipitation or cryogellation cycles and drying of the BG gum. The extractability of BG is influenced by process parameters such as fineness of grind, temperature, ionic strength and pH of the solvent (Mikkelsen et al., 2013). β-Glucanase activity and mechanical damage during extraction have been reported to cause BG depolymerization which modify the BG molecular mass, extraction yield and rheological behavior (Wood, 2010). Thus, valid evaluation of BG yield and quality from different barley genotypes requires identical and standardized extraction conditions. Depending on the method used,

the extractability of barley BGs can vary between 33% and 87% (Izydorczyk and Dexter, 2008). Benito-Román et al. (2011) found BG extraction yields of ~73% and ~62% for hulled and hulless barley, respectively. Amylolytic enzymes are widely used for the degradation of starch in BG extraction (Benito-Roman et al., 2014; Doehlert et al., 2012). Following thermal gelatinization the starch is hydrolyzed into maltooligosaccharides, maltose and glucose by the action of endo- and exo-glucanases such as α- and β-amylases, glucoamylase and debranching pullulanase. α-Amylase (EC 3.2.1.1) and pullulanase (EC 3.2.1.41) act *endo* in a pseudo random mode in the amylose and amylopectin polymer chains of starch and hydrolyze (1→4)-α-D-glycosidic and (1→6)-α-D-glycosidic linkages, respectively. At the non-reducing end of the starch polymer chains, β-Amylase (EC 3.2.1.2) hydrolyses (1→4)-α-D-glycosidic to liberate successive maltose, whereas glucoamylase (EC 3.2.1.3) hydrolyses both (1→4) and (1→6)-α-glycosidic linkages and produces glucose (van Oort, 2010). For complete and efficient starch degradation, enzyme preparations with specific and diverse functionalities are warranted. However, introducing more enzymes to a process increases the risk of hydrolytic side-, or contaminating activities on BGs. Few studies using enzymes as part of their BG isolation have investigated, if the amylolytic enzymes had any effect on the BG itself (Benito-Roman et al., 2014; Doehlert et al., 2012). In the present study we screen widely different barley genotypes for BG extraction yields. A hydrothermal, mechanical enzyme-assisted protocol was optimized and we assess the risk of BG degradation by trace contaminating β-glucanase side activity in commercial bulk enzyme preparations.

2. Materials and methods

2.1. Raw materials, experimental design and data analysis

Grains from eight two-rowed spring barley genotypes having vastly different BG content were included in this work (Fig. 1). The grains of KVL408, KVL1104, CDC Fibar cv., Chameleon cv. and Columbus cv. were provided by Carlsberg Research Laboratory (Denmark) whereas Lys5f and Bomi cv. were from the University of Copenhagen (Denmark). The Lys5f, KVL1104 and KVL408 barley genotypes derive from a larger collection of barley mutants where KVL408 have been mutated in Perga and Lys5f in Bomi standard malt barley (Di Fonzo and Stanca, 1977). KVL1104 derives from the crossing of Lys5f and Bomi. CDC Fibar is a high fiber, 0% amylose hulless barley registered in Canada. Chameleon is a hulless and Columbus a hulled standard malt barley registered in Denmark. Pearling of Columbus was performed on a vertical polishing BSPB (Bühler AG, Switzerland) with pearling 11% of husk. In total, 48 samples were prepared from mashing grains from the eight barley lines with three different enzyme combinations in replicate. Raw grains were investigated for BG content and BG spatial distribution in the grain. BG extracts were analyzed for viscosity, BG yield and molecular mass. Ethanol precipitated BGs from extracts were analyzed for DP3/DP4 ratios. Commercial amylolytic enzymes were examined for BG hydrolytic side activity (Fig. 1).

Data were mined by multivariate data analysis using principal component analysis (PCA) to visualize trends related to genotype, molecular structure and processing. PCA captures the major variation in a data set in a model ($X = T \cdot P' + E$) where the two-dimensional data matrix (X) containing information about samples and variables is decomposed into systematic variation (T and P′) and noise (E). The systematic variation is described by the calculated principal components (PCs) that represent the outer product of scores (containing information about the samples) and loadings (containing information about the variables) (Wold et al., 1987). PCA on the physico-

chemical properties of the 48 BG extracts was performed using Latentix software (LatentiX™ 2.12, Latent5, Copenhagen, Denmark, www.latentix.com). Data were auto-scaled prior to analysis.

2.2. Microscopy

Barley grains were trimmed with a razor blade to aid diffusion of agents, fixed for 24 h in Karnovsky's fixative (5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate buffer at pH 7.3) including a 1 h vacuum treatment, and subsequently washed in cacodylate buffer and water. Samples were dehydrated in a graded acetone series, infiltrated with increasing ratios of Spurr resin:acetone and embedded in Spurr resin within flat molds. The resin was polymerized in an oven at 60°C for 8 h. Semi-thin sections of 2 µm were cut with a histo-diamond knife on a Reichert-Jung supernova ultramicrotome and stained for BGs with 0.01% (w/v) Calcofluor White M2R (Sigma-Aldrich) and imaged with UV excitation and blue emission. Other sections were stained with Periodic Acid Sciffs's (PAS) and counterstained with Amido Black (AB). All images, apart from the overview images, were taken from the central part of the starchy endosperm tissue. Sections were viewed in a Nikon Eclipse 80i light and fluorescence microscope and images were recorded with 4 x dry objective or 100 x objective using immersion oil. Final image processing, cropping and mounting of the images were done with Adobe (San Jose, CA, US) Photoshop CS2 and Illustrator CS2.

