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Effect of pretreatment on arabinoxylan distribution in wheat bran

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ABSTRACT

Arabinoxylan is one of the potential key products of a wheat bran based biorefinery. To develop a suitable process for the isolation of arabinoxylans, the effect of different processing approaches needs to be determined. In this work, chemical analysis was supplemented by immunolocalization of arabinoxylan by confocal microscopy, which proved valuable in the assessment of cell-structural changes occurring upon different chemical and mechanical bran treatments. The influences of acid, lye and hydrogen peroxide treatment, ball-milling, extrusion, fermentation and treatment with esterase, xylanase and a combination thereof were investigated.

Extensive ball-milling showed the best selectivity for harvesting arabinoxylan. Chemical treatments gave the highest yields, but did so at the cost of selectivity. Fermentative and enzymatic treatments were hampered by coextraction of other compounds.

1. Introduction

Wheat bran, the outer layer of the wheat kernel, is an abundant by-product of white flour production. Given its high content of nutritionally valuable and technologically desirable compounds, it is gaining interest as a raw material for biorefineries (Apprich et al., 2014). However, these components are either embedded in a complex and recalcitrant matrix or constitute this matrix themselves. This makes pretreatment imperative for down-stream processing. The choice of an appropriate pretreatment as well as suitable parameters is far from trivial and has to be adjusted according to the objective of the fractionation (Prückler et al., 2014).

Arabinoxylan is the most abundant valuable in wheat bran at about 32% of total dry matter (Maes & Delcour, 2002). As a dietary fiber substituted with hydroxycinnamates, such as ferulic and *p*-coumaric acid, arabinoxylan exhibits both nutritional and rheological benefits (Bauer, Harbaum-Piayda, Stockmann, & Schwarz, 2013; Berlanga-Reyes, Carvajal-Millan, Lizardi-Mendoza, Islas-Rubio, & Rascon-Chu, 2011; Hopkins et al., 2003). There is little consensus on the most advantageous methodology for selective extraction. Approaches range from chemical treatments, such as lye-based or oxidizer-assisted (Bataillon, Mathaly, Cardinali, & Duchiron, 1998; Maes & Delcour, 2001; Sun, Cui, Gu, & Zhang, 2011), over enzymatic treatments (Swennen, Courtin, Lindemans, & Delcour, 2006) to mechanical treatments, such as ball-milling (Van Craeyveld et al., 2009).

Few studies have been undertaken to compare the effectiveness of different treatments. Zhou et al. (2010) have compared peroxide-assisted alkaline extraction with enzymatic extraction and found the former to be more effective by 50%. However, they also found significant structural differences in the isolated products, which has to be taken into consideration for the intended application. Given the complex matrix of wheat bran, high yields usually only come at the cost of extensive treatment times or harsh conditions, which cause degradation of product and residual material, and challenges economic and ecological feasibility. Regardless whether the aim is to isolate arabinoxylan or to remove it in the purification of other target compounds, tracking its course is mandatory.

The aim of this study was to evaluate the efficacy of a wide spectrum of pretreatments with regard to their impact on arabinoxylan distribution and extractability. Evaluated pretreatments were acid, lye and hydrogen peroxide treatment, ball-milling, extrusion, fermentation and treatment with esterase, xylanase and a combination thereof. The effect of the pretreatments was assessed based on the carbohydrate content and distribution after

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thorough washing of the solid residue. An increase in extractability corresponds to a reduced amount of the solid sample. Three analytical approaches were chosen: quantification of the carbohydrate content by methanolysis, generating profiles of the carbohydrate distribution by enzymatic peeling and imaging of the arabinoxylan distribution by fluorescence labeling.

