Carbohydrate Polymers 115 (2015) 104–111

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Viscosity based quantification of endogenous β-glucanase activity in flour

Anne Rieder*, Simon Ballance, Svein Halvor Knutsen

Nofima, Norwegian Institute for Food, Fisheries and Aquaculture Research, PB 210, N-1431 Aas, Norway

ARTICLE INFO

Article history:
Received 28 April 2014
Received in revised form 8 July 2014
Accepted 13 August 2014
Available online 3 September 2014

Keywords:
Cereal β-glucan
β-Glucanase
Quantification
Molecular weight
Activity
Viscosity

ABSTRACT

High molecular weight (MW) is a key parameter for cereal β-glucans physiological benefits like decreased serum cholesterol and attenuated post prandial blood glucose. However, the activity of endogenous flour enzymes during bread production results in a decrease of β-glucan MW. The depolymerization of a standard β-glucan solution by different flour extracts (wheat, barley and rye) was followed by measuring the viscosity decrease with a Rheometer. The slope of the inverse viscosity ($1/\eta$) against degradation time was used to quantify β-glucanase activity by comparison with slopes obtained with known concentrations of the β-glucanase Lichenase. Results correlated well with depolymerization rates estimated by HPSEC. The viscosity based method is rapid (20 min per sample), accurate (≤6% variation), and a powerful screening tool for identifying flour fractions with low β-glucanase activity, treatments that can inactivate β-glucanases in flour, or the development of β-glucanase inhibitors for the use in e.g. bread making.

1. Introduction

Cereal β-glucans are linear polysaccharides composed of β(1,3) and β-(1,4) linked glucose monomers (hereafter referred to as β-glucans). They are the main components of the endosperm cell walls of barley and oat, while rye and wheat endosperm cell walls are dominated by arabinoxylans (Biliaderis & Izydorczyk, 2007). Consequently, the highest β-glucan contents among cereals have been found in barley (2.5–11.3%) and oat (2.2–7.8%) followed by rye (1.2–2.9%) and wheat (0.4–1.4%) (Biliaderis & Izydorczyk, 2007). Beta-glucans from barley and oat have been shown to reduce serum cholesterol levels (AbuMweis, Jew, & Ames, 2010; Maki et al., 2003; Tiwari & Cummins, 2011) and post-prandial glycemic responses (Behall, Scholfield, & Hallfrisch, 2006; Tosh, 2013) in humans, which has created an increased interest for the use of β-glucans or β-glucan rich cereals (barley and oat) as functional food ingredients in human nutrition.

The ability of β-glucans to decrease serum cholesterol levels and glycemic responses is correlated to the potential of the formation of viscous solutions in the small intestine (Tosh, Brummer, Wolever, & Wood, 2008; Wolever et al., 2010; Wood et al., 1994). Since the viscosity of a β-glucan solution is a function of its molecular weight (MW) and concentration, the MW and amount of β-glucan that is extractable into solution in a given food product will influence the extent of its physiological effect (Brummer, Duss, Wolever, & Tosh, 2012; Makelainen et al., 2007; Tosh, 2013; Tosh et al., 2008; Wolever et al., 2010).

In order to retain the full physiological effect of β-glucan it is therefore crucial to reduce its depolymerization during food processing. This is a particular problem during the production of β-glucan enriched bread, since the activity of endogenous flour β-glucanases in combination with the long contact time during mixing, fermentation and proofing causes a substantial decrease in β-glucan MW (Aman, Rimsten, & Andersson, 2004; Andersson et al., 2004; Trogh et al., 2004). It seems therefore warranted to survey β-glucanase activity in flour and other ingredients used in bread making in order to pick ingredients with low enzyme activity were possible and/or develop strategies to inhibit β-glucanase activity.

Previously published β-glucanase activities in flour fractions have essentially been determined with two different methods. The most common method is based on a commercial β-glucanase assay kit from Megazyme (Megazyme International, Bray, Ireland) developed to measure malt and bacterial β-glucanases (Andersson

Abbreviations: HPSEC, high performance size exclusion chromatography; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; MW, molecular weight; M_w, weight average molecular weight; M_p, molecular weight at peak; DNS, 3,5 dinitrosalicylic acid.

* Corresponding author. Tel.: +47 64970175; fax: +47 64970333.
E-mail addresses: anne.rieder@nofima.no, anne.christine.rieder@gmail.com (A. Rieder), simon.ballance@nofima.no (S. Ballance), svein.knutsen@nofima.no (S.H. Knutsen).

http://dx.doi.org/10.1016/j.carbpol.2014.08.075
0144-8617/

et al., 2003; Vatandoust, Ragaee, Wood, Tosh, & Seetharaman, 2012; Wang, Zhang, Chen, & Wu, 2004). The degradation of a standard azo-barley β-glucan (color marked β-glucan) by β-glucanases releases colored β-glucan fragments. The addition of precipitant solution, consisting mainly of methoxyethanol, which is potentially toxic, removes the remaining polymeric azo-barley β-glucan. Hence, the color of the supernatant depends on the concentration of colored β-glucan fragments (not removed by precipitation), which depends on the β-glucanase activity, and can be quantified spectrophotometrically. Beta-glucanse activites are determined relative to a standard curve based on malt β-glucanase (included in the kit) and usually expressed in U/kg. Since the β-glucanase activities in flour are much lower than in malted barley the extraction and incubation conditions of the kit must be adapted to the very low activities found in flour (Vatandoust et al., 2012). The other approach is based on the use of high performance size exclusion chromatography (HPSEC) with post column Calcofluor addition to monitor shifts in MW distribution of a standard β-glucan solution during incubation with β-glucanases (Tosh, Ahmadi, Yip, Roudsari, & Wood, 2012). Peak MWs at different time points (four points over 1–2 h) are determined and used to calculate the different rates of depolymerization. In addition to that, Izydorczyk, Storsley, Labossiere, MacGregor, and Rossnagel (2000) have employed a capillary viscometer (Ubbelohde) to monitor changes of relative viscosity of a control solution of purified β-glucans upon addition of barley extracts over a 4 h time period. The authors report β-glucanase activity as change of relative viscosity of control β-glucan per minute upon addition of barley extracts. However, the different methods have not been compared to each other and the reproducibility has never been tested systematically. In a very recent paper, McCleary et al. (2014) introduced novel colorimetric substrates for the measurement of endo-1,4-β-glucanases consisting of 2-chloro-4-nitrophenol marked cellotriosyl or cellotetraosyl oligosaccharides, showing high reproducibility. However, the absence of β-1,3-linkages, and the different rate of hydrolysis dependent on the degree of polymerization of the oligosaccharides observed for some 1,4-β-glucanases, (McCleary et al., 2014) makes these substrates less suitable for the quantification of β-glucanase activities in flour samples, where the specificity of the present enzymes is not characterized and likely to vary among flour types.

