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Separation and Purification of Soluble Polymers and Cell Wall Fractions from Wheat, Rye and Hull less Barley Endosperm Flours for Structure-Nutrition Studies

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ABSTRACT: The nutritional values associated with the cell walls of cereal endosperm flours are due to a combination of solubilized arabinoxylan and (1-3,1-4)-β-D-glucan as well as residual nonsolubilized cell wall material. In order to investigate structure-nutrition relationships, an appropriate method for the complete functional and structural characterization of cell wall polysaccharides in various cereal endosperm flours is described. This involves the separation of soluble polymers and the residual cell wall fraction without using organic solvents, and the fractionation of soluble polymers into arabinoxylan- and (1-3,1-4)-β-D-glucan-rich fractions for subsequent analysis. This methodology is applied to endosperm flours from wheat, hull-less barley and rye, and could be extended to include studies on the effects of food processing with respect to yield and characteristics of the three fractions in order to better understand the structural basis for nutritional functionality.

KEYWORDS: *arabinoxylan, β-glucan, cell wall, soluble and insoluble fiber, endosperm, non starch polysaccharides, wheat, barley, rye*

INTRODUCTION

Cereal grains are important for human nutrition as they are usually the largest single component of the diet in energy terms. They mainly consist of starch, protein, and non-starch polysaccharides (NSP). NSP's are a major component of dietary fiber and are found in cereal endosperm cell walls, as well as the aleurone layer, the bran and the husk. The endosperm consists of two tissues, the outermost layer that differentiates into highly specialized tissues, the aleurone layer (approximately 7% of the dry grain weight), and the starchy endosperm that is the largest morphological component in all cereals (approximately 80% of the dry grain weight).[1] Several of the nutritional properties of dietary fiber are linked to the extent of solubilization and its consequent ability to modify rheology and interactions with enzymes and bile salts in the digestive tract.

Rye and wheat endosperm cell walls contain approximately 70% arabinoxylans and approximately 20% (1-3,1-4) β-D-glucans (β-glucans), and 10% other components.[2] In contrast, the cell walls of barley and oat endosperms are generally rich in β-glucans with much smaller amounts of arabinoxylans.[3] The endosperm cell walls typically do not contain lignin, but may contain small amounts of glucomannans, cellulose, protein, and phenolic acids.[4] β-glucans and arabinoxylans from cereal endosperm cell walls can be divided into two functional categories, namely water-soluble (or water extractable; WE) and water insoluble (or water unextractable; WU). The proportion of water-soluble and insoluble fractions from endosperm flour depends on the botanical origin and processing conditions.[5] Insoluble NSP's are defined as those polymers which cannot be extracted from the cell walls of the endosperm flour. Water-soluble NSP's are also derived from the cell walls, but represent polymers that are only loosely associated with the cell wall matrix. Fructans may also be present as non-cell wall polymers.

There are two main types of water-soluble nonstarch polysaccharides in wheat and rye, namely arabinoxylans (AX) and highly branched arabinogalactans (AG),[6] and they are typically coextracted in water extraction methods[1,7,8] along with a small amount of β-glucan. For example, in wheat flour, AG is approximately 0.3−0.4 g/100g of original flour and water-extractable arabinoxylan (WEAX) is approximately 0.3−1g/100g.[7] Water insoluble endosperm arabinoxylans require harsher chemical conditions (typically alkali) to solubilize them, due to their associative bonding within the cell walls with other polymers. The insoluble forms of arabinoxylans are rich in bound phenolic acids which are predominantly based on ferulic acid and may form oxidative cross-links.[1,9,10]

NSP isolation procedures broadly involve degrading and removing starch with enzymes, typically amyloglucosidase[8] or α-amylase,[11] recovering cell wall material with ethanol and/or centrifuging and/or filtering (to recover insoluble NSP), then removing soluble protein using enzymes, or clay,[8,12] and heating to help precipitate protein, leading to a solution of soluble NSP. Subsequently, selective precipitation with different aqueous alcohol solutions can be used to recover WEAX and WEBG (water extractable β-glucan) fractions.[13−17] Many extraction methods[15,17,18] use 60%-90% ethanol to inactivate endogenous enzymes and help remove protein from endo-

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sperm flours. However, higher residual protein amounts in wheat endosperm extractions have been found when using aqueous ethanol solubilization compared to clay absorption.[11,19] Soluble wheat AX extractions have been studied extensively, and are well documented.[5,20,21] However, barley (in particular hull-less barley) and rye endosperm flours have been studied to a lesser extent. Vinkx et al.[22] have reported both soluble and insoluble fraction isolation methods,[22] and the chemical structures of AX from rye flour.[12,22−24]

β-glucans from various cereal grain sources will precipitate or extract differently depending on the conditions used. For example, ≤20% of the total (1-3,1-4)-β-D-glucan can be extracted from barley flour at 40 °C using water as a solvent.[14,25] However, as the water temperature increases to 65 °C, the soluble extracted fraction increases to 50−70%.[25,26] Approximately 70−75% of oat β-glucan can be extracted in hot water at 80 °C.[27,28] On the other hand, only ≈10−20% (at most) of rye flour NSP can be extracted with hot water.[29−31] Alkali treatment can lead to much higher extraction levels. A maximum yield of 74% without apparent significant depolymerisation ($M_w \approx 1 \times 10^6$) was obtained using 1 M NaOH.[31] Other methods for extracting rye β-glucan with 2 M NaOH and barium hydroxide followed by water at pH 5−6 resulted in an extraction yield of 45−50%,[32,33] but at the expense of considerable depolymerisation.[30,31] Rye β-glucan extracted using alkali methods can be further purified from arabinoxylan using phenol, ammonium sulfate and/or xylanase.[31,34]

Wheat (1-3,1-4)-β-D-glucan is also very difficult to solubilize, being practically unextractable with water at 65 °C.[35,36] This may possibly be because of physical entanglement in phenolic cross-linked arabinoxylans.[36,37] Consequently, very little work has been done on wheat β-glucan extractions. However, Cui and Wood,[38] have extracted β-glucans from wheat bran using 1.0 M NaOH and then treated with xylanase to remove arabinoxylan. The authors reported that partial depolymerisation did occur but was unavoidable due to the need for wheat β-glucan to be extracted by alkali methods. Also, Beer and Wood[27] reported that 1.25 M NaOH for 16 h at room temperature led to reduced molecular weight.

Thus, choice of extraction methodology used may alter some molecular structures and lead to depolymerisation. Alkali treatment and subsequent neutralization may not only cause depolymerisation, but also swelling of cell wall material due to alkali-induced ionization and subsequent deswelling on neutralization, potentially to a different architecture. Likewise, alcohol or other organic solvents may promote aggregation of polysaccharides within cell wall material (analogous to precipitation from solution), that may not be reversed on subsequent hydration. Alkali or organic solvent treatments may therefore alter the rheological properties of both the extracted molecules and the residual cell wall material, critical factors in developing a greater understanding of structure-nutrition relationships. Therefore there is a need for extraction methods that allow further characterization of structural and rheological properties of each of solubilized arabinoxylan and β-glucan, as well as the residual insoluble cell wall fraction of cereal endosperm flours. In order to draw inferences on potential behavior in the digestive tract, the segregation of soluble and insoluble fractions should avoid organic solvents and alkali treatments, as both of these can lead to changes in solubility that would not occur during digestion. Once polymers have been solubilized away from insoluble cell wall material, separation of arabinoxylan and β-glucan is desirable so that properties such as molecular size distribution and rheological properties can be determined for defined polymers. Selective precipitation has been suggested to achieve this[13,16−18,39] as arabinoxylans typically precipitate at lower ethanol content in aqueous alcohol solutions than β-glucan[14,40] (e.g., 60% cf 75%).

The cell wall extraction and isolation method described in this paper involves dry heating to inactivate endogenous enzymes, and then removal of starch using α-amylase from *Bacillus lichenformis* and protein using Pronase E from *Streptomyces griseus*, leaving predominantly arabinoxylans and β-glucans. The avoidance of organic or alkaline solvents prior to separation of insoluble and soluble fractions means that direct comparison with the extent of solubilization under in vitro and in vivo digestion conditions can be made. Similarly, the avoidance of NSP-degrading enzymes allows the detailed characterization of each of the AX and β-glucan fractions recovered by selective precipitation in aqueous ethanol solutions of the soluble fraction.

MATERIALS AND METHODS

Wheat endosperm flour was supplied from the Macro Food Company (Sydney; NSW), rye endosperm flour (*Bevy*) from Laucke Mills (Strathalbyn; SA), barley hull-less endosperm flour (*Finniss*) was from the University of Adelaide, Waite Campus; Urrbrae, SA. Wheat AX (29cSt), barley β-glucan (28cSt) and rye AX (33cSt) were from Megazyme (Bray, Co Wicklow, Ireland).

Amyloglucosidase from *Aspergillus niger* (A7420, 31.2 U/mL, EC 3.2.1.3), *Bacillus licheniformis* α-amylase (type XII-A, A-3403, 17 980 units/mL [one unit will liberate 1 mg of maltose from starch in 3 min at pH6.9 at 20 °C]), protease E *Streptomyces griseus* (P5147, Type XIV, >3.5 U/mg), porcine α-amylase (A6255, 700−1400 U/mg protein, Type 1-A), bentonite clay, DMSO-*d6* (methyl sulfoxide-D_6, 99.9% atom), TFA (trifluoroacetic acid), TSP (trimethylsilyl propanoic acid), D_2O (deuterium oxide), lithium bromide analytical grade (LiBr), tris (hydroxymethyl aminomethane), maleic acid, calcium chloride analytical grade ($CaCl_2$), sodium hydroxide analytical grade (NaOH) were all purchased from Sigma−Aldrich, St Louis, MO.