Carbohydrate content can be quantified after acidic cleavage of polysaccharides into monosaccharides. The favored method is methanolysis combined with GC–MS or GC–FID analysis, due to low degradation of the comparatively frail pentoses (Sundberg, Sundberg, Lillandt, & Holmbom, 1996; Willför et al., 2009). The composition of the carbohydrate fraction of the sample is gained and expressed as monosaccharide content. Being a mild method, methanolysis cannot decompose crystalline cellulose. Therefore, the glucose measured in this analysis is derived from residual starch, β-glucan and amorphous cellulose only. However, it cannot resolve arabinoxylan spatially which is a major requirement in the assessment of a pretreatment.

Hence, samples were also subject to an enzymatic peeling in order to obtain a cross-sectional profile of polysaccharide distribution (Sjöberg, Potthast, Rosenau, Kosma, & Sixta, 2005). The washed solids were treated with specific enzymes and the released monosaccharides in the supernatant were quantified. Thus, it was determined whether the effect of the pretreatment was only superficial or was affecting the whole sample.

The effects of the pretreatments were further elucidated by imaging the arabinoxylan distribution by immunolocalization in combination with confocal microscopy. Fluorescent-labeled monoclonal antibodies raised against specific polysaccharide epitopes allow for the localization of cell wall components, such as arabinoxylan, on a microscopic scale (McCartney, Marcus, & Knox, 2005). This technique has been used to map the polysaccharide distribution in cell walls of different wood types and to gain insight into the formation of cell walls (Donaldson, 2009; Donaldson & Knox, 2012). So far, immunolocalization has found little application in wheat bran research. To our knowledge it has been employed exclusively to follow xylanase-mediated degradation of arabinoxylan (Beaugrand et al., 2004a; Beaugrand, Reis, Guillon, Debeire, & Chabbert, 2004b).

2. Materials and methods

2.1. Sample preparation

Samples are classified in three treatment categories: chemical, mechanical and fermentation/enzymatical treatment. Chemical treatments were acid treatment (sample ID 1.1), lye treatment (1.2) and hydrogen peroxide treatment (1.3). Mechanical treatments were ball milling for 5 min (2.1) and 60 min (2.2) and extrusion (2.3). Fermentation and enzymatic treatments were fermentation with *Lactobacillus plantarum* (3.1); treatment with esterase (3.2); xylanase (3.3); a combination of esterase and xylanase (3.4); and a combination of *Lactobacillus brevis*, esterase and xylanase (3.5).

2.1.1. Chemical treatments

10 g of wheat bran were stirred at 60 °C for 4 h in one of the following: 200 mL of 1 M sulfuric acid, 1 M sodium hydroxide or 2% hydrogen peroxide solution adjusted with sodium hydroxide to pH 11.5 (all Sigma-Aldrich, St. Louis, MO, USA). The resulting slurries were filtrated and the residues were washed three times with 100 mL of water. Samples were freeze-dried before further analysis.

2.1.2. Mechanical treatments

Wheat bran was extruded using a Bühler Twin Screw Extruder BCTL 42/20D model (Bühler Group, Uzwil, Switzerland) without preconditioner. SME (specific mechanical energy) input was 179 Wh/kg at a maximum temperature of 133 °C.

Wheat bran was ball-milled with a Retsch MM 2000 ball-mill (Retsch, Haan, Germany) using 25 mL containers at about 10% loading with a single 25 mm stainless steel ball at 20 Hz. Milling times were 5 and 60 min.

2.1.3. Fermentation and enzymatic treatments

Wheat bran was inoculated with 2% w/w of bacterial culture for fermentation or 1% w/w of enzyme. After addition of 50% v/w of physiological sodium chloride solution samples were kept at room temperature for five days and then dried at 40 °C. Samples were treated with esterase Sternzym FSR 22010 (SternEnzym, Ahrensburg, Germany) and xylanase Pentopan Mono BG (Novozyme, Bagsvaerd, Denmark). Bacterial cultures were *L. brevis* DSM 20054 (DSMZ GmbH, Braunschweig, Germany) and *L. plantarum* WCFS1. For different combinations applied to samples see Table 1.