Viscosity measurements are a powerful tool to study depolymerization kinetics of polyscaccharides and have been applied to study acid hydrolysis or free radical depolymerization of alginate and carboxymethylcellulose amongst others by using capillary viscometers (Hjerde, Kristiansen, Stokke, Smidsrod, & Christensen, 1994; Smidsrød, Haug, & Larsen, 1967). For linear, single stranded polymers like alginate, cellulose derivatives or cereal β-glucans subjected to a random depolymerization, the inverse of the molecular weight should increase linearly with depolymerization time (Hjerde et al., 1994; Tanford, 1961). The Mark–Houwink–Sakurada equation ($[\eta] = K_\eta \cdot M^a$) describes the relationship between the intrinsic viscosity $[\eta]$ and molecular weight M of a polymer in solution (Kulicke & Clasen, 2004). For polymers were the exponent a in the Mark–Houwink–Sakurada equation is close to unity, also the inverse of the intrinsic viscosity should increase linearly with depolymerization time (Smidsrød et al., 1967). Instead of the intrinsic viscosity also the specific viscosity η_{sp} can be used for the plot due to the proportionality between the two at low polymer concentrations (Hjerde et al., 1994). The slope of the plot of the inverse specific viscosity against depolymerization time is proportional to the actual rate of the depolymerization reaction (i.e., the number of bonds broken in the polymer) (Smidsrød et al., 1967). In the case of a random attack by an endo-β-glucanase, the inverse of the specific viscosity of the β-glucan solution plotted against reaction time should be linear and the slope will correspond to the enzyme activity. In fact this technique has been used to quantify carrageenase activity of fermentation broth using a capillary viscometer (Ostgaard, Indergaard, Markussen, Knutsen, & Jensen, 1993).

Instead of using a capillary viscometer, we have in this study applied viscosity measurements using a Rheometer with a total measurement time of 10 min to follow the degradation of a standard β-glucan solution upon addition of different flour extracts. A standard curve for β-glucanase activity was constructed with the specific β-glucanase Lichenase (Megazyme), which is commonly used for β-glucan quantification. The β-glucanase activities obtained by viscosity measurements for different flours were compared to degradation rates obtained with HPSEC with Calcofluor detection. Furthermore, different extraction procedures and flour to buffer ratios were tested and the reproducibility of the viscosity method was evaluated by repeated measurements with new β-glucan solutions on different days.

2. Materials and methods

2.1. Flour samples and extraction

Two commercial sifted wheat flours (industri and bakeri), wholegrain barley flour (fiberbygg fullformalt), wholegrain rye flour (rugfin) and barley flakes (fiberbygg flak) were all obtained from Lantmannen (Lantmannen Cerealia, Oslo, Norway). The flakes were milled on a laboratory mill (Retsch, Model ZM1, Retsch GmbH, Haan, Germany) with a 0.5 mm mesh. Barley malt was a gift from Mack brewery, Tromsø, Norway.

Different methods were tested to extract β-glucanases from flour. A 20 mM sodium phosphate buffer (pH 6.5) was used for the extractions. The flour to buffer ratio varied from 1:3 (sifted wheat flour) to 1:8 (wholegrain rye flour). Extraction tubes were vortex mixed for 5, 10 or 15 min, alternatively, extraction tubes were placed in a shaking incubator (Innova 40R, New Brunswick Scientific, Edison, NJ, USA) at 200 rpm and 30 °C for 15 min after an initial vortex mixing. Tubes were then centrifuged at 4000 rpm for 10 min (Hereaus Multifuge 4KR, DJB Labcare Ltd., Newport Pagnell, UK). The extracts were stored at room temperature (RT) and assayed for β-glucanase activity. No activity decrease could be observed during one day storage of the extracts. All extractions for viscosity measurements were performed in duplicates.

In an attempt to inactivate β-glucanases, wholegrain barley flour was treated in an oven (WTB binder). Four grams of the flour (the amount needed for β-glucanase extraction) were spread in parallels onto aluminum trays and heated at 130 °C for 90 min or at 150 °C for 3 h.

Oat β-glucan (High viscosity, 100cSt) was obtained from Megazyme, Ireland and as a gift from Swedish oat fiber, Bua, Sweden. Beta-glucan solutions were prepared at 1% (w/v) by addition of β-glucan to Milli Q water in a Duran bottle under constant stirring with a magnetic stirring bar, followed by 120 min incubation in a boiling water bath. The solutions were filtered through 0.8 μm syringe filters (Millipore) to remove some potential undissolved β-glucan. The actual β-glucan concentrations in the different batches of filtered β-glucan solution were 0.77 ± 0.1% and the β-glucan solution used for the experiments presented in Figs. 1–4 had a concentration of 0.75% as determined with HPAEC-PAD detection of oligosaccharides released by Lichenase (Rieder et al., 2012). For measurement of β-glucanase activity 7 mL of the β-glucan solution (always with a nominal concentration of 1% (w/v)) was pipetted with a positive displacement pipette (Eppendorf) into a centrifuge tube and vortex mixed with 3 mL flour extract, phosphate buffer (enzyme blank) or different dilutions of Lichenase (Megazyme, Lot 60101a) in phosphate buffer for 3 s. Air bubbles were removed by centrifugation at 4000 rpm for 1 min (Hereaus

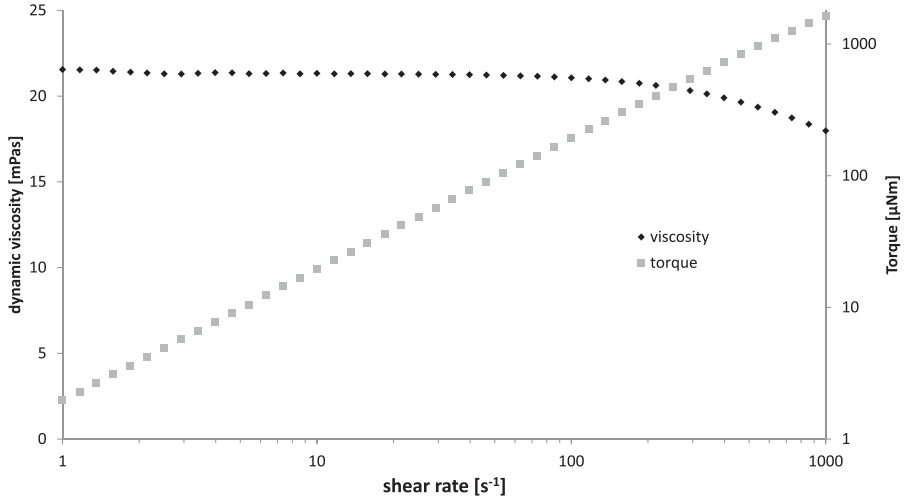

Fig. 1. Dynamic viscosity of the β-glucan solution/buffer mixture (7 mL + 3 mL) and the corresponding torque values as function of shear rate.