10 mM Tris buffer was prepared by making a 0.2 M tris-maleate solution and adding 1.21g of Tris (hydroxymethyl aminomethane) and 1.16g of maleic acid to 50 mL of water. Once dissolved, approximately 45−48 mL of 0.2 M NaOH was added to give a resultant pH of 6.9 and then diluted to 1L. Weighed amounts of 0.11g of $CaCl_2$ (1 mM) and 0.5 of NaCl (10 mM) were then added and the solution mixed thoroughly using a magnetic stirrer for 10 min. The final pH was checked and adjusted to 6.9 if necessary with 0.1 M NaOH or 0.1 M HCl.

Protein Analysis of WEAX and Cell Wall extracts. The protein content of the final extract sample (N × 5.7 wheat flour and N × 6.25 for rye and barley flour) was determined by nitrogen combustion (AACC, Method 46-30).

Monosaccharide Analysis and β-glucan DP3/DP4 ratio Analysis Using HPLC. *Monosaccharide HPLC Analysis.* Monosaccharide analysis was performed by HPLC analysis following the method described by Burton et al.[41] Samples of 20 mg were hydrolyzed using 1 mL 1 M sulphuric acid, for 3 h at 100 °C. Hydrolysates were diluted 20× with distilled water and derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP); 10 μL of 0.5 mM 2-deoxy glucose was added to each sample as an internal standard. PMP-monosaccharides were separated

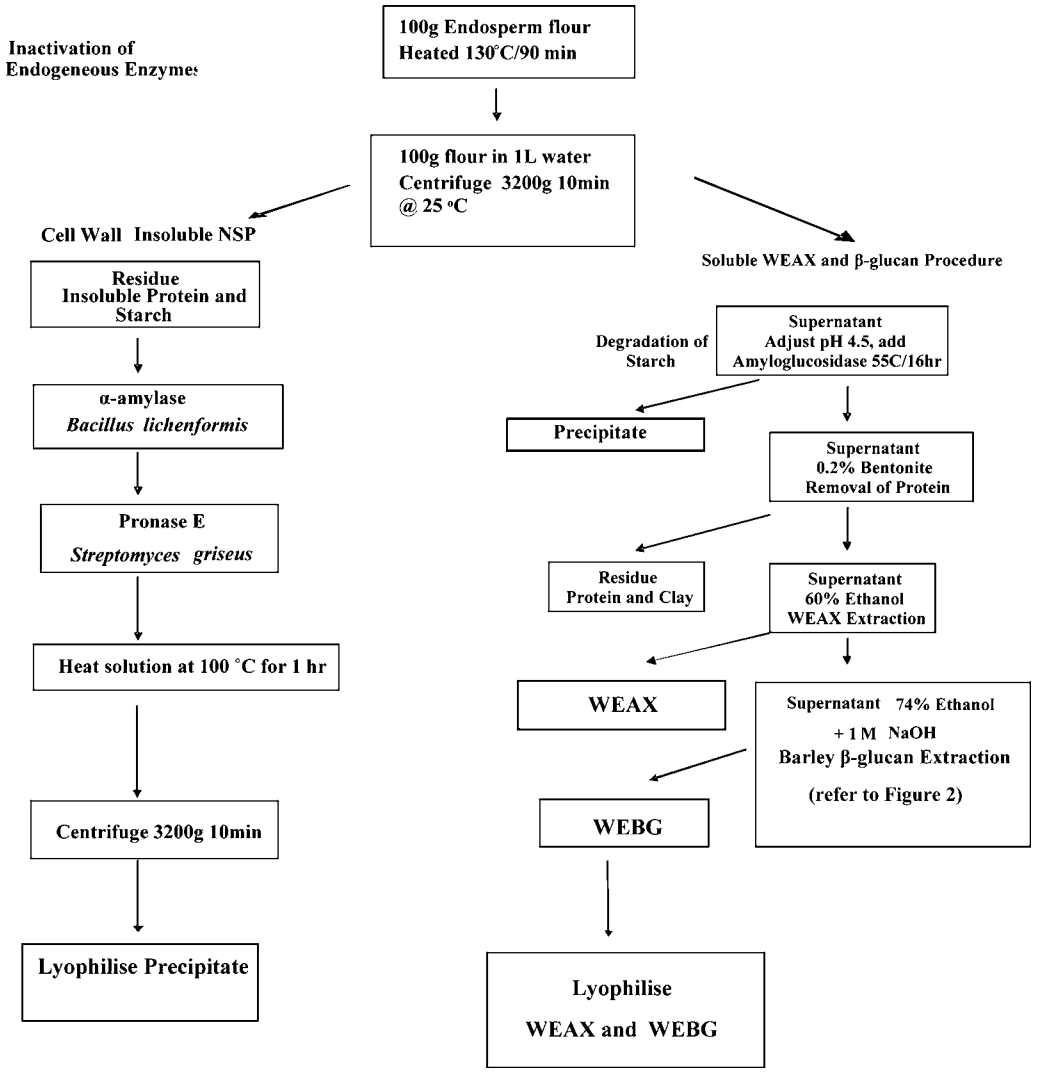

Figure 1. Overall insoluble NSP, WEAX, and WEBG fractionation procedure.

using RP-HPLC on an Agilent 1200LC. Five or 6 μL were injected onto a Phenomenex Kinetex 2.6 μm C18 100 × 3 mm 100A column, operated at a flow rate of 0.6 mL/min and 30 °C. The eluents used were (A) 10% acetonitrile, 40 mM ammonium acetate (pH ~6.8), and (B) 70% acetonitrile. The gradient was 8 to 16% (B) over 12 min, with absorbance detection at 250 nm. Area under the peaks was compared to standard curves of xylose, arabinose, mannose, glucose, and galactose.

DP3/DP4 Ratios Using HPLC. β-glucan oligosaccharide samples were obtained following digestion by lichenase as per the mixed-linkage β-glucan assay kit (AOAC Method 995.16) from Megazyme (Wicklow, Ireland).

The β-glucan oligosaccharides were derivatized in a similar manner to the monosaccharides, with the following changes. Ten μL of internal standard (0.25 mM talose) solution was added to 10 μL of appropriately diluted lichenase digests. 18 μL of PMP reagent (0.5 M PMP in methanol/1 M ammonium hydroxide aq. in the ratio of 5:4 v/v) was added. Derivatised solutions were acidified with 5 μL of 10 M formic acid. PMP- β-glucan-oligosaccharides were separated using RP-HPLC on an Agilent 1200LC. Fifteen μL was injected onto a Phenomenex Kinetex 2.6 μm XB-C18, 100 × 3 mm, 100A column, operated at a flow rate of 0.9 mL/min and 40 °C. The eluents used were (A) 40 mM H_3PO_4, 32 mM NH_4OH (pH~2.7) and (B) 70% acetonitrile. The gradient was 25 to 29% (B) over 5 min, with absorbance detection at 250 nm. The area under the peak was compared to a BG-OS (β-glucan oligosaccharides) DP3 and DP4 standard curve.[41]

Total β-Glucan Analysis. Total β-glucan amounts were determined using a mixed-linkage β-glucan assay kit (AOAC Method 995.16) from Megazyme (Wicklow, Ireland). Analyses were performed in duplicate and the enzymes and standards from the Megazyme β-glucan assay kit were used.

Sample Preparation for SEC (Size Exclusion Chromatography). Duplicate samples were prepared by dissolving 2 mg of sample extract into 1 mL of DMSO + 0.5% LiBr solution. The samples were then placed into an 80 °C thermomixer overnight, and were then injected into the Agilent 1100 Series SEC system (PSS GmbH. Mainz, Germany), using a GRAM preColumn and two 100 columns (PSS GmbH. Mainz, Germany) in series, in a column oven at 80 °C, and eluted with DMSO containing 0.5% w/w LiBr at a flow rate of 0.3 mL/min. The system was equipped with multiangle laser light scattering (MALLS) (BIC-M_wA7000, Brookhaven Instrument Corp., New York) followed by parallel flow into a refractive index detector (RID) (Shimadzu RID-10A, Shimadzu Corp.,

Japan) and a viscometric detector (ETA-2010, PSS GmbH, Mainz, Germany). [42]

The AX concentration used for the SEC columns was 2 mg/mL. To determine that the detectors were giving correct responses, pullulan standards were used (PSS GmbH, Mainz, Germany), with a molecular weight range of 342−1.66 × 10^6. A differential refractive index (dn/dc) value of 0.0869 mL g−1 for pullulan in DMSO/LiBr (0.5% w/w) was used, and previous data for β-glucan and arabinoxylan used for molecular size calibration.[42] Data analysis used WinGPC software.

Soluble and Insoluble Cell Wall Fraction Calculations. The yield of both the cell wall and soluble fractions was determined by weighing the extracts in duplicate and repeating extractions and comparing reported values. Averaged values over repeated extractions are reported.

Sample Preparation and Analysis Using ^{1}H NMR. Extract samples were prepared by placing approximately 6 mg of sample into a 2 mL Eppendorf tube and then adding 690 μL of d6-DMSO (methyl sulfoxide-D_6, 99.9% atom D). The Eppendorf tubes were then placed into a thermoshaker set at 80 °C and left overnight. Once samples had been dissolved, they were cooled to room temperature and 10 μL of TSP/D_20 solution (1.2 mg/mL) was added. Sample contents were then pipetted into a labeled Wilmad 5 mm thin wall 7" 500 MHz NMR tube, and then 40uL d1-TFA was added to each NMR tube.