2.3. Extraction and enzymatic treatment

BG extraction yield was optimized in a mashing pre-study with focus on milling particle size, solvent:flour ratio, pH, temperature and extraction time using the Lys5f line as model. Enzymes were dosed as suggested by the manufacturer (1.5% w/w, enzyme/flour). Production of high BG wort was performed using a Lochner electronic mashing device with 8 beakers. Barley grains were milled immediately before mashing with an EBC mill adjusted to 0.5 mm. The water:flour ratio was

1:15 per weight equivalent to 27 g barley flour mashed-in with 400 ml standard brewing water in a 500 ml metal beaker and the pH was adjusted to 5.5 with phosphoric acid. To facilitate starch hydrolysis three different enzyme treatments were used: Termamyl® SC containing thermostable α-mylase (**T**), a combination of Termamyl® SC and Attenuzyme®, the latter containing glucoamylase (**TA**) or a combination of Termamyl® SC and Attenuzyme® Flex, the latter containing glucoamylase and pullulanase (**TAF**). All enzymes were purchased from Novozymes A/S, Denmark. Following 45 min incubation at 65°C, the temperature was linearly increased to 90°C for 25 min, and finally kept at 90°C for 30 minutes. The mash was centrifuged at 3500 rpm for 10 min to remove insoluble spent grain material. From the 340 ml wort sample, 100 ml were withdrawn and subjected to ethanol precipitation by adding 100 ml ethanol at room temperature, incubating for precipitation 30 min and draining BG gums. The BG gums were lyophilized for 24 h and ground prior to DP3/DP4 analysis of pure BG samples free of maltose and glucose. The remaining wort was used directly for analyses of viscosity, BG content and molecular mass.

2.4. β-Glucan content and viscosity

The content of BGs in wort and barley grains was measured by the fluorimetric calcofluor-method (Brewing EBC standards, 1994). Calcofluor has the capacity to form fluorescent products with BGs larger than approx. 10-30 kDa present in solution and its fluorescence proportionally increases with the content of BG bound (Rieder et al., 2015). The instrument used was a BG Carlsberg System 5700 Analyzer with flow injection (Tecator, Sweden). The viscosity was measured by a viscometer Vibro SV-10 (A&D Company Limited, Tokyo) at 20°C within 1 h after centrifugation of the wort. The viscometer cup was filled up with 10 ml of wort and the viscosity recorded as mPas at 30 Hz constant frequency and less than 1 mm amplitude.

2.5. Molecular mass and oligomer block structure

The molecular mass analysis was conducted by size exclusion chromatography (SEC). Prior to analysis the wort samples were heated at 80 °C for 30 min and diluted 1:4 in 50 mM ammonium formate buffer of pH 5. The solutions were mixed, heated at 80°C for 30 min, centrifuged and filtered (0.45 µm) before injection of 50 µl. Separation was performed using an Asahipak GS 520HQ (7.5 × 300 mm) column (Shodex, US). The column was calibrated with five BG standards; barley 650,000 kDa, oat 391,000 kDa, oat 265,000 kDa, barley 229,000 kDa, oat 70,600 kDa and oat 35600 kDa (Megazyme, Ireland). Elutions were performed with 50 mM of ammonium formate buffer (pH 5) and 0.01% NaN_3 at 60°C with a constant flow rate of 0.5 ml/min. The separation was performed using a GPC system (viscotek 270max, Malvern) equipped with an online degasser, a pump and a differential refractometer controlled at 40°C. Data for molecular mass determinations was analyzed by Omnisec software (version 4.7.0.406, Malvern) based on conventional calibration of homopolymers. Results are reported as weight average molecular mass (M_w).

The DP3/DP4 analysis was based on lichenase digestion of BG precipitated from wort prepared as described above. BG powder (2.5 mg) was wetted with 10 µl 50% EtOH, and the slurry was suspended in 500 µl of 10 mM NaH_2PO_4/Na_2HPO_4 buffer. The solution was heated at 96°C for 2 h, cooled to 60°C and 10 U of lichenase (Megazyme, Ireland) were added. The samples were incubated with mixing overnight at 60°C. Subsequently, the enzyme was inactivated at 100°C for 30 min and the samples were lyophilized. The BG fragments were labeled with the fluorophore Aminobenzamide (2-AB) by a reductive amination procedure (Walther et al., 2015). To the lyophilized samples was added 150 µl of 1 M 2-AB in DMSO/AcOH (7:3) and 150 µl of 1 M NaBH3CN in DMSO/AcOH (7:3). Samples were mixed and incubated for 4 h at 60ºC, cooled to room temperature, centrifuged and diluted 400-fold with a mixture of Milli Q water and acetonitrile (22:78). The samples were directly analyzed by hydrophilic interaction liquid chromatography (HILIC) using maltotriose and maltotetraose as standards. 5 µl aliquots were injected onto a Waters Acquity UPLC System equipped with a fluorescence detector (excitation wavelength of 350 nm and

emission wavelength of 420 nm) and columns: a VanGuard BEH glycan 1.7 µm, 2.1 × 5 mm pre-column and an Acquity UPLC BEH glycan 1.7 µm, 2.1 × 150 mm column. Measurements were performed at room temperature. Data were processed using MassLynx V4.1.