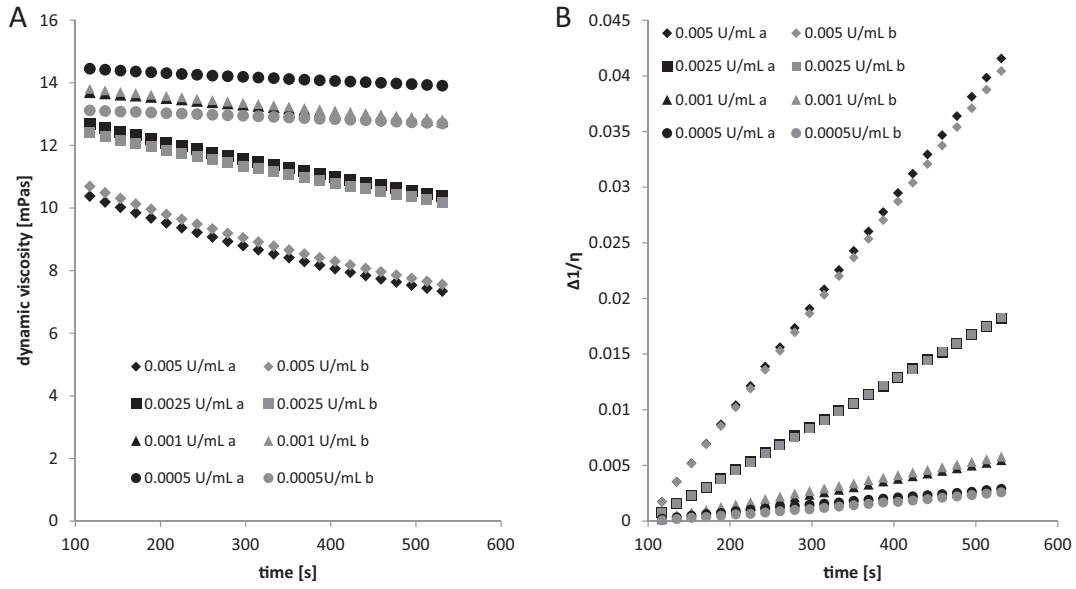

Fig. 2. Degradation of β-glucan by different concentrations of Lichenase as a function of time. Experiments were conducted in duplicates (a and b). (A) Decrease in dynamic viscosity. (B) Delta inverse viscosities (time 90 s subtracted).

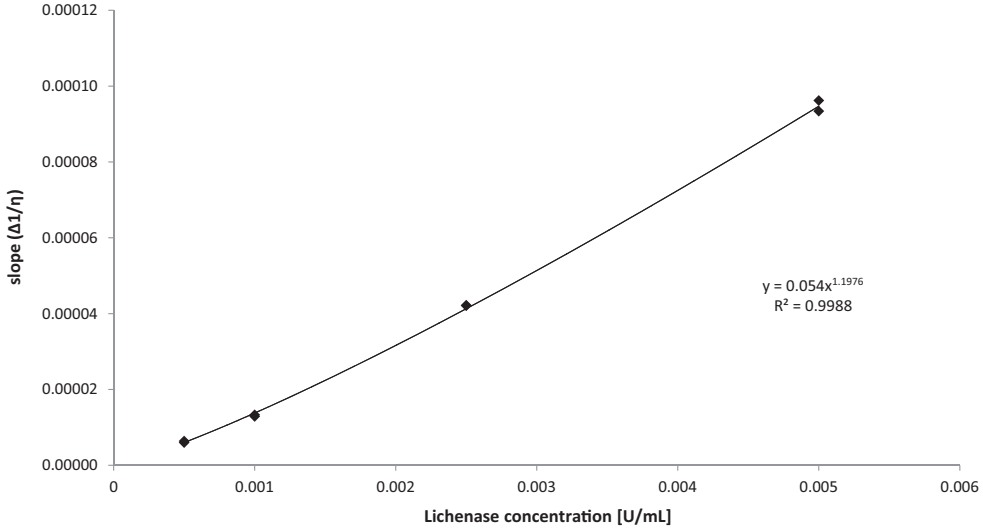

Fig. 3. Standard curve for Lichenase activity. The slopes of the delta inverse viscosities ($\Delta 1/\eta$) are shown as a function of the Lichenase activity in U/mL.

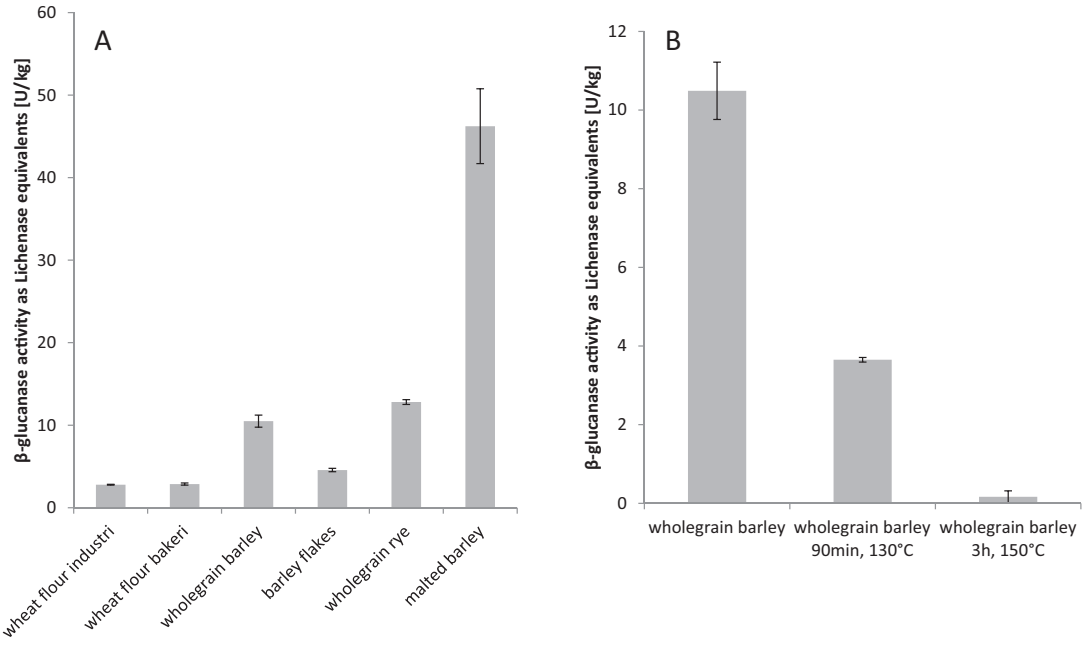

Fig. 4. Beta-glucanase activity of flour extracts in Lichenase equivalents per kg flour. Data represent the average ± standard deviation of 2 different extractions. (A) Different flour and malt extracts, (B) wholegrain barley flour subjected to heat treatment before extraction.