NMR spectra were obtained using a Bruker Biospin Avance 500-MHz NMR spectrometer, with a 5 mm TXi5 1H/D probe equipped with shielded z gradient and controlled by XWIN-NMR software (Zürich, Switzerland). The same sample preparation and NMR protocols were used for both the β-glucan and AX purified fractions. Each ^{1}H NMR spectrum was obtained under the following conditions: 64 scans, 45°pulse, an acquisition time of 3.57 s, and a relaxation delay of 20 s to ensure quantitative responses. Spectral width was 6 kHz, time domain 43 K. TSP was used as an internal chemical shift standard (0.0 ppm) and D2O acted as the lock for the spectrometer. The NSP ^{1}H NMR spectrum has peaks in the range 3−6 ppm, with diagnostic anomeric signals at 4.3−5.5 ppm. TFA was used to shift the hydroxyl peaks from within this range to approximately 8 ppm.[43]

Rheology−Intrinsic viscosity. An Advanced Rheometric Expansion System TA GA12 (ARES, TA Instruments; New Castle, DE) was used to investigate the viscoelastic characteristics of β-glucan (WEBG) and arabinoxylan (WEAX) solutions of varying concentrations. A range of concentrations from 0.1% to 2.5% (w/v) (0.1%, 0.3%, 0.5%, 0.8%, 1%, 2.5% (w/v)) of soluble β-glucan and arabinoxylan was prepared by dissolving in deionized water at 80 °C for 8 h in an oil bath. Parallel plate geometry (60 mm diameter) was used at 37 °C, and the linear visco-elastic region was determined for each sample using a dynamic strain sweep at 10 Hz frequency. This was followed by frequency sweep experiments for 0.1−100 Hz at a suitable strain within the linear viscoelastic region. The steady-shear experiments were performed for a range of frequencies from 1 to 1000 s^{-1}. Intrinsic viscosity values were calculated.[44,45]

FRACTIONATION PROCEDURES FOR INSOLUBLE CELL WALL AND SOLUBLE NSP'S (ARABINOXYLANS AND β-GLUCANS)

Figure 1 gives an overview of the extraction and purification procedures used for the insoluble cell wall fractions and soluble arabinoxylan and β-glucan.

Cell Wall Fractionation Procedure and Purification of Cell Wall Residue. One hundred grams of dry endosperm flour was spread out evenly onto a tray (50 × 30 cm) and heated at 130 °C for 90 min, in order to inactivate endogenous enzymes.[8,17,46−48] In order to determine if this treatment inactivated arabinoxylan and beta-glucan degrading enzymes, viscosities of 1.25% w/v wheat arabinoxylan (Megazyme) and barley beta-glucan (Megazyme) were compared after 16 h treatment at room temperature with (a) boiled (110 °C for 90 min) aqueous flour extract, (b) aqueous extract after dry heating of flour, and (c) aqueous extract from unheated flour, for each of the wheat, hull-less barley, and rye. Boiled and dry heated extracts all showed very similar and essentially unaltered steady shear viscosities while the unheated extract showed greatly decreased viscosities (results not shown), consistent with the presence of arabinoxylan and β-glucan degrading enzymes in each of the unheated flour extracts, and the effective inactivation of enzymes by either boiling unheated flour extract or dry heating flour prior to extraction.

Once the flour had cooled to RT, it was added to 1L of deionized water, the slurry was mixed using a magnetic stirrer on a hot plate for 90 min at 40 °C, and then centrifuged at 3200g for 10 min. The supernatant (soluble NSP and protein) was then decanted, and the residue (insoluble NSP with starch and protein flour residue) was recovered, as shown in Figure 1. Approximately 10 g of residue was weighed into a beaker and made up to a 10% w/v suspension by adding Tris buffer (10 mM tris-maleate buffer pH 6.9).[49,50] The sample mixture was then heated to 70 °C and 1 mL *Bacillus lichenformis* α-amylase was added and incubated at 70 °C for 4 h. [23]For barley flour, due to the increased dissolution of β-glucans at temperatures above 55 °C, amyloglucosidase from *Aspergillus niger* EC 3.2.1.3 (31.5 U/mL) at 55 °C and pH 4.5 for 4 h, or porcine amylase (25 mg) 700−1400 U/mg protein) at 40 °C and pH 6.9 for 2 h, was used to digest starch.

The pH was then adjusted to 7.5 using 0.1 M NaOH and 0.5 mg/1 mL of Pronase E from *Streptomyces griseus* was added. The mixture was incubated at 55 °C for 2 h[51] and then cooled to RT and centrifuged at 3220g for 10 min. The solution was then heated to 100 °C for 60 min to inactivate enzymes. The supernatant was decanted and the residue washed twice with 70% ethanol, and then twice with acetone. Samples were then air-dried for two days, vortex mixed with minimal amounts of distilled water, frozen, and lyophilized.

Extractable Arabinoxylan (WEAX) and β-Glucan (WEBG) Isolation. The WEAX fraction from the wheat, hull-less barley, and rye endosperm flours was isolated according to the method described by Ganguli and Turner[8] with modifications. The water-extractable β-glucans (WEBG) were also isolated separately from the WEAX using differential precipitation with ethanol. The individual steps used were as follows and are outlined in Figure 1.

Degrading Starch. After separation from the cell wall residue (Figure 1), the flour slurry supernatant was adjusted to pH 4.5 with 0.1 mol L^{-1} HCl and 2 mL of distilled water containing 31.5 U mL^{-1} of amyloglucosidase from *Aspergillus niger* was added to degrade starch by incubating at 55 °C for 16 h.[8,46] After starch hydrolysis, the solution was heated in a boiling water bath for 30 min to inactivate the amyloglucosidase and to precipitate soluble proteins.[8]

Removing Protein. The extract was then centrifuged (3220g for 10 min) and the filtrate was adjusted to pH 5.0 immediately with 0.1 mol L^{-1} NaOH, then a 0.2% bentonite

aqueous solution was added to absorb the contaminating protein.[8] The 0.2% bentonite solution was added at a rate of 10 mL per 100 mL of filtrate, while being stirred for 30 min. It has been reported by Hartmann[46] that if just using aqueous ethanol,[46,52] or heating the supernatant at 90 °C[46,48,52] proteinaceous material will still remain in the WEAX extracts causing purity issues. Therefore bentonite was chosen as a more suitable alternative for removing protein. The reason why aqueous ethanol is not a universally effective means of removing protein for cereal extractions lies in the diverse protein types and the different solubility characteristics of the four major cereal protein classes 1. albumins (soluble in water or dilute salt), 2. globulins (soluble in dilute salt, but insoluble in water), 3. gliadins (soluble in aqueous alcohol), and 4. glutenins (insoluble in aqueous alcohol).[53] For example, in wheat flour about 15% of the protein is soluble in water or aqueous salt solutions (albumins or globulins) and the remaining 85% are insoluble storage protein (gliadins and glutelins).[54] Others have also supported the use of clay as a more effective means of removing protein from wheat endosperm extractions.[8,11,12,19,46,55]

Precipitation of Arabinoxylan (WEAX). The bentonite suspension was centrifuged at 3220*g* for 10 min to separate protein that was adsorbed to the bentonite. The filtrate was then adjusted to pH 7.0 by addition of 0.1 mol L^{-1} NaOH. To selectively precipitate WEAX, ethanol was added while stirring to a final concentration of 60% (v/v). The solution was stirred for an additional 30 min and left to stand overnight at 25 °C. Precipitated WEAX was recovered by decanting most of the supernatant and subsequent centrifugation at 3220*g* for 10 min. The supernatant was kept for subsequent *β*-glucan precipitation. Precipitated WEAX was then washed once with 60% ethanol, twice with acetone, and air-dried. A 1% aqueous solution of the precipitate was then prepared, and the solution was lyophilized and powdered. [8]

Precipitation of Soluble *β*-Glucan. Ethanol was added to the supernatant after precipitation of WEAX to obtain a 74% final v/v concentration. The solution was stirred for 60 min and left to stand overnight at 25 °C. Water extractable *β*- glucan was recovered by decanting the supernatant and subsequent centrifugation at 3220*g* for 10 min. Precipitated samples were washed once with 70% ethanol, twice with acetone, and air-dried. A 1% aqueous solution of the precipitate was then prepared, and the solution was lyophilized and powdered.

Purification of Soluble *β*-Glucan. During the 74% ethanol precipitation, other components were coprecipitated with the barley *β*-glucan. To enable the isolation of pure *β*-glucan, the precipitate was suspended in 1 M NaOH[15,31,56,57] at 25 °C[15] for 12 h (solid to liquid ratio 100 mg:5 mL).[58,59] The alkaline extract was neutralized with 2 M HCl and centrifuged for 20 min at 5000*g* and 25 °C. A precipitate was obtained when the pH was between 8.5 and 7.0 upon neutralization with 2 M HCl. An analysis of the precipitate composition by ^{1}H NMR showed arabinoxylan (and arabinogalactan) with a minor amount of *β*-glucan (results not shown). The supernatant was removed after centrifugation and the residue was washed twice with distilled water. The washings were recovered and combined with the supernatant (from the previous centrifugation step), frozen and lyophilized, as shown in Figure 2.