2.6. NMR spectroscopy

The possible presence of trace β-glucanase activity in the three different commercial enzyme mixtures applied in the wort production was tested using medium viscosity barley BG (200 kDa, Megazyme, Ireland). The BG was dissolved by gentle heating and whirl-mixing to concentrations of 1 mg in 600 µl of 50 mM potassium phosphate buffer (pH 6) in D_2O (Cambridge Isotope Laboratories, Andover, MA, USA). Buffer of the desired pH had been prepared by lyophilization and re-dissolution in D_2O. To three substrate samples 0.5 µl of enzyme solution (Termamyl® SC, Attenuzyme®, or Attenuzyme® Flex) were added, respectively. Reactions were followed *in situ* by high resolution nuclear magnetic resonance (NMR) spectroscopy for 240 min at 18°C to slow down the reaction relative to process conditions. Spectra were acquired as a time series *in situ* by sampling 16,384 complex data points during an acquisition time of 1.57 sec, summing 32 transients and using a recycle delay of 2 sec.

The site-specific action of trace β-glucanase activity was investigated using a Lys5f BG sample extracted solely by Termamyl® SC under real process conditions, i.e. at 65°C. Homonuclear ^{1}H -^{1}H DQF COSY spectra were recorded on enzyme-treated samples using a 800 MHz Bruker Avance II (Fällanden, Switzerland) NMR spectrometer equipped with a TCI cryoprobe and 18.7 T magnet (Oxford Magnet Technology, Oxford, UK). Spectra were acquired as a matrix of 2048 × 256 complex data points sampling 512 ms × 64 ms in the two proton dimensions. Spectra were processed with extensive zero filling in both dimensions. BG structures formed by exposure to Attenuzyme® Flex were analyzed. Assignments of cleavage site signals from intermediates and products were conducted through comparison of the 2D spectra using

reference assignments from previous work (Petersen et al., 2013). All spectra were acquired, processed and analyzed using Topspin 2.1 (Bruker).

3. Results and discussion

3.1. Raw materials

Barley raw materials showed significant variation in the content of BG (Fig. 1) with Lys5f and KVL408 ranging 11.8-15.3 % (dry mass, dm), KVL1104 and CDC Fibar ranging 8.0-8.9 % (dm) and Bomi, Chameleon, Columbus and Columbus pearled ranging 3.9-4.9 % (dm). Accordingly, the barley lines were categorized into high, medium or low BG genotypes. The pearling of Columbus grains increased the BG content from 3.9 to 4.5 % (dm).

The distribution and appearance of BG within the barley outer layers and starchy endosperm was investigated by histochemical analyses of the eight genotypes (Fig. 2). Three kernels of each genotype were prepared for light microscopy and the results shown are consistent for all repetitions. BG is known to be the main cell wall constituent (70%) of barley starchy endosperm and is also part (20%) of the aleurone layer, and can be stained with calcofluor. Calcofluor also labels cellulose, which however only makes up 2% of the barley grain cell walls (Burton et al., 2010). As deduced from the calcofluor fluorescence (Fig. 2), the cell walls in the starchy endosperm appear significantly thicker in Lys5f and KVL408 (Fig. 2C, D) compared to the other lines. The cell wall signal is also fairly strong in KVL1104, CDC Fibar and Bomi (Fig. 2E, F, G), compared with Chameleon, Columbus and Columbus pearled (Fig. 2H-J). Thus, the visual appearance of the BG in the microscope is consistent with the content of BG in the different lines. Microscope samples of Lys5f and Bomi were furthermore stained for the presence of insoluble polysaccharides and counter stained with a protein stain. Starch granules from Lys5f were of similar size and distribution as those of Bomi however empty voids were frequently seen in the center of Lys5f starch granules (Fig

2K). No differences in protein amount could be noted. The empty center of starch granules in Lys5f might be related to the general decreased starch content in this mutant line (Munck et al., 2004).

3.2. β-Glucan extractability and oligomer block structure

To meet the requirements of the BG health claims of FDA and EFSA a BG extract should provide 3 g BG/day from at least 1 g/serving. This implies that a concentration of 4 g BG/l from 3 × 250 ml typical portion sizes sufficiently will supply the recommended dose. From Fig. 3A it can be seen that Lys5f, KVL408, KVL1104 and CDC Fibar extracts exceed this threshold whereas Bomi, Chameleon and Columbus extracts, regardless of the enzyme treatment, contain lower concentrations. Benito-Roman et al. (2014) extracted 2-4 g BG/l (239 kDa) from barley bran using ultrasound extraction, enzymatic starch hydrolysis and membrane filtration. In comparison, our extraction process provides higher BG solution concentrations from simple hot water extraction, enzymatic starch hydrolysis and centrifugation.