Multifuge 4KR) directly before measuring with a Physica MCR 301 Rheometer (Anton Paar, Stuttgart, Germany). The detection limit of the Rheometer was 0.1 μNm torque and a Double Gap geometry (DG 26.7) was used to further increase sensitivity. The sample size of the DG 26.7 is approximately 7 mL, so the application of 10 mL sample resulted in a desired overfill of the DG geometry to avoid undesirable surface tension and capillary effects. A Peltier element (C-PTD200) around the cup made sure that the measuring temperature remained at 30 °C. Measurements were performed at a constant shear rate of 20^{-1} s for 8 min. Data collection started after temperature equilibration of 90 s.

2.2. Lichenase as a β-glucanase standard

Lichenase (EC 3.2.1.73; Megazyme) was selected as β-glucanase standard for the construction of a standard curve, because it is central in the mixed-linkage β-glucan assay kit for cereal β-glucan quantification (Megazyme), which is a very common method. Lichenase is supplied at a standardized activity of 1000 U/mL (barley β-glucan, pH 6.5, 40 °C) by Megazyme. The activity of Lichenase against β-glucan (1 mg/mL, high viscosity oat β-glucan, 100 cSt, Megazyme) was determined in this study at 30 °C and pH 6.5 by measuring the increase in reducing sugars in the β-glucan solution with 3,5 dinitrosalicylic acid (DNS) over time. One unit was defined as the release of 1 μmol glucose equivalents per minute.

2.3. Molecular weight changes by HPSEC with Calcofluor detection

To validate the results obtained by viscosity measurements, 7 mL of the oat β-glucan solution 1% (w/v) were incubated with 3 mL of the different flour extracts or Lichenase (directly after extraction) at 30 °C. Samples of 1 mL were taken at 15, 30, 60 and 120 min and immediately placed in a boiling water bath for enzyme inactivation. Samples were filtered (0.8 μm syringe filter, Millipore) and diluted 1:40 with water containing 0.02% NaN_3 before injecting 50 μL into the HPLC system consisting of two pumps (DIONEX P680,), a Spectaphysics AS3500 auto injector, a pre-column (Tosoh PWXL), two serially connected columns (Tosoh TSK-gel G5000 PWXL and G6000PWXL, maintained at 40 °C) and a fluorescence detector (Shimadzu RF-10A, Shimadzu Europa, Duisburg, Germany). The eluent (50 mM Na_2SO_4) was delivered at a flow rate of 0.5 mL/min. Calcofluor (Megazyme) solution (25 mg/L in 0.1 M tris(hydroxymethyl)aminomethane) was delivered post-column through a T-valve at a flow rate of 0.25 mL/min. Fluorescence detection of the formed Calcofluor/β-glucan complexes occurred at λ_{ex} = 415 nm and λ_{em} = 445 nm. A calibration curve for β-glucan MW was constructed with in house β-glucan MW standards and standards purchased from Megazyme with peak MW from 100,000 to 1,080,000. A second order polynomial regression was fitted to the retention time plotted against the peak MW using PSS WinGPC Unichrome software (PSS Polymer Standard Service, Mainz, Germany).

3. Results and discussion

3.1. Selection of viscosity measurement conditions

The measurement temperature was set at 30 °C to mimic standard dough temperature. A shear rate of $20\,s^{-1}$ was selected based on the results from a shear rate ramp test (1–1000) of the β-glucan/buffer solution mixture (7 mL + 3 mL) as shown in Fig. 1. A shear rate of $20\,s^{-1}$ lies in the Newtonian range, where the viscosity is independent of shear rate. In order to increase the torque and thereby the sensitivity of the instrument, the shear rate of $20\,s^{-1}$, corresponding to ≈40 μN m torque in this experiment, and not an even lower shear rate in the Newtonian range was selected.

3.2. Lichenase standard curve

The activity of the Lichenase solution was determined as described in Section 2.2. The initial release of glucose equivalents (linear up to 5 min) was 0.31 μmol/min, which corresponded to an activity of 6200 U/mL of the undiluted enzyme solution. The activity information provided by the manufacturer was 1000 U/mL, but the full details of the assay are not provided. Hence, the difference between the two activities may be explained by different assay conditions (e.g. temperature) or a different definition of units. In the

following all information on Lichenase activity refers to the activity of 1000 U/mL provided by Megazyme. However, our inclusion of the protocol to measure Lichenase activity enables the comparison of the presented data with data obtained by other enzymes than Lichenase as long as their activity is measured in a similar way.

Fig. 2A shows the viscosity decrease of the β-glucan solution upon incubation with different concentrations of Lichenase. Higher enzyme concentrations resulted in lower initial viscosities and a faster decrease of viscosity with time. The inverse of the dynamic viscosities was calculated for all samples/measurements and the inverse viscosity of the start of the measurement was subtracted from all data points in order to compensate for the different starting viscosities (different extent of degradation during mixing, centrifugation and temperature equilibration depending on enzyme concentration). The delta (Δ) inverse viscosities given as a function of time (Fig. 2B) therefore solely depict the differences in degradation rate. All measurements follow straight lines, which is in agreement with a random depolymerization of cereal β-glucan by Lichenase, a endo-β-glucanase that specifically binds to β-1,3 linkages in mixed linkage cereal β-glucans and hydrolyzes adjacent β-1,4 linkages (Wood, Weisz, & Blackwell, 1991). The slopes of these straight lines correspond to the depolymerization rate of β-glucan and hence the enzyme activity. The slopes were therefore used to construct a standard curve for Lichenase activity as shown in Fig. 3. The best fit for the standard curve was obtained by fitting a power function to the data. The slight deviation from linearity seems to be due to the lowest Lichenase concentrations and maybe due to a slight adsorption of enzyme to the plastic centrifugation tubes used to make the dilution series. It should be noted here that the Lichenase solution obtained from Megazyme has a concentration of 1000 U/mL and several dilution steps are necessary to reach the concentration range, which is relevant for flour β-glucanases. However, there were no great differences in back calculated Lichenase Units using the power function depicted in Fig. 3 or a linear regression. The advantage of the use of a power function maybe the fact that it will always go through the origin and thereby helps to prevent the generation of false positive results (no change in viscosity should equal no enzyme activity).