2.2. Methanolysis

In order to remove mobilized arabinoxylan before methanolysis, 200 mg of bran sample were stirred in 10 mL water for 1 h, filtrated, washed twice with 10 mL of water and dried in vacuo at room temperature.

2.2.1. Procedure and derivatization

Methanolysis was performed according to Sundberg et al. (1996). For silylation, dried samples after methanolysis were left to equilibrate in 400 μL of pyridine for 1 h. 200 μL of BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) containing 10% TMCS (trimethylchlorosilane; both Sigma-Aldrich) were added and the samples kept at 70 °C for 2 h. Samples were diluted with 600 μL of ethyl acetate, filtrated through a 0.45 μm PTFE syringe filter and analyzed by GC–MS.

2.2.2. GC–MS

GC–MS was performed as follows. 0.2 μL of silylated sample was injected (260 °C, splitless) on a 30 m/0.25 mm HP-5 column (film thickness 0.25 μm) in an Agilent 6890N Series GC System with an Agilent 5973 Series Mass Selective Detector. The temperature program was 140 °C (1 min); 4 °C/min to 210 °C (0 min); 30 °C/min to 260 °C (5 min). The carrier gas was helium (0.9 ml/min, constant flow). Detector conditions were 70 eV with a scan range from 45 to 950 Da. Data was acquired and processed with MSD Chemstation E.2.01.1177 software from Agilent Technologies.

2.3. Enzymatic peeling

Procedure for enzymatic peeling was adopted from Sjöberg et al. (2005) with modifications to the enzyme mix to suit wheat bran polysaccharides.

2.3.1. Preparation of enzyme mix

The enzyme mix was composed of 0.5 g cellulase from *Trichoderma reesei* (Sigma-Aldrich), 3 g xylanase Pentopan Mono BG (Novozyme) and 2 mL of GH 43 α-L-arabinofuranosidase from *Bifidobacterium adolescentis* (Megazyme Internartional, Wicklow, Ireland). Cellulase activity is given as ≥1 U/mg, xylanase as 2.5 FXU/mg (farbe xylanase units) and α-L-arabinofuranosidase activity as 102 U/mg. The enzyme mix was filtrated through Whatman filter paper grade 4 and then desalted and concentrated by ultrafiltration to 1% of its original volume with a molecular weight cutoff at 1000 Da and then filled up to 50 mL with water.

2.3.2. Enzymatic peeling procedure

Twenty milligrams of washed sample (see Section 2.2) were stirred in a sodium acetate buffer adjusted to pH 4 at 40 °C for 1 h before the addition of 100 μL (20 mg/mL) of sorbitol as internal standard and 700 μL of enzyme mix. The suspension was kept at 40 °C and 0.2 mL aliquots were taken after 5, 10, 30, 60 and 120 min. Aliquots were filtrated through a 0.45 μM PTFE filter and incubated for 48 h at 40 °C for complete enzymatic hydrolysis. Samples were used for HPLC in a dilution of 1:10. A blank was recorded to correct for residual sugars in the enzyme mix.

2.3.3. HPLC analysis

Sugars were quantified on a Dionex UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) equipped with a Phenomenex 300 × 7.8 mm Rezex™ ROA-Organic Acid H+ (8%) column kept at 80 °C (Phenomenex, Aschaffenburg, Germany) and a HP 1100 Series G1362A RID Refractive Index Detector (Hewlett-Packard, Palo Alto, CA, USA). Mobile phase was 5 mM sulfuric acid at a flow of 0.4 mL/min. Calibration was performed externally relative to sorbitol, which was used as an internal standard. All standards were purchased from Sigma-Aldrich. Data was recorded with Dionex Chromeleon 6.8.

2.4. Immunolocalization

2.4.1. Sample preparation

Samples from Section 2.1 were prepared for immunolocalization by pouring the wheat bran flakes/powder into molten paraffin wax and letting it harden. The wax was trimmed to blocks and cut with a microtome so that cross sections of wheat bran were located at the surface of the block. Labeling with antibody, mounting and microscopy were performed on the block of wax.