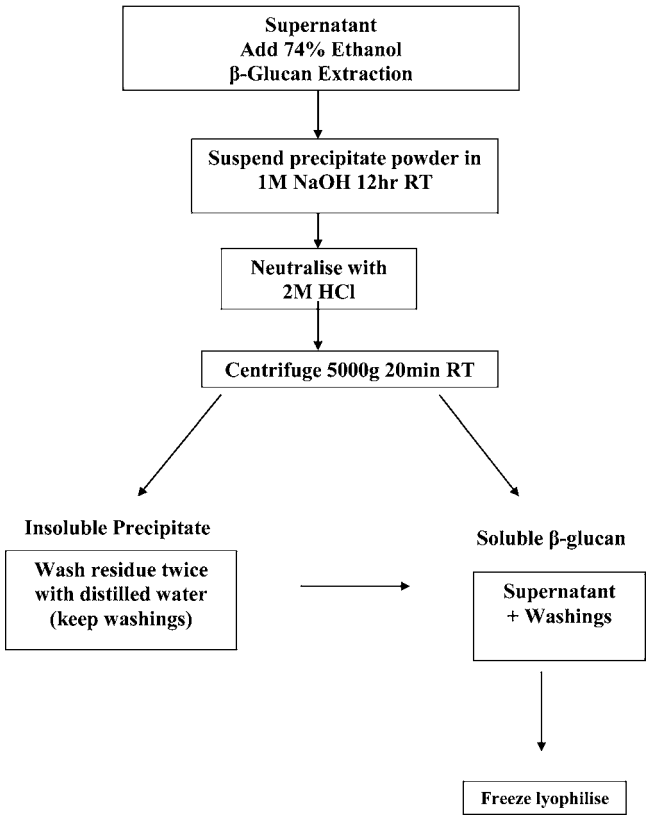

Figure 2. Purification of water extractable hull-less barley *β*- glucan.

RESULTS AND DISCUSSION

Composition of Original Endosperm Flours. In order to isolate both soluble and insoluble NSP's from the same original endosperm flour, the starch and protein, being the main components in the flour, need to be degraded and removed. The contents of glucose (primarily from starch), protein, total arabinoxylan and total *β*-glucan (Table 1) were determined so that the appropriate amounts of enzymes and clays could be selected. Clay is a proven method used to absorb the protein from the supernatant fraction during extraction of soluble NSP's.[8] The amount of clay used will depend on the amounts of protein in the endosperm flours. Protein amounts vary with differing cereal flours (Table 1).The enzymes *α*-amylase (and/or amyloglucosidase) and Pronase E are used frequently[13,46,51,60] to degrade starch and protein respectively from cell wall material while keeping the cell wall apparently intact. Cytoplasmic proteins are efficiently eliminated by the use of Pronase.[61] Morrison[61] has shown that Pronase, a mixture of endo- and exopeptidases is able to degrade proteins to amino acids, and is a more efficient agent for removal of intracellular proteins in grass samples compared to pepsin and sodium lauryl sulfate. Pronase has been used by others[24,62−64] for isolating purified cell walls from protein rich sources. The enzyme exhibits maximum activity at pH 7.5 and 55 °C.[51]

Total *β*-glucan levels for the *Finniss* hull-less barley reported in Table 1, are 7 times that of the levels in the wheat endosperm flour, and are comparable to the *Bevy* rye endosperm flour *β*-glucan amounts. Zheng, Holtekjølen, and Lee,[65−67] reported total *β*-glucan amounts in hull-less barley to be 2.48−2.95% (flour), and is higher than the values found here, but studies on *β*-glucan contents of endosperm hull-less barley flours have been limited.

The total AX endosperm flour results (Table 1) show that rye AX levels are much higher than wheat and barley, as expected.[1] In general, hull-less barley contains comparable AX

Table 1. Protein Composition, Arabinoxylan (AX) (Monosaccharide HPLC), and β-Glucan Analysis of Endosperm Flours

		HPLC Monosaccharide Release (%w/w)						
	protein content (%)	mannose	glucose	galactose	xylose	arabinose	total AX[a]	% β-glucan Megazyme (AOAC 995.16)
Barley Finnis								
Barley Finnis endosperm flour #1	6.9	0	84.3	0.03	0.8	0.6	1.3	1.4
Barley Finnis endosperm flour #2	7.2	0	77.9	0.03	0.8	0.6	1.2	1.7
Wheat Macro								
Wheat Macro endosperm flour #1	15	0.2	83.6	0.3	1.4	0.9	2	0.2
Wheat Macro endosperm flour #2	15.2	0.2	75.5	0.3	1.3	0.8	1.8	0.2
Rye Bevy								
Rye Bevy endosperm flour #1	10.4	0	76.3	0.04	3.2	2.4	4.9	1.2
Rye Bevy endosperm flour #2	10.6	0	71.5	0.04	2.9	2.2	4.6	1.2

[a]Total arabinoxylan contents have been calculated by adding the arabinose and xylose sugars and multiplying by 0.88 to account for the loss of the water molecule.

Table 2. Soluble Arabinoxylan (WEAX) Extract Results from Endosperm Flours

					HPLC monosaccharide release (%w/w)					
	SEC average molecular weight (daltons)	intrinsic viscosity η (dL/g)	WEAX yield/100 g flour	protein content (%)	mannose	glucose	galactose	xylose	arabinose	total AX[a]
Barley Finnis										
Barley WEAX RT #1	5.63×10^5		0.47	1.7	0.1	9.4	3.4	52.7	33.6	75.9
Barley WEAX RT #2					0.1	9.8	3.7	50.3	32.4	72.8
Barley WEAX 40 °C #1	4.56×10^5	4.57	0.42	1.7	0.2	9.8	3.9	51.1	32.3	73.5
Barley WEAX 40 °C #2		4.64			0.1	9.9	4.5	50.8	33.1	73.8
Wheat Macro										
Wheat WEAX #1	6.24×10^5	4.84	0.56	1.8	0.0	4.2	0.6	61.3	35.4	85.1
Wheat WEAX #2		4.62			0.0	3.1	0.4	58.8	33.7	81.4
Wheat AX Megazyme 29cSt	3.80×10^5	5.04								
Rye Bevy										
Rye WEAX #1	3.25×10^5	6.21	1.64	2.8	0.0	2.2	1.3	63.2	33.3	85.0
Rye WEAX #2		6.15			0.0	2.4	1.3	59.1	31.0	79.3
Rye AX Megazyme 33cSt	3.50×10^5	6.23								

[a]Total arabinoxylan contents have been calculated by adding the arabinose and xylose sugars and multiplying by 0.88 to account for the loss of the water molecule.

Table 3. Analysis of Purified Soluble β-glucan WEBG from Hull Less Barley Endosperm Flour Extract

					HPLC monosaccharide release (%w/w)					
	SEC weight average MWt (daltons)	intrinsic viscosity (dL/g)	WEBG yield/100 g Flour	protein content (%)	mannose	glucose	galactose	xylose	arabinose	% beta-glucan Megazyme (AOAC 995.16)
Before 1 M NaOH treatment										
Barley Finnis										
Barley BG 40C #1					3.2	35.6	19.9	14.2	26.0	
Barley BG 40C #2					3.1	36.6	18.4	15.5	26.1	
After 1 M NaOH Treatment										
Barley Finnis										
Barley BG RT #1	3.10×10^5	5.84	0.49	0.3	0.4	80.4	0.4	0.6	0.8	74.8
	6.95×10^4									
Barley BG 40C #1	3.10×10^5	5.96	0.47	0.2	0.4	91.2	0.4	0.7	0.7	93.0
	6.50×10^4				0.4	87.3	0.6	0.9	1.0	88.7
Barley BG Megazyme 28cSt	3.10×10^5	6.71								

levels to wheat,[68] but much higher (1-3, 1-4)-β-D-glucan levels than wheat,[69,70] as indicated in Table 1. Trogh et al.[71] reported total AX contents of hull-less European barley flours to be 1.16−1.53% which is in agreement with results found in this study.

Composition of Separated Purified Soluble NSP Results. *Purified Water Extractable Arabinoxylan (WEAX)*

Table 4. Purified Insoluble Cell Wall Composition Results

			HPLC monosaccharide release (%w/w)						
	cell wall yield/100 g flour	protein content (%)	mannose	glucose	galactose	xylose	arabinose	total AX[a]	% β-glucan Megazyme (AOAC 995.16)
Barley Finnis									
Barley Cell Wall #1	3.3	1.5	2.7	72.9	0.0	12.8	10.1	20.2	68.2
Wheat Macro									
Wheat Cell Wall #1	2.3	1.6	3.9	19.4	0.8	47.7	28.3	66.9	19.3
Wheat Cell Wall #2			3.2	19.5	0.7	47.7	28.2	66.8	19.1
Rye Bevy									
Rye Cell Wall #1	6.2	1.7	2.3	11.5	0.5	43.1	23.5	58.6	10.1
Rye Cell Wall #2			2.5	10.3	0.4	42.7	22.6	57.5	11.2

[a]Total arabinoxylan contents have been calculated by adding the arabinose and xylose sugars and multiplying by 0.88 to account for the loss of the water molecule.