3.3. Flour extracts

Different extraction procedures were tested in order to find optimal conditions. All extractions were performed with 20 mM phosphate buffer pH 6.5, which is recommended for Lichenase in the mixed-linkage β-glucan assay kit for cereal β-glucan quantification by Megazyme. Besides, pH 6.5 is close to the average pH of 6 found in the dough of wheat, wheat/barley and wheat/oat breads (own unpublished results). Different flour to buffer ratios were used for the different flour types in order to obtain β-glucanase activities in the extracts within the range of the standard curve. Furthermore, a higher buffer to flour ratio for the wholegrain flours (1:8 for rye and 1:6 for barley compared to the 1:3 for wheat) did not only reduce the β-glucanase activity per mL extract, but also the viscosity of the flour extracts, ranging from 1.1 mPa s for wheat to 3.5 mPa s for rye (data not shown). This is practically important, since very viscous flour extracts may confound the viscosity based measurement of β-glucanase activity.

In an initial extraction trial, flour and buffer were briefly vortex mixed in a centrifugation tube and then placed in a shaking incubator at 30 °C and 200 rpm for 15 min. However, for the samples with high buffer to flour ratios, the shaking was insufficient to prevent sedimentation of the flour and consequently reproducibility between parallels was poor. As an alternative, the tubes with flour and buffer were continuously vortex mixed at room temperature for 5, 10 or 15 min before centrifugation. The increase in mixing time from 5 to 10 or 15 min did not result in significantly greater changes in viscosity of the β-glucan solution and 5 min vortex mixing was therefore selected due to practical reasons. The obtained extracts remained stable with regards to measured β-glucanase activity over at least 12 h at room temperature.

Viscosity measurements of β-glucanase solution/flour extract mixtures were performed as described above. The inverse of the dynamic viscosities as a function of time of the different samples all follow straight lines, indicating the presence of enzymes capable of random attacks and hydrolysis of glycosidic linkages in the β-glucan chain (data not shown). The slopes of the straight lines were used to calculate the hydrolytic activities in Lichenase equivalents according to the standard curve from Fig. 3. The values were expressed as Lichenase equivalent units per kg of flour and the results for the different flour samples are presented in Fig. 4A. The two wheat flour samples showed the lowest β-glucanase activities of 2.8 and 2.9 U/kg, followed by the barley flakes with 4.6 U/kg. Among the flour samples the wholegrain rye flour showed the highest β-glucanase activity (12.8 U/kg), closely followed by wholegrain barley (10.5 U/kg). The β-glucanase activity of the malted barley (46 U/kg) was dramatically higher than that of the flour samples.

The endogenous β-glucanase activity of different wheat flour fractions has been investigated by Vatandoust et al. (2012), while Andersson et al. (2003) have determined β-glucanase activity of different barley milling fractions. Both investigations used the Megazyme kit for malt β-glucanase for β-glucanase quantification. However, since only Vatandoust et al. (2012) adapted the assay extraction procedure to the low β-glucanase activities in flour, it is difficult to compare their results. Nevertheless both studies found an increased amount of β-glucanase in the bran and wholegrain flour fractions compared to white endosperm flour, which is in agreement with the results from the present study. There are two possible explanations for the lower β-glucanase activity of the barley flakes compared to the wholegrain barley flour. The wholegrain barley flour was obtained by milling the barley grains including the husk in order to increase the total dietary fiber content of the resulting flour. To obtain the barley flakes, however, the grains were first pearled, removing the husk, before flaking, which may have removed some of the β-glucanase enzymes located in the outer parts of the kernel. In addition to that the flaking process involves a heat treatment (personal communication with the manufacturer), which also may have decreased β-glucanase activity. Germination of both wheat (Vatandoust et al., 2012) and barley grains (Wang et al., 2004) has been demonstrated to increase β-glucanase activity. Wang et al., 2003 reported an 8-fold increase in β-glucanase activity during malting. This is in agreement with the 4–10 times higher β-glucanase activity of the tested malt compared to the wholegrain barley and barley flakes, respectively.

In an attempt to inactivate the endogenous β-glucanases, the wholegrain barley flour was subjected to heat treatments. The heat treatment at 130 °C for 90 min was selected according to the conditions applied by Comino, Shelat, Collins, Lahnstein, and Gidley (2013) to inactivate endogenous enzymes in rye, wheat and hull less barley flour. As shown in Fig. 4B this treatment led to a reduction (about 3 fold) in β-glucanase activity but not to a complete inactivation. The harsher treatment of 150 °C for 3 h on the other hand was successful in reducing the β-glucanase activity substantially to under 0.15 U/kg, corresponding to a fold reduction of at least 70. Since Comino et al. (2013) reported no differences in viscosities of barley β-glucan solutions treated with boiled (110 °C, 90 min) or dry heated (130°, 90 min) flour extracts the absence of complete β-glucanase inactivation of wholegrain barley flour by dry heat treatment of 130° for 90 min in our experiments was surprising. However, the barley flour employed by Comino et al. (2013) was from hull-less barley and may have had a substantially lower starting β-glucanase activity. The results show that inactivation of endogenous flour enzymes, especially for wholegrain flours with

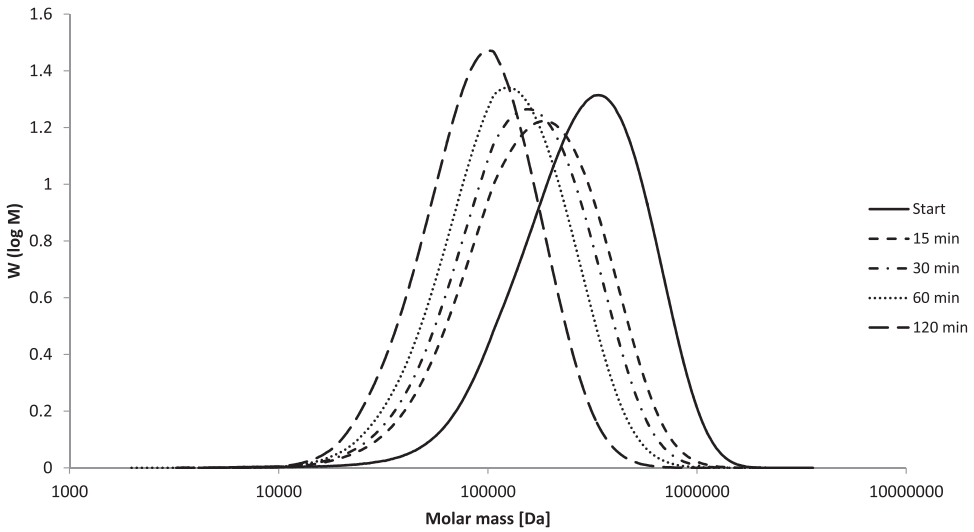

Fig. 5. Molar mass distributions of standard β-glucan solution initially (Start) and after, 15, 30, 60 and 120 min incubation with wholegrain barley flour extract.

relatively high starting activities, by dry heating require quite harsh time and temperature regimes, which may compromise the taste and nutritional properties of the flours (the barley flour treated at 150 °C for 3 h smelled slightly burned) and hence possible food products thereof. However, the β-glucanase assay, presented in this paper may enable the study of many different inactivation protocols within a relatively short time.