2.4.2. Labeling

Immunolocalization of arabinoxylan was performed by placing a droplet of a 1:20 dilution of primary rat monoclonal antibody hybridoma cell culture supernatant LM11 (PlantProbes, Leeds, UK) in 0.1 M PBS (pH 7, containing 1% w/v acetylated bovine serum albumin as a blocking agent and 5 mM sodium azide) on a wax block over night at 4 °C so that the top of the wax block was entirely covered. Wax blocks were rinsed five times with PBS and dried before being covered with a droplet of fluorescently labeled secondary antibody Alexa647 (goat anti-rat; Invitrogen, Paisley, UK) at a 1:100 dilution in PBS (20 μg/mL) for 2 h under light exclusion at room temperature. Blocks were washed five times with PBS, mounted in SlowFade at pH 9 (Molecular Probes Inc., Eugene, Oregon, USA) and measured on the same day. A control sample was prepared accordingly with the omission of the primary antibody. Each sample was measured with and without immunolabeling. The overlap of fluorescence from the antibody and autofluorescence from the sample was negligible.

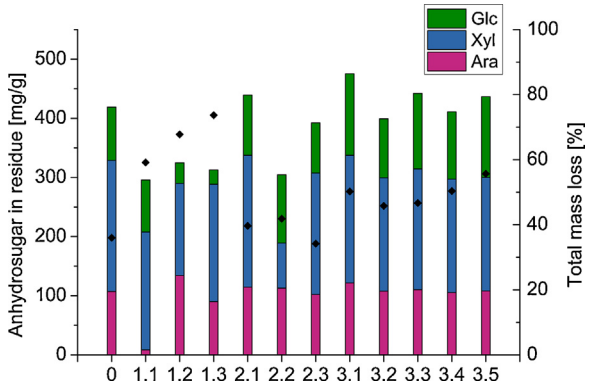

Fig. 1. Total mass losses and residual sugar content of samples after washing. **Glc** = glucose, **Xyl** = xylose, **Ara** = arabinose, ♦ = total mass loss. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase.

2.4.3. Confocal laser scanning microscopy

Confocal laser scanning microscopy was performed on a Leica SP5 II. Three channels were recorded in parallel. For autofluorescence, excitation was 488 nm (shown as green in Fig. 3) and 561 nm (shown as red) and emission was recorded at 500–535 nm and 570–620 nm. The fluorescent antibody was excited at 633 nm (shown as blue) and recorded at 650–750 nm. For all images, only overall exposure was adjusted when necessary. The relative exposure of the channels was kept constant throughout. All images are shown as maximum intensity projections.

3. Results and discussion

3.1. Methanolysis

Washing of untreated wheat bran resulted in a 36% weight loss and gave a residue composed of 11% arabinose-, 22% xylose- and 9% glucose-based polysaccharides. Due to the methodology, glucose values do not reflect cellulose content but only starch and β-glucan. For the assessment of cellulose accessibility and spatial distribution after treatments see Section 3.2. Total mass as well as specific polysaccharide losses of treated samples are given relative to untreated wheat bran and refer to solid residues after washing, i.e. negative values for residual sugars indicate an increase in solubilization. Methanolysis results and mass losses are shown in Fig. 1 (absolute values) as well as Table 1 (values relative to untreated wheat bran).

3.1.1. Chemical treatments

Acid treatment is a common pretreatment in the biorefinery of lignocellulosic biomass and is advantageously followed by

Table 1
Total mass losses and residual sugar contents of samples after washing as determined by methanolysis. Values are given relative to untreated wheat bran. Negative values for residual sugars indicate an increase in solubilization.