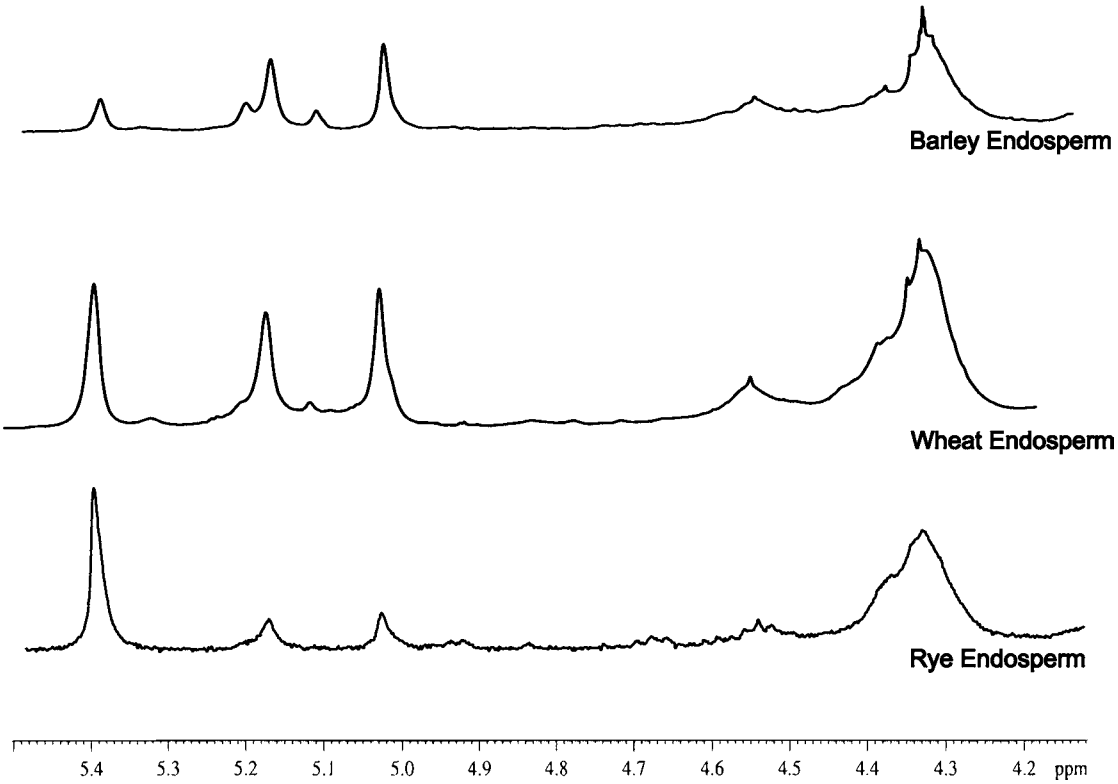

Figure 3. 1H-NMR spectra of WEAX fractions from hull-less barley, wheat, and rye endosperm flours. Monosubstituted arabinose O-3 at 5.40 ppm; disubstituted arabinose O-2,O-3 at 5.03 ppm, and 5.17 ppm, and xylose at 4.23−4.60 ppm. Traces of arabinogalactan can be seen in the hull-less barley and wheat endosperm spectra at 5.12 ppm.

Composition Results. For the WEAX or soluble extraction method, amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3) and bentonite clay were used to degrade the starch, and remove the protein, respectively. Optimal concentrations of bentonite clay needed to remove protein from wheat flour slurries (100g flour/1L water) have previously been found to be approximately 0.2% w/w.[8] Purity of the soluble extracts was evaluated by HPLC for monosaccharide analysis which showed that arabinose and xylose were the dominant sugars (Table 2).

SEC analysis gave weight average molecular weights ranging from 324 kDa to 625 kDa. Reported $M_w$ts of WEAX for wheat, barley, and rye are typically in the range of 200−300 kDa.[1] All of the WEAX extracts from the current method (Table 2) have higher values than this, which suggests that minimal depolymerisation occurred with the wheat, hull-less barley and rye WEAX polymers. WEAX average molecular weights are comparable to those of commercial (Megazyme) products. [42]

The WEAX yields obtained from this study ranged from 0.4 to 1.6 g/100g for the wheat, hull-less barley, and rye endosperm flours. Generally, wheat WEAX yields are reported to be approximately 0.3−1 g/100g.[7,72] Rye WEAX yield values are about 3 times that of wheat, being 1.0−1.5 g/100g flour.[5] Similar results were found in this study (Table 2). Holtekjølen[65] reported soluble AX results for various hull-less barley types to be 0.62−5.03%, while Trogh[71] found WEAX contents of European hull-less barley flours were 0.15−0.31%. This study indicates WEAX contents of 0.42−0.47%, in *Finniss* hull-less barley flour (Table 2).

Protein contents of the hull-less barley β-glucan and wheat, hull-less barley and rye arabinoxylan soluble extracts and insoluble cell wall extracts were analyzed and found to all be

less than 3% and typically less than 2% (Table 2, Table 3, and Table 4) compared to starting flour levels of 7−15% (Table 1), demonstrating that the enzyme and clay methods for the removal of protein were effective, bearing in mind that some protein is expected to be associated with the arabinogalactan found in the WEAX fraction and with the insoluble cell wall material.

Glucose contents for all extracts were determined using HPLC for monosaccharide analysis. The glucose contents for the barley WEAX, cell wall, and purified β-glucan extracts are higher than wheat and rye as barley contains higher levels of β-glucan, a polymer of glucose. The (1-3,1-4) β-glucan contents of the extracts were also measured to determine if the glucose detected was due to β-glucan alone and not residual starch. There was close agreement between β-glucan and glucose levels for barley, rye, and wheat cell wall residues (Table 4) showing that starch had been effectively removed from the insoluble residue.

Small amounts of galactose in WEAX extracts (Table 2) suggest the coprecipitation of traces of arabinogalactan. This was confirmed by the presence of a characteristic anomeric proton signal at 5.12 ppm in the ^{1}H NMR spectrum (Figure 3), particularly for barley WEAX.

Signals for mono and disubstituted arabinose residues in WEAX fractions illustrate characteristic differences between rye and wheat AX with the former having a larger relative amount of monosubstituted arabinose.[1,11] Barley WEAX is different from both rye and wheat in having a relatively lower level of monosubstituted arabinose and higher level of disubstitution as in Figure 3. [71]

The hull-less barley WEAX and β-glucan WEBG extractions were performed at both 40 °C and room temperature (RT ca. 20 °C) to test if the temperature difference affected the overall characterization results. The extractions did not show any significant differences in sugar composition or yield (Table 2 and Table 3). Intrinsic viscosities were also determined for the WEAX and hull-less barley WEBG samples at 37 °C. Values were approximately 4.57−6.21 dL/g for wheat, hull-less barley, and rye WEAX samples, and the hull-less barley WEBG (40 °C) samples were approximately 5.84−5.96 dL/g. Others[1,4] have reported intrinsic viscosity values between 2 and 6 dL/g for wheat, barley, oat, and triticale WEAX,[1] and higher values for rye, approximately 4−10 dL/g.[1] Barley β-glucan intrinsic viscosities have been reported to be between 4.6 and 6.9 dL/g,[4] and general cereal β-glucans 0.3−9.6 dL/g.[4,68] The values obtained in this study confirm the high molecular weight nature of the extracts.

FT-IR spectra for the WEAX rye and wheat fractions show excellent agreement with the Megazyme AX rye 33cSt, and Megazyme wheat AX 29cSt products, and barley WEAX also shows similar features (Figure 4). Robert et al.[7] have reported that each particular polysaccharide has a specific band maximum in the 1200−800 cm^{-1} region. For example, the linear and branched (1−4)-β-xylans have a band maximum at about 1047 cm^{-1},[7] as shown in Figure 4. In arabinogalactans, two strong bands are observed at about 1075 and 1045 cm^{-1} which characterize galactopyranose in the backbone and arabinofuranose units in side branches, respectively. [7]

Purified Water Extractable β-glucan (WEBG) Composition Results. Following precipitation of WEAX with 60% ethanol, the soluble β-glucan (WEBG) remains in the supernatant, but can be precipitated using 74% ethanol as shown in Figure 2. However, it appears that significant amounts of arabinoxylan

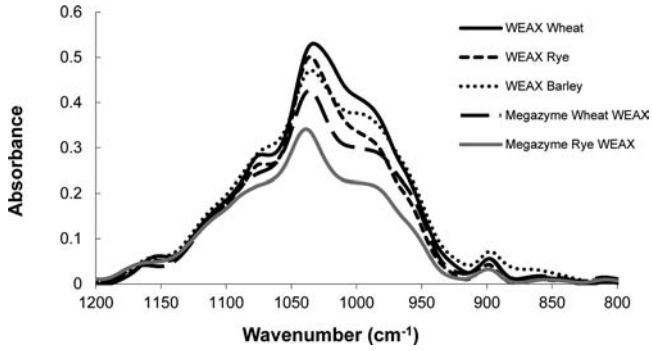

Figure 4. FT-IR spectra of WEAX fractions from wheat, hull-less barley, and rye endosperm flours in comparison with the Megazyme AX wheat 29cSt and Megazyme AX rye 33cSt.

and arabinogalactan coprecipitate with the β-glucans, as shown in ^{1}H NMR spectra (Figure 5, cf. Figure 3 for AX), and HPLC results in Table 3. Characteristic signals for arabinogalactan[47] are at 5.12 and 4.3 ppm, and are the major signals in the 74% ethanol precipitate from wheat (Figure 5).

To help remove arabinogalactan and arabinoxylan from the residue after precipitation with 74% ethanol, 1 M NaOH was used (Figure 2) which resulted in a purer form of β-glucan, as shown by ^{1}H NMR (Figure 5), and the HPLC results showing the % (w/w) monosaccharide before and after treatment with 1 M NaOH in Table 3. This purification is achieved through the precipitation of arabinoxylan (and arabinogalactan) during neutralization after the alkaline treatment. As a validation of this method, a mixture of pure wheat arabinoxylan and barley β-glucan was dissolved, precipitated with 74% ethanol and subjected to the alkali treatment shown in Figure 2. ^{1}H NMR analysis showed that the precipitate obtained on neutralization after alkaline treatment was largely arabinoxylan with a small amount of β-glucan, and the supernatant was essentially pure β-glucan. The relatively high solution concentration (20 mg/mL) and salt concentration (1 M) are probably the key factors which result in arabinoxylan precipitation.