3.4. Investigation of assay accuracy and day to day variability

In general, variation between the two parallel extractions of the same flour was quite low (see standard deviations in Fig. 4) and ranged from 1.8 to 7%. The two parallels were measured with the same batch of β-glucan solution. This was also the same batch of β-glucan solution that was used to construct the Lichenase standard curve and the experiments were performed on the same day. The measured β-glucan concentration of the solution was 0.75% (w/v). We have observed a slight variation in the viscosity of different batches of β-glucan solutions and a corresponding variation in β-glucan concentration measured by quantification of Lichenase released oligosaccharides with HPAEC-PAD (0.77 ± 0.1% (w/v)). The β-glucan concentration in separately prepared solutions may vary slightly due to small errors in weighing and pipetting. However, the main variation is likely to arise from difficulties to completely dissolve cereal β-glucan of molecular weight above 200,000 at such high concentrations as 1% (w/v). The extent of β-glucan dissolution may also be slightly different, even under presumably strictly similar conditions, which in turn may influence the amount of β-glucan removed during filtration and hence the final concentration in the test solution. Also the filtered β-glucan solutions are not stable over longer time periods and gelling was sometimes observed in β-glucan solutions kept for longer than 4 days at 18 °C (room temperature).

We have therefore examined the assay performance with β-glucan solutions prepared on different days using the wheat flour industri sample. In addition to that a new dilution of Lichenase (0.0025 U/mL) was tested on different days. Furthermore, the wheat flour sample was extracted with 12 and 24 mL extraction buffer. The results of these experiments are presented in Table 1. The use of 24 mL extraction buffer instead of 12 mL did not have a major effect on the measured β-glucanase activity (3.09 vs 2.78 U/kg). The slight increase in measured activity with 24 mL buffer may point toward a slightly better extraction of β-glucanase due to increased buffer volume. However, the difference may also reflect the assay variability. Extractions at day 2 and 3, which were measured with different β-glucan solutions, showed a higher variability and results ranged from 2.59 to 3.29 U/kg. Due to the different β-glucan solutions the slopes obtained with the Lichenase dilutions (0.0025 U/mL) on days 2 and 3 also varied from the one obtained at day 1 (data not shown). To compensate for this variation, the quotients of the slopes of Lichenase (0.0025 U/mL) at days 2 and 3 and day 1 (day of the standard curve) were calculated. These quotient factors were then used to correct the slopes obtained for the wheat flour sample at days 2 and 3 by multiplication, before the conversion to Lichenase equivalent units with the standard curve from day 1. With this correction, more homogenous data were obtained and the percentile variation of the average dropped from 10.6 to 5.6%, while the absolute value of the average remained the same with 2.94 (uncorrected) and 2.91 (corrected) Lichenase equivalents U/kg (Table 1).

Table 1
Assay accuracy and day to day variability.

Wheat flour industri	β-Glucanase activity by standard curve from day 1 [U/kg]	Corrected β-glucanase activity [U/kg]
12 mL extraction day 1	2.78 ± 0.05	2.78 ± 0.05
24 mL extraction day 1	3.09 ± 0.03	3.09 ± 0.03
12 mL extraction day 2	3.29 ± 0.02	3.01 ± 0.02
12 mL extraction day 3	2.59 ± 0.03	2.76 ± 0.03
Average	2.94 ± 0.31	2.91 ± 0.16
% Variation of average	10.6%	5.6%

3.5. MW changes by HPSEC with Calcofluor detection

Depolymerization of the standard β-glucan solution by flour extracts was also evaluated by following the shifts in the MW distribution with HPSEC over time. Fig. 5 illustrates the change in MW distribution of the standard β-glucan solution upon addition of wholegrain barley flour extract. The change in weight average MW (M_w) of all the tested flour samples and two concentrations of Lichenase are shown in Fig. 6A. The data have not been corrected for the use of different volumes of extraction buffers and therefore only two groups can be detected amongst the flour samples. The two wheat flours (flour to buffer ratio 1:3) and the barley flakes (1:6) show higher M_w than wholegrain barley (1:6) and wholegrain rye (1:8). There is also a dramatic difference between the

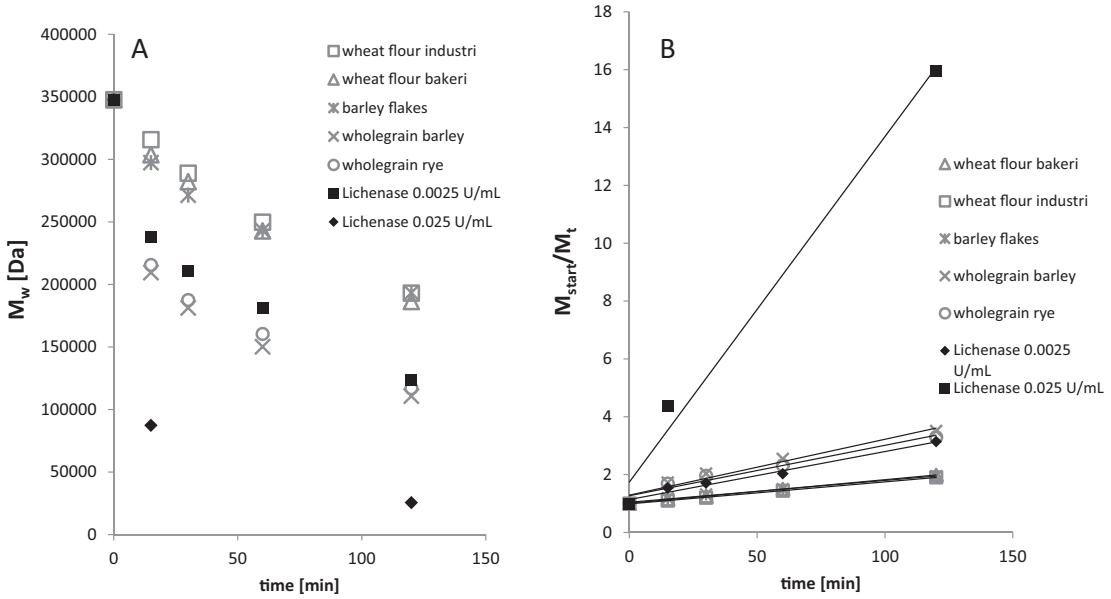

Fig. 6. Changes in MW of standard β-glucan solution during incubation with different flour extracts and different concentrations of Lichenase. (A) Weight average MW. (B) Quotient of peak MW at the beginning (M_{start}) to peak MW after time t (M_t).