Sample ID	Type of treatment	Mass loss [%]	Arabinose [%]	Xylose [%]	Glucose [%]
1.1	Acid treatment	+64	−92	−10	−2
1.2	Lye treatment	+88	+25	−30	−61
1.3	Hydrogen peroxide treatment	+105	−16	−10	−73
2.1	Ball-milling 5 min	+10	+7	+1	+13
2.2	Ball-milling 60 min	+16	+5	−66	+28
2.3	Extrusion	−5	−5	−7	−6
3.1	*Lactobacillus plantarum*	+40	+13	−3	+53
3.2	Esterase	+27	+/−0	−13	+10
3.3	Xylanase	+30	+3	−8	+42
3.4	Esterase + Xylanase	+40	−2	−13	+26
3.5	*Lactobacillus brevis* + esterase + xylanase	+55	+1	−13	+51

enzymatic hydrolysis to yield monosaccharides (Eggeman & Elander, 2005; Gomathi et al., 2012; López-Arenas, Rathi, Ramírez-Jiménez, & Sales-Cruz, 2010). For wheat bran, acid treatment increased total solubles by 64%, but was not very specific towards polysaccharides. It only slightly increased the amount of soluble xylose- and glucose-based polysaccharides (−10% and −2%) but drastically solubilized arabinose (−92%). Arabinose occurs as singe-unit side chains in arabinoxylan and acid preferentially cleaves off such terminal monosaccharides.

Lye treatment is regarded as a selective method for the extraction of arabinoxylan from biomass (Cui, Wood, Weisz, & Beer, 1999; Sun et al., 2011). However, we found treatment with sodium hydroxide not to be specific towards arabinoxylan. Despite an increase of 88% in total solubles, it solubilized only average amounts of xylose-based polysaccharides (−30%), and also high amounts of glucose (−61%) were solubilized. The seeming increase in arabinose in the residue (+25%) is a relative increase due to the removal of other components. Still, it points to a favored extraction of non-substituted or lowly substituted over highly branched arabinoxylan.

Hydrogen peroxide is often combined with alkaline conditions in order to achieve simultaneous delignification and selective extraction of arabinoxylan (Hollmann & Lindhauer, 2005; Maes & Delcour, 2001). We found this treatment to give the highest increase in solubilization (by 105%), but could not attest good specificity towards arabinoxylan. Arabinose content in the residue decreased by 16%, xylose content by 10% and glucose content by 73%.

3.1.2. Mechanical treatments

Extensive ball-milling is known to lead to fragmentation of arabinoxylan into oligosaccharides and thus to an increase in solubility (Van Craeyveld et al., 2009). In our study, 5 min and 60 min of ball-milling increased overall solubility by 10% and 16%, respectively, but only after 60 min we observed a strong preference for xylan-based polysaccharides (−66%) over glucose (+28%). Five minutes of ball-milling showed no observable effect on sugar composition and the improved extractability was thus probably only caused by an increase in accessible surface area due to decreased particle size. According to microscopy, it was estimated that ball milling reduced the particle sizes to be generally smaller than 250 μm after 5 min and to be generally smaller than 20 μm after 60 min. the fragments of the employed bran are noticeably larger: 37% of the particles were smaller than 250 μm and only 17% were smaller than 125 μm.

Extrusion is commonly used for the preparation of convenience foods such as pasta or snacks from whole grain or white flour (Gajula, Alavi, Adhikari, & Herald, 2008). Despite comparatively low yields, the treatment holds potential for the isolation of arabinoxylan from wheat bran due to a smaller environmental footprint than alkaline extraction (Jacquemin, Zeitoun, Sablayrolles, Pontalier, & Rigal, 2012). After extrusion, we observed a slight decrease in overall extractability by 5%, but also a preference for polysaccharides: arabinose, xylose and glucose moieties decreased by 5%, 7% and 6%, respectively, in the residual material.