The SEC data for the β-glucan WEBG purified extracts, gave a peak apparent molecular weight of 3.1 × 10^5 Da, identical to that for the barley β-glucan 28cSt Megazyme product. All WEBG purified extracts showed the same peak apparent molecular weight, but also contained small but varying amounts of a lower molecular size fraction averaging 6−7 × 10^4 Da. We conclude that this smaller fraction represents the products of alkali degradation which occurred to varying extents for repeat treatments. The purification using 1 M NaOH therefore appears to have reduced the molecular weight of a portion of the β-glucan.[15,27] Others have reported molecular weight values for cereal β-glucans in the range of 65−3100 × 10^3 for oats, 31−2700 × 10^3 for barley, 21−1100 × 10^3 for rye, and 209−487 × 10^3 for wheat.[1,73] The fact that the intrinsic viscosity values for the β-glucans isolated in this study (5.84, 5.96, Table 3) are only slightly lower than that for the commercial standard with the same peak molecular weight (6.71, Table 3) suggests that the extent of degradation is not major.

The purified soluble barley β-glucan WEBG yield results in Table 3 are approximately 0.48 g/100g flour. Lee et al.[67] reported soluble β-glucan 2.65% (insoluble β-glucan 1.73%) in Bowman hull-less barley milled groats. Collins et al.[4] described water extractable β-glucan levels of 1.7−2.6% in barley cereal grain. However, specific reports on water extractable β-glucan contents in endosperm hull-less barley flour have not been

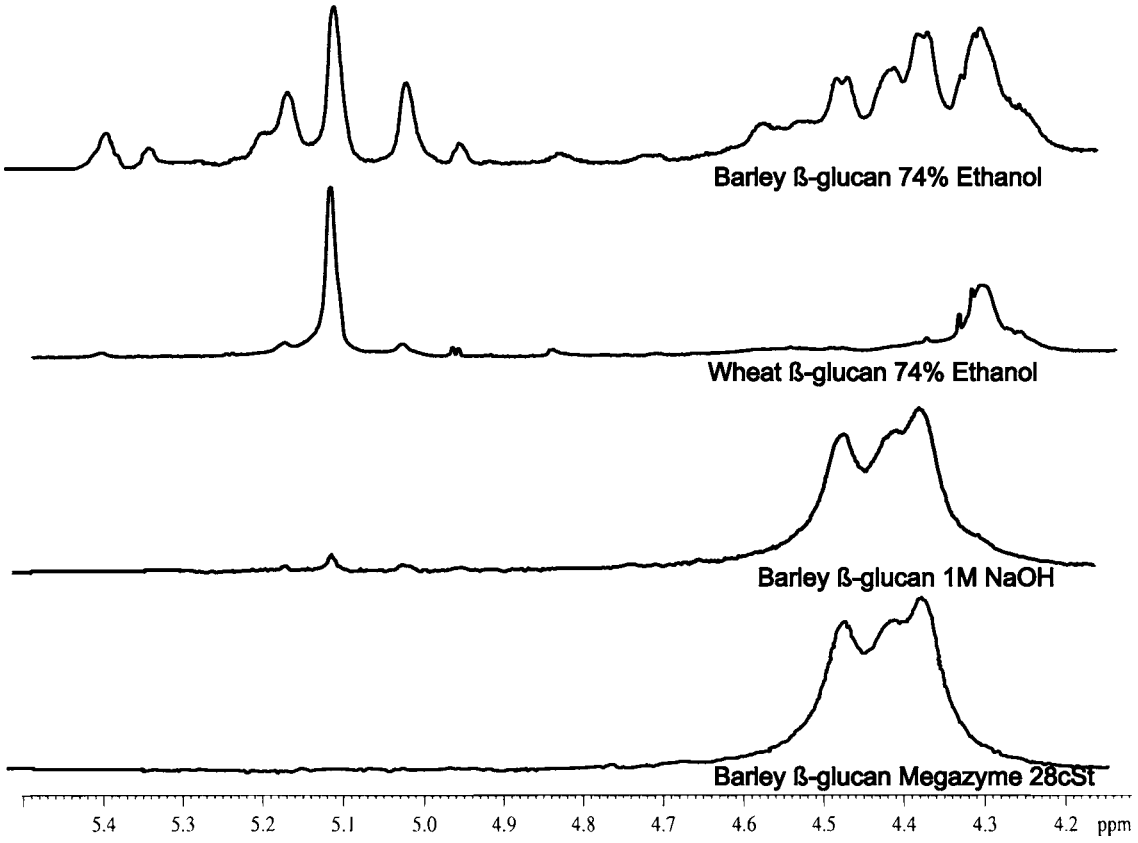

Figure 5. 1H-NMR spectra for hull-less barley β-glucan purified with 1 M NaOH treatment after 74% ethanol precipitation, compared with barley and wheat after 74% ethanol precipitation. The Megazyme barley β-glucan 28cSt product was used for comparison.

found. The FT-IR results shown in Figure 6, show the characteristic (1−4)-β-glucan absorption band at around 1030

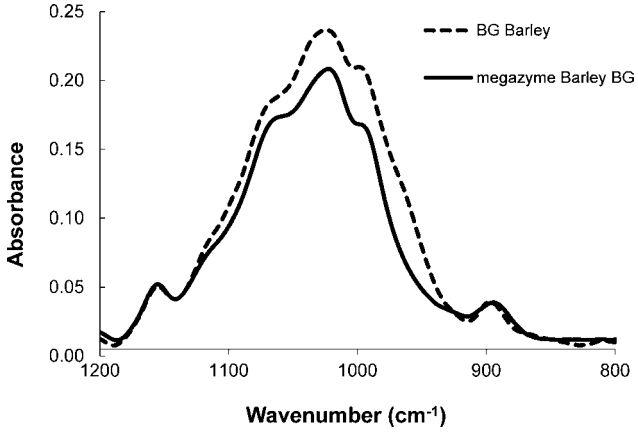

Figure 6. FT-IR spectra of purified hull-less barley and Megazyme barley β-glucans.

cm^{-1}.[7] Both the purified β-glucan extract and the Megazyme barley β glucan have similar FT-IR spectra, clearly different to AX (Figure 4).

Composition of Purified Insoluble Cell Wall Residues. Previous reports[2−4,7] indicate that AX amounts in the endosperm cell walls of wheat and rye are approximately 70%, 65%, respectively, and barley endosperm cell walls contain approximately 20%. Conversely the β-glucan levels in the endosperm cell walls of wheat and rye are approximately 20% and 12%, respectively, and that of barley and oats is approximately 70 and 80%, respectively. The results in Table 4 indicate that the rye and wheat endosperm insoluble cell walls contain approximately 60 and 70% AX, respectively, and the hull-less barley endosperm cell wall has approximately 20%. There are no previous reports of AX contents in the cell walls of endosperm hull-less barley flours. Table 4 also indicated approximately 68% β-glucan levels in the endosperm cell wall of hull-less barley and approximately 10% and 19% β-glucan in the cell walls of rye and wheat respectively. This is in line with other reports on endosperm cell wall contents.[2−4,7] The similarity in relative amounts of AX and β-glucan in total endosperm and water-unextractable fractions is not surprising given the relatively low percentage solubility values for wheat, rye and barley endosperm flours found in this study. The fact that the water-unextractable fraction (and the endosperm cell wall itself) contains predominantly AX and β-glucan (Table 4) is reflected in FT-IR spectra (Figure 7).

Bands at 1030 cm^{-1} for (1−4) β-glucans can be seen in the barley water unextractable fraction (endosperm cell wall) FT-IR spectra. While the FTIR spectra for the wheat and rye cell walls are very similar, the major signal at 1047 cm^{-1} found for purified soluble arabinoxylan from rye and wheat (WEAX) extractions in Figure 4 does not appear to be as pronounced. Also a feature present at approximately 928 cm^{-1} was not found in either AX or BG spectra and needs further investigation. Although Robert et al.[7] have investigated the main individual cell wall polymers as water-soluble subfractions namely arabinogalactan, arabinoxylans and β-glucans, the present results suggest that when these polymers are integrated within cell walls, the FTIR spectra are not a simple sum of relevant NSP polymers.

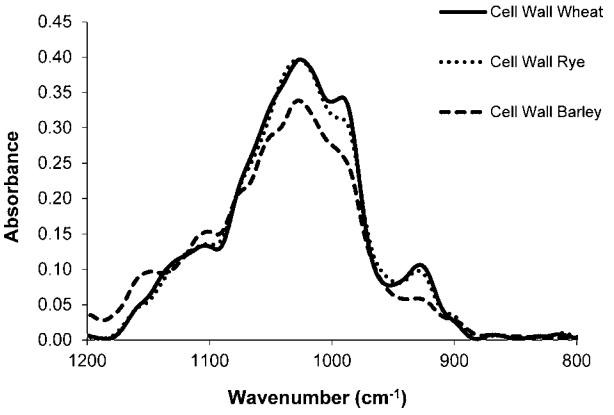

Figure 7. FT-IR of purified insoluble cell wall fractions from wheat, rye, and hull-less barley endosperm flours.

Applicability in Structure–Nutrition Studies. The approach described satisfies the criteria of (a) separation of soluble and insoluble phases without using organic solvents or other treatments that would be expected to modulate polymer solubility, (b) efficient removal of both starch and protein from each of soluble and insoluble fractions, and (c) separation of solubilized arabinoxylan- and β-glucan for detailed structural analysis including molecular size. The water extractable and water-unextractable (cell wall) components from the endosperm flours of rye, wheat, and hull-less barley have been successfully separated, purified, and characterized under conditions that are suitable for investigating structure–nutrition relationships.[74] Water-soluble extracts have been further separated into relatively pure arabinoxylan and β-glucan preparations by precipitation with 60% and then 74% ethanol with subsequent alkali treatment, respectively. Molecular sizes of purified soluble polymers were sufficiently high to suggest that depolymerisation was avoided (apart from limited depolymerisation following alkali treatment), and characteristic substitution differences were observed between wheat, rye, and barley arabinoxylans. Water unextractable fractions were also shown to be free from starch and low in protein. FTIR spectroscopy can be used to quickly and easily characterize both purified polymers and the mixtures present within the water insoluble fraction without any sample loss due to preparation, although cell wall spectra may not be a simple sum of component spectra due to apparent wall architecture effects. However to adequately verify extraction purity and specific NSP polymer structure, the FTIR should be used in conjunction with other more specific analyses such as ^{1}H NMR and HPLC monosaccharide analysis. Because this methodology avoids the use of organic solvents or alkali prior to the separation into soluble and insoluble fractions, it is also suitable for use in studies of the effects of food processing on structure-nutrition relationships.