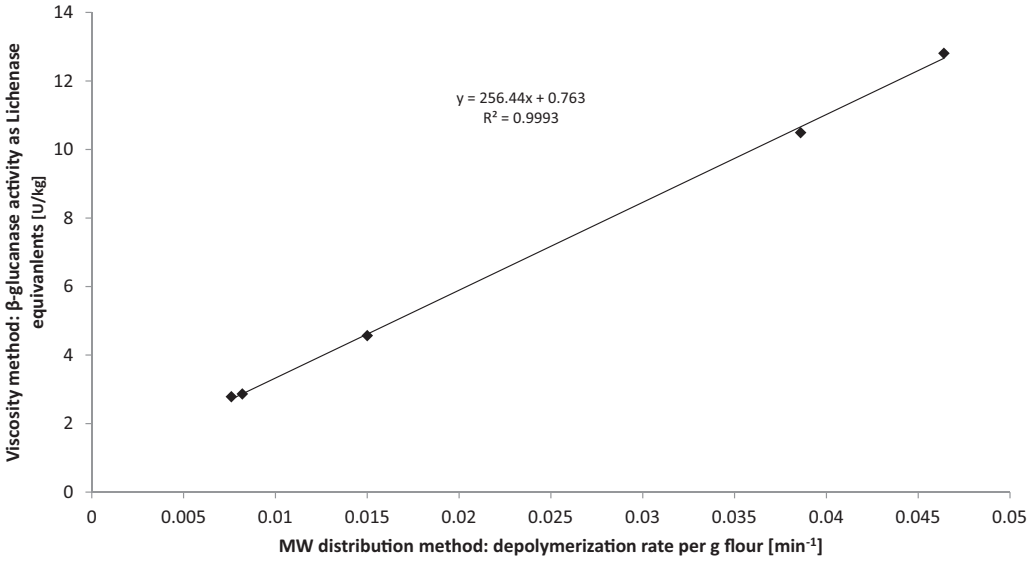

Fig. 7. Correlation between the viscosity based measurement of β-glucanase activity and the depolymerization rates estimated by HPSEC of the 5 different flour extracts.

two Lichenase concentrations, with the higher concentration given much lower M_w.

In order to estimate the depolymerization rate from the MW data, the ratio of the peak molecular weight at start (M_{start}) to the peak molecular weight at time t (M_t) was calculated and shown as a function of time in Fig. 6B. Linear regression lines were fitted to each data series and the slope of each line corresponds to the depolymerization rate. In this plot it becomes even more obvious that the higher Lichenase concentration (0.025 U/mL) resulted in a very strong depolymerization of the standard β-glucan solution. For the flour samples, the same groups as in Fig. 6A were obtained.

In order to correct for the different amounts of extraction buffer and enable a comparison with the β-glucanase activities obtained by the viscosity method, the depolymerization rates per g flour were calculated. The data for the 5 flour samples obtained with viscosity measurement and HPSEC were compared in Fig. 7. Even though the values cannot be directly compared there was a clear linear relationship with a linear correlation coefficient of $R^2 = 0.9993$ between the two methods. This indicated that similar differences in β-glucanase activity between the flour samples can be observed by both methods and confirms the accuracy of the developed viscosity based method. It should, however, be noted that it is only 5 samples that form the basis of this correlation.

4. Conclusions

The use of a double gap geometry enabled accurate viscosity measurements of relatively low viscosity β-glucan solutions treated with β-glucanase containing flour extracts and thus formed the basis of the presented viscosity based measurement of β-glucanase activity. The method correlated well with a known HPSEC method, but has the advantage of short investigation time as one sample can be measured within 20 min including the extraction of the flour and cleaning of the DG, opposed to several hours

(60–120 min incubation of the β-glucan solution with the extract plus roughly 1 h run time for HPSEC for each time point) for the HPSEC method. In addition the presented viscosity based method does not require the use of toxic solvents. The direct comparison of wheat, barley and rye flour samples clearly showed higher β-glucanase activities in barley and rye flour compared to wheat and confirmed previous results showing higher β-glucanase activities in wholegrain flour and bran versus sifted flour. For the construction of the standard curve, Lichenase was selected since the enzyme is widely used for β-glucan quantification, highly standardized and stable in the supplied concentration. This may encourage the use of Lichenase as calibration standard also in other β-glucanase assay methods and can thereby increase the direct comparability of results obtained by different groups. Furthermore, the simple application in combination with the short investigation times of the viscosity based method and it's high accuracy (less than 6% variation) enable it's use as a powerful screening tool for flour fractions with low β-glucanase activity, heat- or other treatments that can inactivate β-glucanases in flour, or the development of β-glucanase inhibitors that can be used e.g. during bread making. These will be extremely important in order to limit β-glucanase activity during the production of β-glucan enriched bread thus protecting β-glucan from degradation, preserving it's MW and thereby most importantly preserving it's beneficial physiological effects.

Acknowledgements

The work is supported by the research project Optifiber (KPN-NFR 224819/E40) financed by the Norwegian Research Levy on Agricultural Products (FFL) and The Agricultural Agreement Research Fund of Norway (JA).

References

AbuMweis, S. S., Jew, S., & Ames, N. P. (2010). beta-glucan from barley and its lipid-lowering capacity: A meta-analysis of randomized, controlled trials. *European Journal of Clinical Nutrition*, *64*(12), 1472–1480.

Aman, P., Rimsten, L., & Andersson, R. (2004). Molecular weight distribution of beta-glucan in oat-based foods. *Cereal Chemistry*, *81*(3), 356–360.

Andersson, A. A. M., Armo, E., Grangeon, E., Fredriksson, H., Andersson, R., & Aman, P. (2004). Molecular weight and structure units of (1 -> 3, 1 -> 4)-beta-glucans in dough and bread made from hull-less barley milling fractions. *Journal of Cereal Science*, *40*(3), 195–204.