3.1.3. Fermentation and enzymatic treatments

L. plantarum is a lactic acid producing species that has been shown to possess strain-dependent probiotic properties (Goossens, Jonkers, Russel, Stobberingh, & Stockbrugger, 2006) as well as to be able to utilize wheat bran as a substrate (Naveena, Altaf, Bhadriah, & Reddy, 2005). In addition to carbohydrates, it can also ferment protein (Fadda, Vildoza, & Vignolo, 2010). After fermentation, we observed an increase of insoluble polysaccharides (arabinose +13%, xylose −3% and glucose +53%) despite an increase in overall solubility of 40% compared to untreated wheat bran. This points towards the conclusion that mainly protein and only small amounts of arabinoxylan have been solubilized, concentrating the remaining compounds.

Enzymatic extraction of arabinoxylan is considered to be the mildest form of pretreatment and thus best suited for food and feed applications (Swennen et al., 2006). Esterase and xylanase treatment showed a comparable increase in total solubility (27% and 30%). In the residual polysaccharide analysis, they showed similar amounts of arabinose moieties (0% and +3%). Esterase treatment seemed to be slightly more efficient for solubilization of xylose (−13% against −8%) and showed a smaller increase in residual glucose-based polysaccharides (+10% against +42%).

When combined, however, esterase and xylanase brought overall solubility to 40%. The synergistic effect is presumed to be due to esterase being able to cleave ester bonds between arabinoxylan and phenolics as well as between two arabinoxylan chains, which can be ester-linked through ferulic acid at arabinose side chains. This gives xylanase access to more substrate for depolymerization (Lewis & Yamamoto, 1990). However, the fact that residual polysaccharides stayed within the same range (arabinose −2%, xylose −13% and glucose +26%) implies a concomitant increase in extractability of untracked plant constituents, for example protein.

The addition of *L. brevis* to the enzyme mix of esterase and xylanase caused total solubilization to rise to 55%. *L. brevis* has been shown to be able to utilize xylooligosaccharides, which might account for the observed increase (Michlmayr et al., 2013). Yet again, extraction was accompanied by other compounds so that polysaccharides in the residual material remained to make up a comparable percentage with only glucose-based polymers showing some discrimination (arabinose +1%, xylose −13% and glucose +51%).

3.2. Enzymatic peeling

It is important to note that wheat bran is too resistant to undergo complete enzymatic hydrolysis. Even for isolated, insoluble arabinoxylan, enzymatic hydrolysis rates do not exceed 45% (Sørensen, Pedersen, & Meyer, 2007). Furthermore, only the aleurone layer is readily accessible for enzymes. Thick cell walls and lignification shield the outer layers against enzymatic attack (Benamrouche, Cronier, Debeire, & Chabbert, 2002). Therefore, it must be assumed that the application of an enzymatic peeling to wheat bran can only profile a limited layer of the sample surface.

Untreated wheat bran showed a comparatively low amount of accessible cellulose, especially so in the outer layers (corresponding to the first 30 min of peeling). However, values for xylose release were amongst the highest and showed an enrichment of arabinoxylan in the deeper layers (after 30 min of peeling, see Fig. 2).

Enzymatic peeling profiles for glucose from cellulose and xylose are given in Fig. 2.

3.2.1. Chemical treatments

The chemical treatments showed by far the most pronounced effect regarding the enzymatic release of glucose from cellulose with peroxide and lye treatment exceeding acid treatment. This points towards an improved accessibility of cellulose, which might be due to the removal of inhibitory or protective substances such as lignin (Rahikainen et al., 2011).

In the release of xylose, all chemical treatments fared substantially worse than untreated bran. Peroxide treatment gave an increase in the outer layers, but its release over time was rather stagnant so that little more xylose was accessible in deeper layers. Lye treatment released the smallest amount of xylose of all samples whereas acid treatment performed slightly below average.