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Notes

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ABBREVIATIONS USED

WEAX,water extractable arabinoxylan; WEBG,water extractable β-glucan; AX,arabinoxylan; BG,β-glucan; AG,arabinogalactan; WU,water unextractable; NSP,non starch polysaccharides

REFERENCES

(1) Saulnier, L.; Guillon, F.; Sado, P.; Rouau, X. *Plant Cell Wall Polysaccharides in Storage Organs: Xylans (Food Applications)*; Elsevier Ltd., 2007.

(2) Shewry, P. R. Wheat. *J. Exper. Bot.* **2009**, *60*, 1537–1553.

(3) Cui, S.; Wang, Q. Cell wall polysaccharides in cereals: Chemical structures and functional properties. *Struct. Chem.* **2009**, *20*, 291–297.

(4) Collins, H. M.; Burton, R. A.; Topping, D. L.; Liao, M.-L.; Bacic, A.; Fincher, G. B. Review: Variability in fine structures of noncellulosic cell wall polysaccharides from cereal grains: Potential importance in human health and nutrition. *Cereal Chem.* **2010**, *87*, 272–282.

(5) Saulnier, L.; Sado, P.-E.; Branlard, G.; Charmet, G.; Guillon, F. Wheat arabinoxylans: Exploiting variation in amount and composition to develop enhanced varieties. *J. Cereal Sci.* **2007**, *46*, 261–281.

(6) Girhammar, U.; Nair, B. M. Isolation, separation and characterization of water soluble non-starch polysaccharides from wheat and rye. *Food Hydrocolloids* **1992**, *6*, 285–299.

(7) Robert, P.; Marquis, M.; Barron, C.; Guillon, F.; Saulnier, L. FT-IR investigation of cell wall polysaccharides from cereal grains. Arabinoxylan infrared assignment. *J. Agric. Food Chem.* **2005**, *53*, 7014–7018.

(8) Ganguli, N. K.; Turner, M. A. A simplified method for extracting water-extractable arabinoxylans from wheat flour. *J. Sci. Food Agric.* **2008**, *88*, 1905–1910.

(9) Muralikrishna, G.; Rao, M.; Subba, V. Cereal non-cellulosic polysaccharides: Structure and function relationship—an overview. *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 599–610.

(10) Nyström, L.; Lampi, A.-M.; Andersson, A. A. M.; Kamal-Eldin, A.; Gebruers, K.; Courtin, C. M.; Delcour, J. A.; Li, L.; Ward, J. L.; Fraś, A.; Boros, D.; Rakszegi, M.; Bedő, Z.; Shewry, P. R.; Piironen, V. Phytochemicals and dietary fibre components in rye varieties in the healthgrain diversity screen. *J. Agric. Food Chem.* **2008**, *56*, 9758–9766.

(11) Delcour, J. A.; Rouseu, N.; Vanhaesendonck, I. P. Pilot-scale isolation of water-extractable arabinoxylans from rye. *Cereal Chem.* **1999**, *76* (1), 1–2.

(12) Vinkx, C.; Reynaert, H.; Grobet, P.; Delcour, J. Physicochemical and functional properties of rye nonstarch polysaccharides 5. Variability in the structure of water-soluble arabinoxylans. *Cereal Chem.* **1993**, *70*, 311–317.

(13) Hoffmann, R. A.; Roza, M.; Maat, J.; Kamerling, J. P.; Vliegenthart, J. F. G. Structural characteristics of the cold-water-soluble arabinoxylans from the white flour of the soft wheat variety Kadet. *Carbohydr. Polym.* **1991**, *15*, 415–430.

(14) Woodward, J.; Fincher, G.; Stone, B. Water-soluble (1-3), (1-4)-β-D-glucans from barley (hordeum vulgare) endosperm. II. Fine structure. *Carbohydr. Polym.* **1983**, *3*, 207–225.

(15) Carr, J. M.; Glatter, S.; Jeraci, J. L.; Lewis, B. A. Enzymic determination of β-glucan in cereal based food products. *Cereal Chem.* **1990**, *67*, 226–229.