The effect of the different enzyme treatments was evaluated in terms of extractability defined as the recovery of BG from the total amount found in the barley grain. For most barley lines the intensification of enzyme treatment did not influence the genotypic BG extractability significantly. The extractability for T, TA and TAF samples was in the range of 41-81 %, 47-79 % and 60-100 %, respectively (Fig. 3B). These numbers are similar or slightly higher (for Lys5f and KVL408 TAF extracts) compared to extraction yields reported elsewhere (Benito-Roman et al., 2011; Izydorczyk and Dexter, 2008). Unlike Benito-Román et al. (2011), we did not find lower extractability for the hull-less barley lines (CDC Fibar and Chameleon) and we did not see an increase in BG yield from the pearling treatment of the Columbus grains, which have been indicated by others (Baik and Ullrich, 2008). From Fig. 3B, a clear tendency of higher extractability from barley lines containing BGs with low DP3/DP4 ratios can be seen. This is in good agreement with the general understanding of the effect of BG non-repetitive oligomer block structure on polymer solubility

(Burton et al., 2010). For Lys5f, KVL408 and KVL1104 with high DP3/DP4 ratios of 3.8, 3.8 and 3.2, respectively, the T and TA BG yields reached only 41-68 % of the potential extraction levels indicating a lower releasability of BG from these grains. Nevertheless, when comparing all barley lines and enzyme treatments, the combination of either Lys5f or KVL408 high BG raw materials with the TAF enzyme treatment resulted in the overall highest extractabilities. Thus, the Lys5f and KVL408 TAF extractions meet important material and method requirements for preparing an extract having a high content of natural BG.

3.3. Molecular mass and viscosity

The M_w of extracted barley BGs calculated from equivalent external BG standards is shown in Fig. 3C. The highest M_w values were found for Lys5f, KVL408 and KVL1104 samples with values ranging 570-580 kDa (T), 415-535 kDa (TA) and 40-110 kDa (TAF). In comparison, the CDC Fibar, Bomi, Chameleon and Columbus BGs from T, TA, and TAF extractions showed significantly lower M_w values of 155-415 kDa, 130-270 kDa and 15-35 kDa, respectively. In a previous study (Mikkelsen et al., 2013), we extracted Lys5f and Bomi BGs in large scale using thermostable α-amylase, protease, wet milling, repeated heat cycles (up to 125°C), decanting plus centrifugation and ethanol precipitation and found the BG M_w from the two barley lines to be similar (200-300 kDa). In the present study, the genotypic variation in BG M_w observed by others (Irakli et al., 2004) is supposedly better preserved due to the more gentle extraction conditions. It is obvious that extraction with some amylolytic enzyme preparations resulted in BG depolymerization (Fig. 3C). This effect could either be due to contaminating activities in the commercial enzyme preparations or due to amylolytic enzymes themselves exhibiting nonspecific activities toward the BG (Doehlert et al., 2012). The degree of BG degradation followed the order T < TA < TAF, and a profound reduction in M_w was especially found for the TAF combination, where α-amylase is combined with glucoamylase and pullulanase. The elution profiles of Lys5f and Bomi T, TA, and TAF samples

shown in Fig. 4 represent the general trend in sample polydispersity as affected by the different enzyme combinations. In addition to peaks eluting in the order T > TA > TAF due to decreasing M_w, the polydispersity index (M_w/M_n) increased in the order T < TA < TAF, indicating a broadening of the molecular mass distribution in the samples as a result of intensified enzymatic treatment. In a similar study Doehlert et al. (2012) found starch hydrolytic enzymes to have a large effect on the M_w and polydispersity when used for maximizing the extractability of oat BGs. Hence, efficient BG extractability and recovery must be balanced against M_w loss of the extracted BG when using existing enzyme preparations. High extractability and low M_w is advantageous for beverage applications since high viscosity is unsuited for these applications. The wort viscosity generally followed the M_w of the samples (Fig. 3C). Thus, a significant drop in viscosity was seen for TAF samples (2-12 mPas) as compared to T (5-296 mPas) and TA (4-216 mPas) samples, respectively.

It should be noted that the calcofluor method for BG quantification, albeit being rapid and suitable for liquid samples, is not accurate for low molecular mass (<10-30 kDa) BGs. The assay problem implies that the BG content of the TAF samples, being generally low in BG M_w, could be somewhat underestimated. This might be the reason why CDC Fibar, Bomi, Chameleon and Columbus TAF (17-35 kDa) extractabilities compared to Lys5f, KVL408 and KVL1104 (42-109 kDa) are relatively lower when compared internally to the T and TA extraction yields (Fig. 3B).

The BG extracts generally showed high variability in their physico-chemical properties (supplemental table S1) as illustrated by the PCA bi-plot (Fig. 5). The samples typically distribute along the diagonals of the plot according to high, medium and low BG barley lines or enzymatic treatment (T, TA, TAF). Inspection of the loadings (Fig. 5) shows that the main variance among samples (PC1, 53%) is explained both by differences in structural features like DP3/DP4 ratio and molecular mass and by BG content in the raw materials. Due to general viscosity dependence of M_w