Andersson, A. A. M., Courtin, C. M., Delcour, J. A., Fredriksson, H., Schofield, J. D., Trogh, I., et al. (2003). Milling performance of north European hull-less barleys and characterization of resultant millstreams. *Cereal Chemistry*, *80*(6), 667–673.

Behall, K. M., Scholfield, D. J., & Hallfrisch, J. G. (2006). Barley beta-glucan reduces plasma glucose and insulin responses compared with resistant starch in men. *Nutrition Research*, *26*(12), 644–650.

Biliaderis, C. G., & Izydorczyk, M. S. (2007). *Functional food carbohydrates*. Boca Raton: CRC Press.

Brummer, Y., Duss, R., Wolever, T. M. S., & Tosh, S. M. (2012). Glycemic response to extruded oat bran cereals processed to vary in molecular weight. *Cereal Chemistry*, *89*(5), 255–261.

Comino, P., Shelat, K., Collins, H., Lahnstein, J., & Gidley, M. J. (2013). Separation and purification of soluble polymers and cell wall fractions from wheat, rye and hull less barley endosperm flours for structure-nutrition studies. *Journal of Agricultural and Food Chemistry*, *61*(49), 12111–12122.

Hjerde, T., Kristiansen, T. S., Stokke, B. T., Smidsrod, O., & Christensen, B. E. (1994). Conformation-dependent depolymerization kinetics of polysaccharides studied by viscosity measurements. *Carbohydrate Polymers*, *24*(4), 265–275.

Izydorczyk, M. S., Storsley, J., Labossiere, D., MacGregor, A. W., & Rossnagel, B. G. (2000). Variation in total and soluble beta-glucan content in hulless barley: Effects of thermal, physical, and enzymic treatments. *Journal of Agricultural and Food Chemistry*, *48*(4), 982–989.

Kulicke, W., & Clasen, M. C. (2004). *Viscosimetry of polymers and polyelectrolytes*. Heidelberg: Springer.

Makelainen, H., Anttila, H., Sihvonen, J., Hietanen, R. M., Tahvonen, R., Salminen, E., et al. (2007). The effect of beta-glucan on the glycemic and insulin index. *European Journal of Clinical Nutrition*, *61*(6), 779–785.

Maki, K. C., Davidson, M. H., Ingram, K. A., Veith, P. E., Bell, M., & Gugger, E. (2003). Lipid responses to consumption of a beta-glucan containing ready-to-eat cereal in children and adolescents with mild-to-moderate primary hypercholesterolemia. *Nutrition Research*, *23*(11), 1527–1535.

McCleary, B. V., Mangan, D., Daly, R., Fort, S., Ivory, R., & McCormack, N. (2014). Novel substrates for the measurement of endo-1,4-beta-glucanase (endo-cellulase). *Carbohydrate Research*, *385*, 9–17.

Ostgaard, K., Indergaard, M., Markussen, S., Knutsen, S. H., & Jensen, A. (1993). Carbohydrate degradation and methane production during fermentation of Laminaria-Saccharina (Laminariales Phaeophyceae). *Journal of Applied Phycology*, *5*(3), 333–342.

Rieder, A., Grimmer, S., Aachmann, F. L., Westereng, B., Kolset, S. O., & Knutsen, S. H. (2012). Generic tools to assess genuine carbohydrate specific effects on in vitro immune modulation exemplified by beta-glucans. *Carbohydrate Polymers*.

Smidsrød, O., Haug, A., & Larsen, B. (1967). Oxidative-reductive depolymerization: A note on the comparison of degradation rates of different polymers by viscosity measurements. *Carbohydrate Research*, 482–485.

Tanford, C. (1961). *Physical chemistry of macromolecules*. New York: John Wiley.

Tiwari, U., & Cummins, E. (2011). Meta-analysis of the effect of beta-glucan intake on blood cholesterol and glucose levels. *Nutrition*, *27*(10), 1008–1016.

Tosh, S. M. (2013). Review of human studies investigating the post-prandial blood-glucose lowering ability of oat and barley food products. *European Journal of Clinical Nutrition*, *67*(4), 310–317.

Tosh, S. M., Ahmadi, L., Yip, L., Roudsari, M., & Wood, P. J. (2012). Presence of beta-glucanase activity in wheat and dairy ingredients and use of organic salts as potential enzyme inhibitors. *Journal of Cereal Science*, *56*(3), 538–543.

Tosh, S. M., Brummer, Y., Wolever, T. M. S., & Wood, P. J. (2008). Glycemic response to oat bran muffins treated to vary molecular weight of beta-glucan. *Cereal Chemistry*, *85*(2), 211–217.

Trogh, I., Courtin, C. M., Andersson, A. A. M., Aman, P., Sorensen, J. F., & Delcour, J. A. (2004). The combined use of hull-less barley flour and xylanase as a strategy for wheat/hull-less barley flour breads with increased arabinoxylan and (1 -> 3,1 -> 4)-beta-D-glucan levels. *Journal of Cereal Science*, *40*(3), 257–267.

Vatandoust, A., Ragaee, S., Wood, P. J., Tosh, S. M., & Seetharaman, K. (2012). Detection, localization, and variability of endogenous beta-glucanase in wheat kernels. *Cereal Chemistry*, *89*(1), 59–64.

Wang, J. M., Zhang, G. P., Chen, J. X., & Wu, F. B. (2004). The changes of beta-glucan content and beta-glucanase activity in barley before and after malting and their relationships to malt qualities. *Food Chemistry*, *86*(2), 223–228.

Wolever, T. M. S., Tosh, S. M., Gibbs, A. L., Brand-Miller, J., Duncan, A. M., Hart, V., et al. (2010). Physicochemical properties of oat beta-glucan influence its ability to reduce serum LDL cholesterol in humans: A randomized clinical trial. *American Journal of Clinical Nutrition*, *92*(4), 723–732.

Wood, P. J., Weisz, J., & Blackwell, B. A. (1991). Molecular characterization of cereal beta-D-glucans. Structural analysis of oat beta-D-glucans and rapid structural evaluation of beta-D-glucans from different sources by high-performance liquid chromatography of oligaosaccharides released with Lichenase. *Cereal Chemistry*, *68*(1), 31–39.

Wood, P. J., Braaten, J. T., Scott, F. W., Riedel, K. D., Wolynetz, M. S., & Collins, M. W. (1994). Effect of dose and modification of viscous properties of oat gum on plamsa-glucose and insulin following an oral glucose-load. *British Journal of Nutrition*, *72*(5), 731–743.