(16) Åman, P.; Bengtsson, S. Periodate oxidation and degradation studies on the major water-soluble arabinoxylan in rye grain. *Carbohydr. Polym.* **1991**, *15*, 405−414.
(17) Debyser, W.; Schooneveld-Bergmans, M. E. F.; Derdelinckx, G.; Grobet, P. J.; Delcour, J. A. Nuclear magnetic resonance and methylation analysis derived structural features of water-extractable arabinoxylans from barley (*Hordeum vulgare* L.) malts. *J. Agric. Food Chem.* **1997**, *45*, 2914−2918.
(18) Bengtsson, S.; Åman, P. Isolation and chemical characterization of water-soluble arabinoxylans in rye grain. *Carbohydr. Polym.* **1990**, *12*, 267−277.
(19) Izydorczyk, M. S.; Biliaderis, C. G. Cereal arabinoxylans: Advances in structure and physicochemical properties. *Carbohydr. Polym.* **1995**, *28*, 33−48.
(20) Izydorczyk, M.; Biliaderis, C., Structural and functional aspects of cereal arabinoxylans and β-glucans. In *Developments in Food Science*; Doxastakis, G., Kiosseoglou, V., Eds.; Elsevier, 2000; Vol. *41*, pp 361−384.
(21) Rattan, O.; Izydorczyk, M.; Biliaderis, C. Structure and rheological behaviour of arabinoxylans from canadian bread wheat flours. *Lebensm.-Wiss. Technol.* **1994**, *27*, 550−555.
(22) Vinkx, C.; Delcour, J.; Vannieuwenhove, C. Physiochemical and functional properties of rye nonstarch polysaccharides 3. Oxidative gelation of a fraction containing water-soluble pentosans and proteins. *Cereal Chem.* **1991**, *68*, 617−622.
(23) Vinkx, C.; Delcour, J.; Verbruggen, M.; Gruppen, H. Rye water-soluble arabinoxylans also vary in their 2-monosubstituted xylose content. *Cereal Chem.* **1995**, *72*, 227−228.
(24) Vinkx, C.; Stevens, I.; Gruppen, H.; Grobet, P.; Delcour, J. Physiochemical and functional-properties of rye nonstarch polysaccharides 6. variability in the structure of water-unextractable arabinoxylans. *Cereal Chem.* **1995**, *72*, 411−418.
(25) Fincher, G. Morphology and chemical composition of barley endosperm cell walls. *J. Inst. Brew* **1975**, *81*, 116−122.
(26) Fleming, M.; Kawakami, K. Studies of the fine structure of β-D-glucan of barleys extracted at different temperatures. *Carbohydr. Res.* **1977**, *57*, 15−23.
(27) Beer, M. U.; Wood, P. J.; Weisz, J. Molecular weight distribution and (1-3)(1-4)-β-D-glucan content of consecutive extracts of various oat and barley cultivars. *Cereal Chem.* **1997**, *74*, 476−480.
(28) Colleoni-Sirghie, M.; Fulton, D.; White, P. Structural features of water soluble (1-3)(1-4)-β-D-glucans from high β-glucan and traditional oat lines. *Carbohydr. Polym.* **2003**, *54*, 237−249.
(29) Rimsten, L.; Stenberg, T.; Andersson, R.; Andersson, A.; Aman, P. Determination of β-glucan molecular weight using SEC with calcofluor detection in cereal extracts. *Cereal Chem.* **2003**, *80*, 485−490.
(30) Brummer, Y.; Jones, S.; Tosh, S. M.; Wood, P. J. Extraction and physicochemical characterization of rye β-glucan and effects of barium on polysaccharide molecular weight. *Cereal Chem.* **2008**, *85*, 174−181.
(31) Ragaee, S.; Wood, P.; Wang, Q.; Tosh, S.; Brummer, Y. Extractability, structure and molecular weight of β-glucan from canadian rye (*secale cereale L.*) whole meal. *Cereal Chem.* **2008**, *85*, 283−288.
(32) Nilsson, M.; Saulnier, L.; Andersson, R.; Aman, R. Water unextractable polysaccharides from three milling fractions of rye grain. *Carbohydr. Polym.* **1996**, *30*, 229−237.
(33) Roubroeks, J.; Andersson, R.; Aman, P. Structural features of (1-3),(1-4) β D-glucan and arabinoxylan fractions isolated from rye bran. *Carbohydr. Polym.* **2000**, *42*, 3−11.
(34) Ragaee, S.; Wood, P.; Wang, Q.; Tosh, S.; Brummer, Y.; Huang, X. Isolation, fractionation and structural characteristics of β-glucan from rye (*Secale cereale* L) whole meal. *Cereal Chem.* **2008**, *85*, 289−294.
(35) Beresford, G.; Stone, B. (1-3),(1-4) β-D-glucan content of *triticum* grains. *Cereal Sc* **1983**, *1*, 111−114.
(36) Li, W.; Cui, S. W.; Kakuda, Y. Extraction, fractionation, structural and physical characterization of wheat β-d-glucans. *Carbohydr. Polym.* **2006**, *63*, 408−416.
(37) Maes, C.; Delcour, J. Structural characterisation of water-extractable and water-unextractable arabinoxylans in wheat bran. *J. Cereal Sci.* **2001**, *35*, 315−326.
(38) Cui, W.; Wood, P. J. Relationships between structural features, molecular weight and rheological properties of cereal β-D-glucan. In *Hydrocolloids, Part 1: Physical Chemistry and Industrial Application of Gels, Polysaaccharides, And Proteins*; Nishinari, K., Ed.; Elsevier: Amsterdam, Netherlands, 2000; pp 159−168.
(39) Westerlund, E.; Andersson, R.; Åman, P.; Theander, O. Effects of baking on water-soluble non-starch polysaccharides in white bread fractions. *J. Cereal Sci.* **1990**, *12*, 33−42.
(40) Debyser, W.; Schooneveld Bergmans, M E F; Derdelinckx, G.; Grobet, P J; Delcour, J A Nuclear magnetic resonance and methylation analysis-derived structural features of water-extractable arabinoxylans from barley (*hordeum vulgare L*) malts. *J. Agric. Food Chem.* **1997**, *45*, 2914−2918.
(41) Burton, R. A.; Collins, H. M.; Kibble, N. A. J.; Smith, J. A.; Shirley, N. J.; Jobling, S. A.; Marilyn, H.; Singh, R. R.; Pettolino, F.; Wilson, S. M.; Bird, A. R.; Topping, D. L.; Bacic, A.; Fincher, G. B. Over-expression of specific HvCslF cellulose synthase-like genes in transgenic barley increases the levels of cell wall (1-3;1-4)-β-D-glucans and alters their fine structure. *Plant Biotechnol. J.* **2011**, *9*, 117−135.
(42) Shelat, K.; Vilaplana, F.; Nicholson, T.; Wong, K.; Gidley, M.; Gilbert, R. Diffusion and viscosity in arabinoxylan solutions: Implications for nutrition. *Carbohydr. Polym.* **2010**, *82*, 46−53.
(43) Tizzotti, M. J.; Sweedman, M. C.; Tang, D.; Schaefer, C.; Gilbert, R. G. New ^{1}H NMR procedure for the characterization of native and modified food-grade starches. *J. Agric. Food Chem.* **2011**, *59*, 6913−6919.
(44) Gomez, C.; Navarro, A.; Manzanares, P.; Horta, A.; CarbonelI, J. Physical and structural properties of barley (1-3),(1-4)-β-D-glucan. Part II. Viscosity, chain stiffness and macromolecular dimensions. *Carbohydr. Polym.* **1997**, *32*, 17−22.
(45) Gomez, C.; Navarro, A.; Garnier, C.; Horta, A.; Carbonell, J V Physical and structural properties of barley (1- 3),(1−4)-β-D-glucan—III. Formation of aggregates analysed through its viscoelastic and flow behaviour. *Carbohydr. Polym.* **1997**, *34*, 141−148.
(46) Hartmann, G.; Piber, M.; Koehler, P. Isolation and chemical characterisation of water-extractable arabinoxylans from wheat and rye during breadmaking. *Eur. Food Res. Technol.* **2005**, *221*, 487−492.
(47) Loosveld, A.-M. A.; Grobet, P. J.; Delcour, J. A. Contents and structural features of water-extractable arabinogalactan in wheat flour fractions. *J. Agric. Food Chem.* **1997**, *45*, 1998−2002.
(48) Cleemput, G.; Roels, S. P.; Van Oort, M.; Grobet, P. J.; A, D. J. Heterogeneity in the structure of water-soluble arabinoxylans in european wheat flours of variable bread making quality. *Cereal Chem.* **1993**, *70*, 324−329.
(49) Bunzel, M.; Ralph, J.; Marita, J. M.; Hatfield, R. D.; Steinhart, H. Diferulates as structural components in soluble and insoluble cereal dietary fibre. *J. Sci. Food Agric.* **2001**, *81*, 653−660.
(50) Mares, D.; Stone, B. Studies on wheat endosperm I. Chemical composition and ultrastructure of the cell walls. *Aust. J. Biol. Sci.* **1973**, *26*, 793−812.
(51) Brillouet, J. M.; Rouau, X.; Hoebler, C.; Barry, J. L.; Carre, B.; Lorta, E. A new method for determination of insoluble cell walls and soluble nonstarchy polysaccharides from plant materials. *J. Agric. Food Chem.* **1988**, *36*, 969−979.
(52) Cleemput, G.; Booij, C.; Hessing, M.; Gruppen, H.; Delcour, J. A. Solubilisation and changes in molecular weight distribution of arabinoxylans and protein in wheat flours during bread-making, and the effects of endogenous arabinoxylan hydrolysing enzymes. *J. Cereal Sci.* **1997**, *26*, 55−66.
(53) Hurkman, W. J.; Tanaka, C. K. Extraction of wheat endosperm proteins for proteome analysis. *J. Chromatogr., B* **2007**, *849*, 344−350.
(54) Sievert, D.; Hoseney, R. C.; Delcour, J. A., Bread and other baked products. In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH: Weinheim, Germany, 2007; *Vol 6*, pp 1−60.

(55) Rattan, O.; Izydorczyk, M.; Biliaderis, C. G. Structure and rheological behaviour of arabinoxylans from Canadian bread wheat flours. *Lebensm.-Wiss. Technol.* **1994**, *27*, 550−555.
(56) Knuckles, B. E.; Yokoyama, W. H.; Chiu, M. M. Molecular characterization of barley β-glucans by size-exclusion chromatography with multiple-angle laser light scattering and other detectors. *Cereal Chem. J.* **1997**, *74*, 599−604.
(57) Bhatty, R. S. Laboratory and pilot plant extraction and purification of β-glucans from hull-less barley and oat brans. *J. Cereal Sci.* **1995**, *22*, 163−170.
(58) Cui, W.; Wood, P. J.; Weisz, J.; Beer, M. U. Nonstarch polysaccharides from preprocessed wheat bran: Carbohydrate analysis and novel rheological properties. *Cereal Chem.* **1999**, *76*, 129−133.
(59) Cui, W.; Wood, P. J.; Blackwell, B.; Nikiforuk, J. Physicochemical properties and structural characterization by two-dimensional NMR spectroscopy of wheat β-D-glucan—Comparison with other cereal β-D-glucans. *Carbohydr. Polym.* **2000**, *41*, 249−258.
(60) Ordaz-Ortiz, J.; Saulnier, L. Structural variability of arabinoxylans from wheat flour. Comparison of water-extractable and xylanase-extractable arabinoxylans. *J. Cereal Sci.* **2005**, *42*, 119−125.
(61) Morrison, I. M. The isolation of plant cell wall preparations with low nitrogen contents. *J. Agric. Sci.* **1973**, *80*, 407−410.
(62) Selvendran, R. R. Analysis of cell wall material from plant tissues: Extraction and purification. *Phytochemistry* **1975**, *14*, 1011−1017.
(63) Brillouet, J.-M.; Carré, B. Composition of cell walls from cotyledons of *Pisum sativum*, *Vicia faba* and *Glycine max*. *Phytochemistry* **1983**, *22*, 841−847.
(64) Diisterhoft, E.-M.; Voragen, A. G. J. Non-starch polysaccharides from sunflower (*Helianthus annuus*) meal and palm kernel (*Elaeis guineenis*) meal-preparation of cell wall material and extraction of polysaccharide fractions. *J. Sci. Food Agric.* **1991**, *55*, 411−422.
(65) Holtekjølen, A. K.; Uhlen, A. K.; Bråthen, E.; Sahlstrøm, S.; Knutsen, S. H. Contents of starch and non-starch polysaccharides in barley varieties of different origin. *Food Chem.* **2006**, *94*, 348−358.
(66) Zheng, X.; Li, L. a.; Wang, Q. Distribution and molecular characterization of β-glucans from hull-less barley bran, shorts and flour. *Int. J. Mol. Sci.* **2011**, *12*, 1563−1574.
(67) Lee, C. J.; Horsley, R. D.; Manthey, F. A.; Schwarz, P. B. Comparisons of β-glucan content of barley and oat. *Cereal Chem. J.* **1997**, *74*, 571−575.
(68) Izydorczyk, M. S.; Dexter, J. E. Barley β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products-a review. *Food Res. Int.l* **2008**, *41*, 850−868.
(69) Bhatty, R. The potential of hull-less barley. *Cereal Chem.* **1999**, *76*, 589 −599.
(70) Trogh, I.; Courtin, C. M.; Andersson, A. A. M.; Aman, P.; Sørensen, J. F.; Delcour, J. A. 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)-β-D-glucan levels. *J. Cereal Sci.* **2004**, *40*, 257−267.
(71) Trogh, I.; Courtin, C. M.; Delcour, J. A. Isolation and characterization of water-extractable arabinoxylan from hull-less barley flours. *Cereal Chem.* **2004**, *81*, 576−581.
(72) Van Der Borght, A.; Goesaert, H.; Veraverbeke, W. S.; Delcour, J. A. Fractionation of wheat and wheat flour into starch and gluten: Overview of the main processes and the factors involved. *J. Cereal Sci.* **2005**, *41*, 221−237.
(73) Lazaridou, A.; Biliaderis, C. G. Molecular aspects of cereal β-glucan functionality: Physical properties, technological applications and physiological effects. *J. Cereal Sci.* **2007**, *46*, 101−118.
(74) Gidley, M. J. Hydrocolloids in the digestive tract and related health implications. *Curr. Opin. Colloid Interface Sci.* **2013**, *18*, 371−378.