359 the clustering of these variables was expected and the apperent co-variance between high BG
360 content in raw materials and high DP3/DP4 ratios of the extracted BG has been suggested by others
361 (Burton et al., 2011). The main target of the Lys5f mutation is suggested to be in starch
362 biosynthesis, decreasing starch content in the endosperm (Patron et al., 2004) and the effects on BG
363 deposition have been explained as pleitropic. This implies that a redirection of glucose
364 incorporation into alternative carbohydrate biosynthesis pathways may take place. In addition to the
365 thicker cell walls found in Lys5f compared to its mother line Bomi (Fig. 2), more BGs were
366 allocated to the outer parts of the grain. Here, the more recalcitrant BGs with high DP3/DP4 ratio
367 are typically found (Izydorczyk and Dexter, 2008), which may explain the overall higher DP3/DP4
368 ratio found for the Lys5f BGs. The minor variance along PC2 (33%) explained by extractability and
369 M_w is spanned by TAF and T/TA samples. This finding confirms that BG depolymerization events,
370 especially originating from the Attenuzyme® Flex enzyme side activity, modify the BG extractable
371 amount.
372
373 *3.4. Side activity from amylolytic enzymes*
374 The molecular mass profiles of the BGs were affected by the amylolytic enzymes, especially by the
375 Attenuzyme® Flex mixture. Hence, NMR spectroscopy was used to investigate the products
376 generated by the β-glucanase activities in the commercial enzyme preparations. Using pure medium
377 viscosity barley BG from Megazyme as the substrate, the highest β-glucanase side activity was
378 found in Attenuzyme® Flex (Fig. 6A) followed by Attenuzyme®. No significant β-glucanase
379 activity was observed in Termamyl® SC. Enzyme activity was judged by the emergence of
380 reducing end signals other than glucose (present in the enzyme mixtures), as indicated in Fig. 6A.
381 The assignments in Fig. 6B show that the vastly predominating cleavage site signals can be
382 attributed to β–(1-3) reducing end signals, formed by cleavage with an *endo*-1,3-1,4-β-D-glucanase
383 activity in Attenuzyme® Flex and Attenuzyme® preparations. The substrate sample (BG from

Lys5f) was largely devoid of pullulan and amylopectin substrates due to Termamyl® SC (amylase) pretreatment and subsequent ethanol precipitation in the preparation of the dry BG powder fraction. Thus, the use of high-resolution NMR spectroscopy provides atomic resolution to validate residual β-glucanase activity in Attenuzyme® Flex and Attenuzyme® preparations, which can be, from cleavage site structures (Petersen et al., 2013), identified as a Lichenase (EC 3.2.1.73) activity (Fig. 6C). The presence of this activity rationalizes the rapid decay of BG molecular masses due to its endoglucanase activity. In this specific case, the presence of minor β-endoglucanase activity is considered advantageous, since is permits a controlled minor degradation of the barley BGs to increase extractability and decrease viscosity.

4. Conclusion

The genotypic variation in barley grain BG content and molecular structure in combination with enzymatic modifications directs its potential as an ingredient in a natural extract high in BG. Lys5f and KVL408 high BG lines extracted with combined α-amylase, glucoamylase and pullulanase enzymes meet optimal material, methods and product requirements. The extracts demonstrate extraordinary high BG yields meeting the criteria for the EFSA and FDA barley BG health claims, low viscosity (~10 mPa s) and molecular mass values (~100 kDa) comparable to commercial barley BG products. Lichenase side activity from the amylolytic enzymes cause controlled BG degradation, which increases the extractable amount and provides desired viscosity.

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Fig. 1.

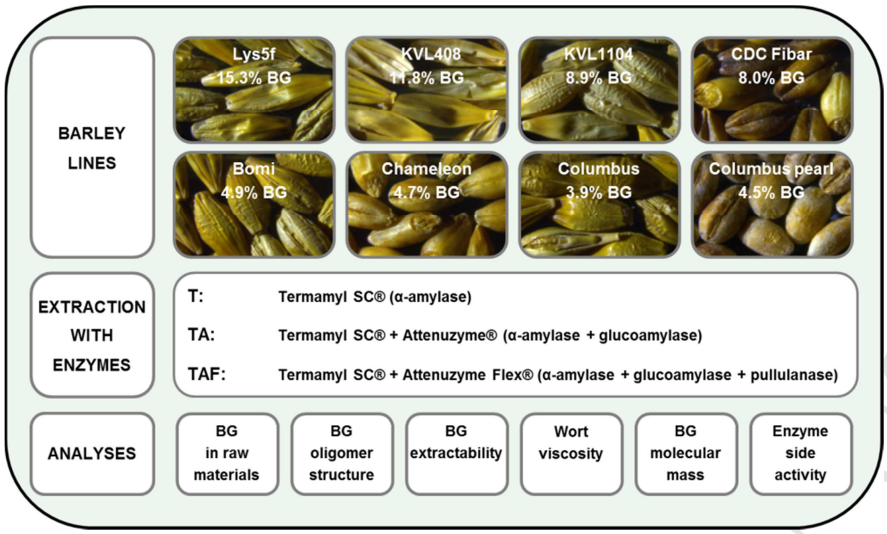

Fig.1. Study overview including eight barley genotypes, three enzyme combinations and six analysis methods.

Fig .2.

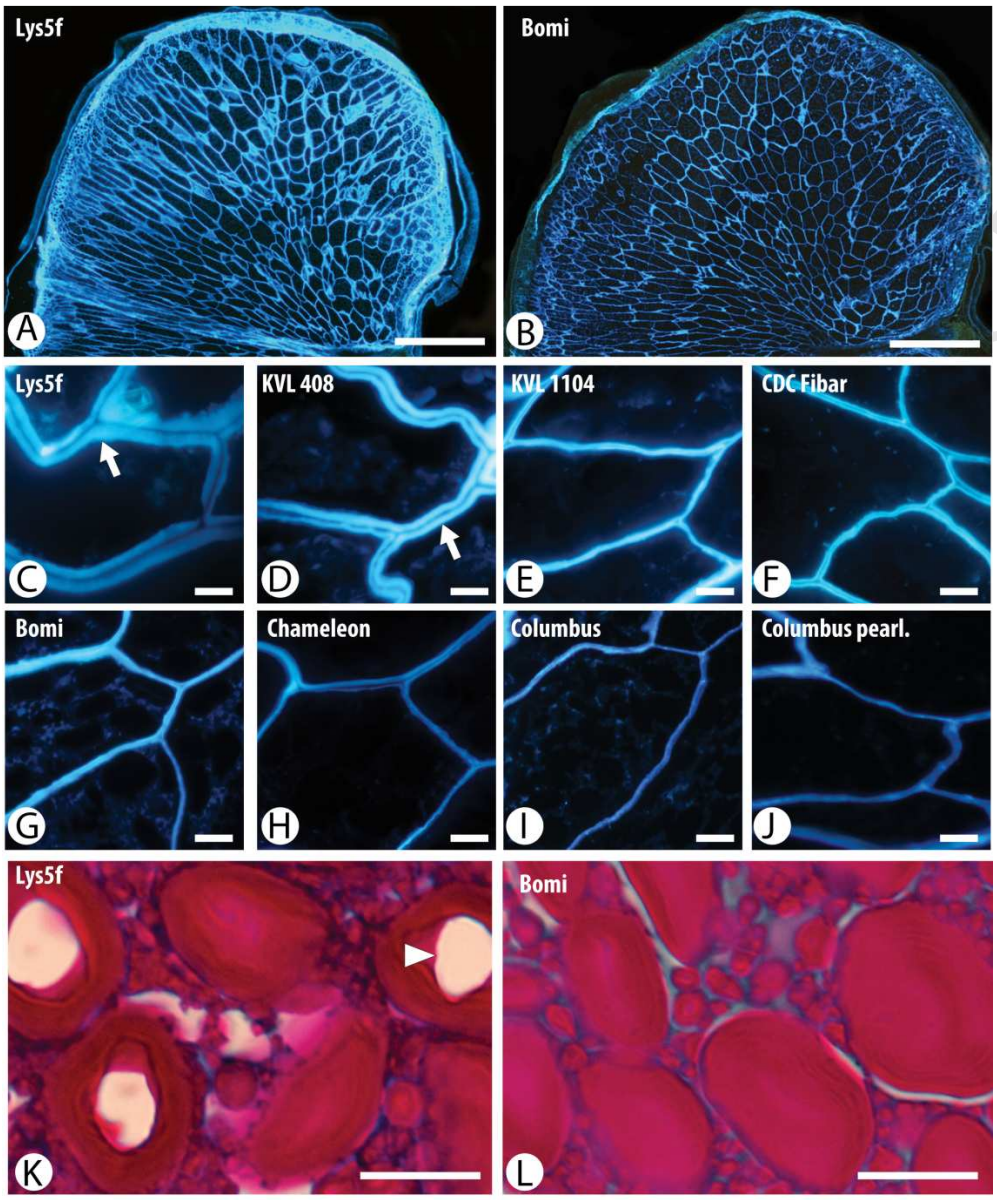

Fig. 2. Histochemical analysis of the eight barley genotypes used for extraction experiments. A and B show half kernels of Lys5f and Bomi in low magnification. C-J show details from the starchy endosperm cell wall from all the genotypes. Note the thick and less dense cell walls in Lys5f and KVL408 (arrow). In K and L the differences in starch granule structure is shown for Lys5F and Bomi.

Note the empty voids in Lys5F (arrowhead). A-J are calcofluor stained, K and L are stained with PAS/AB. Bar = 300 µm (A,B), 10 µm (C-L).

Fig. 3.

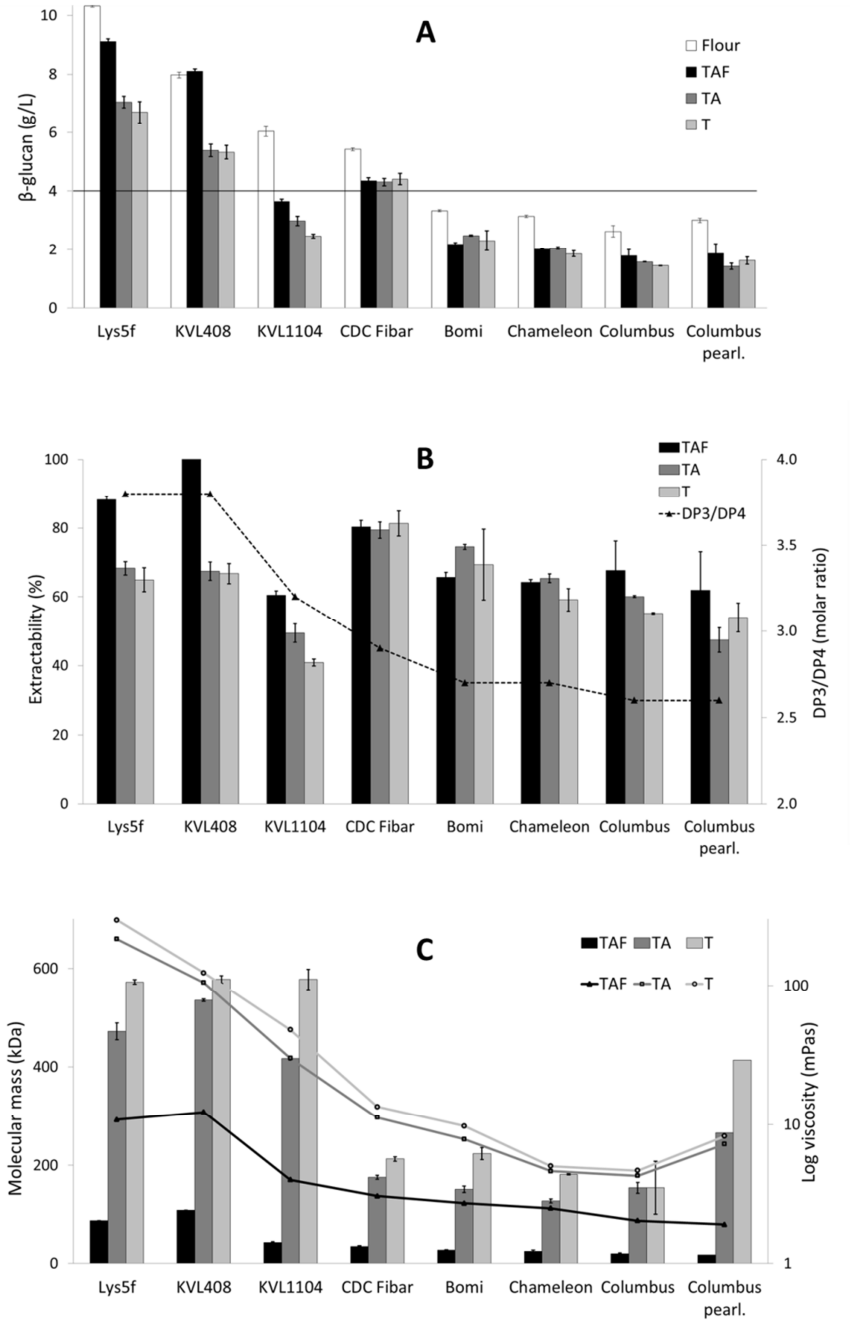
A
β-glucan (g/L)
Flour
TAF
TA
T
Lys5f
KVL408
KVL1104
CDC Fibar
Bomi
Chameleon
Columbus
Columbus pearl.
B
Extractability (%)
DP3/DP4 (molar ratio)
DP3/DP4
C
Molecular mass (kDa)
Log viscosity (mPas)

Fig. 3. (A) Potential and real extraction level of BG from different barley genotypes. Bar = 4 g BG/l. (B) Extractability (%) of BG from different barley genotypes in relation to oligomer block structure (DP3/DP4). (C) Molecular mass (M_w) of BG from different wort in relation to wort viscosity. Mean values ± SD, n = 2.

Fig. 4.

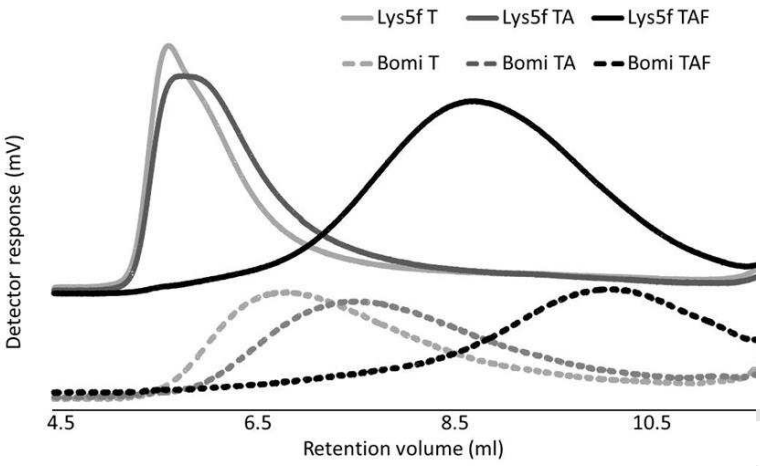

Fig. 4. The effect of T, TA and TAF amylolytic enzymes on Lys5f and Bomi BG molecular mass profiles as determined by size-exclusion chromatography. The peak widths calculated as polydispersibility indices, M_w/M_n are provided in the supplemental table S1.

Fig. 5.

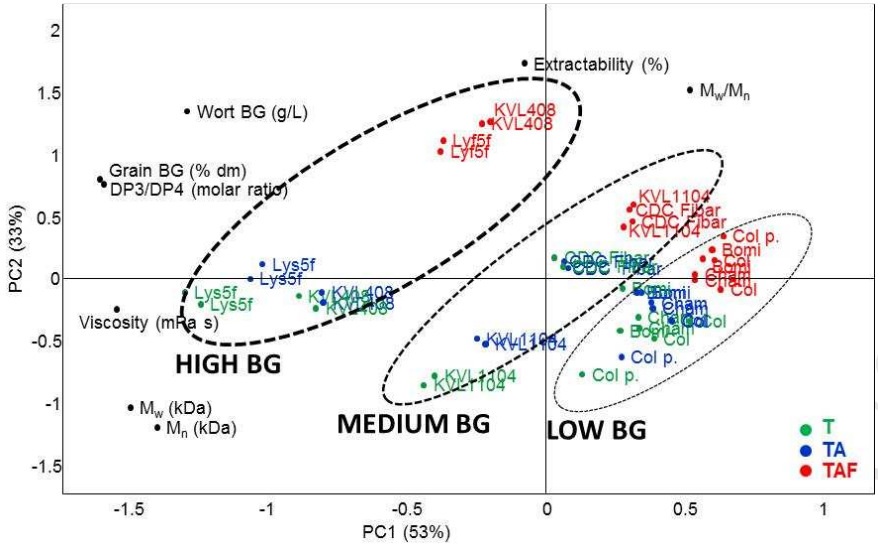

Fig. 5 PCA bi-plot based on the auto-scaled physico-chemical properties of the 48 BG extracts (supplemental table S1). The first two principal components explain 87% of the data variance. Samples cluster according to high, medium and low BG barley lines. DP3/DP4 = triosyl/tetraosyl molar ratio, M_w = Weight average molecular mass, M_n = Number average molecular mass, M_w/M_n = Polydispersibility index.

Fig. 6.

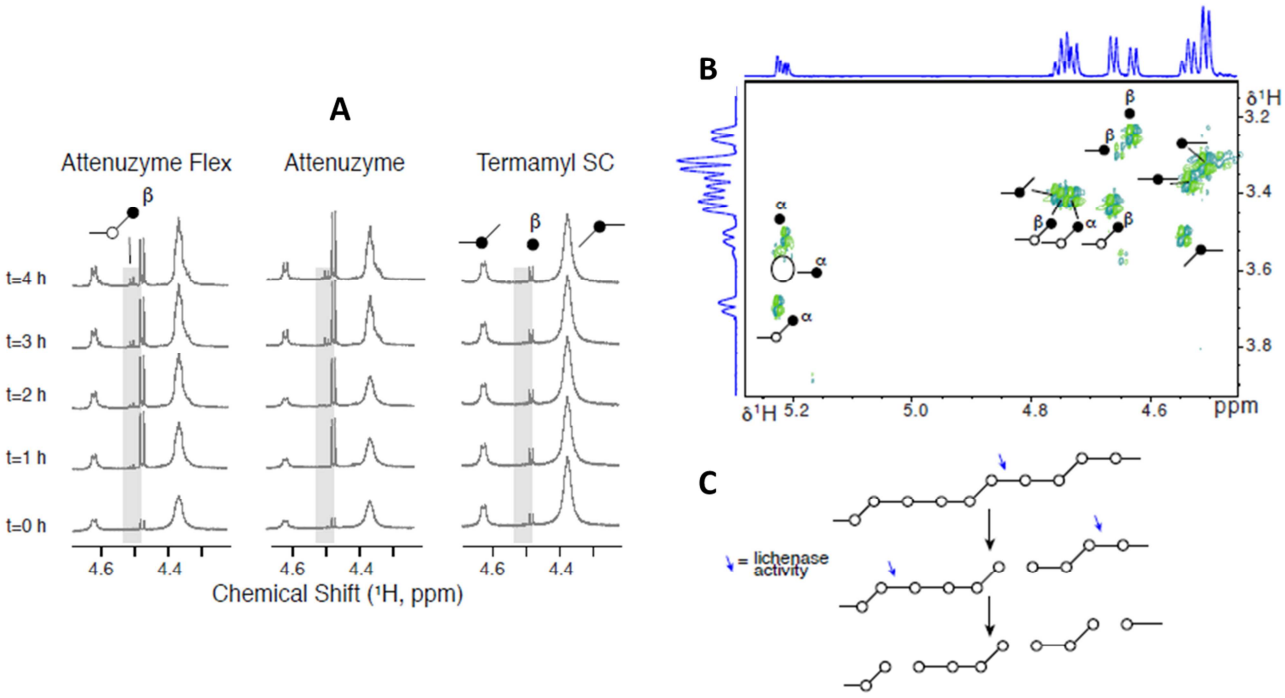

Fig. 6. (A) End products of BG degradation at 18˚C by Attenuzyme® Flex, Attenuzyme® and Termamyl® SC enzyme mixtures using medium viscosity barley BG from Megazyme as the substrate. (B) ^{1}H-^{1}H COSY spectrum of Lys5f BG degraded by the Attenuzyme® Flex side activity at 65˚C. (C) Lichenase cleavage pattern on mixed linkage BGs.

Highlights

- Screening of barley genotypes for high β-glucan (BG) extraction yields
- High BG grain content correlate with high BG triosyl/tetraosyl (DP3/DP4) molar ratio
- Lichenase side activity from amylolytic enzymes cause controlled BG degradation
- Lys5f and KVL408 barley extracts meet EFSA and FDA BG health claim criteria