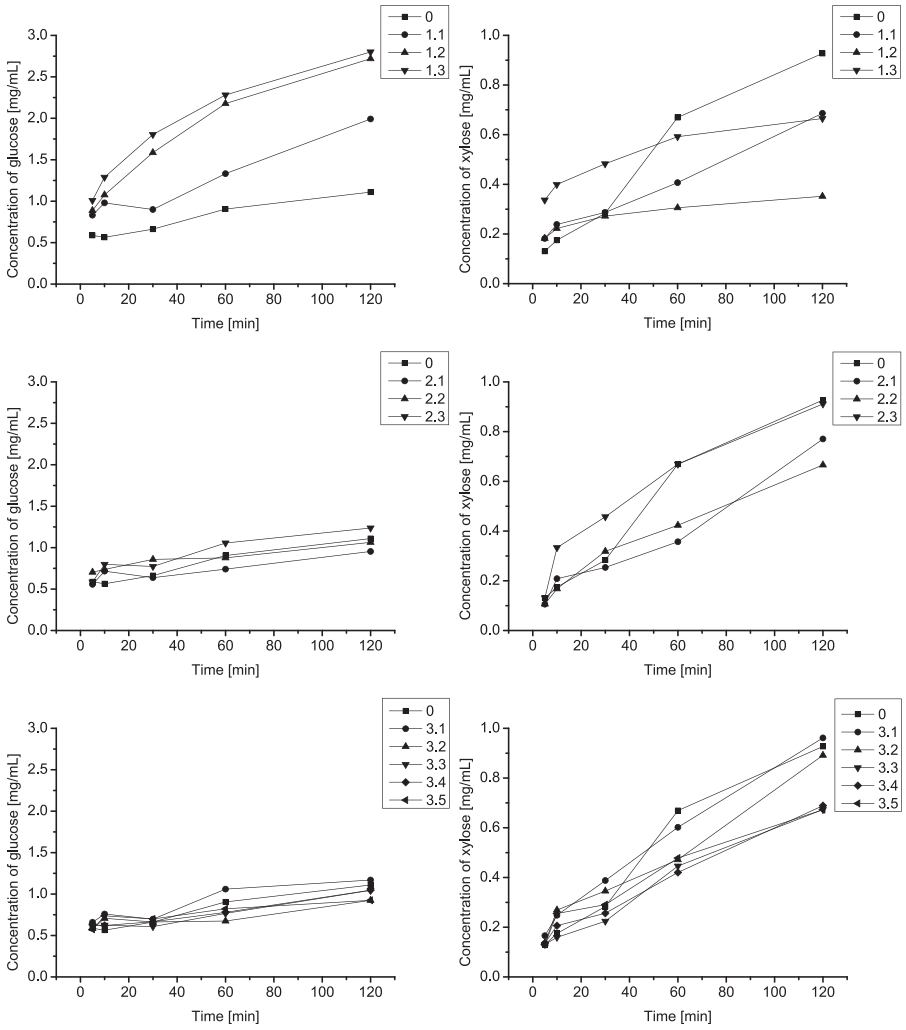

Fig. 2. Release of glucose (left) and xylose (right) over time during enzymatic peeling. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase.

3.2.2. Mechanical treatments

Ball-milling for 60 min released slightly more glucose over time than 5 min of treatment, but both gave overall release values marginally lower than untreated wheat bran. The 60 min treatment seemed to expose cellulose in the outer layers and therefore most of the glucose was released within the first 30 min of peeling. As for xylose, both ball-milling treatments revealed substantially less accessible substrate than untreated wheat bran. Again, 60 min of ball-milling led to increased exposure of xylose moieties in the outer layer whereas 5 min of treatment led to a delayed, albeit eventually more pronounced release of xylose during peeling (see Fig. 2).

Extrusion showed an increased accessibility of cellulose in the outer layers and released overall more glucose than untreated wheat bran. Also for xylose a high solubilization was observed in the first 30 min and overall levels were comparable to untreated wheat bran.

3.2.3. Fermentation and enzymatic treatment

Glucose release for fermented and enzymatically treated samples suggested a distribution of cellulose very similar to that of untreated wheat bran. *L. plantarum* and esterase treatment framed the upper and lower limit of effect reached after 60 min of peeling.

Differences were more prominent for the release of xylose. Here, *L. plantarum* showed high amounts of accessible arabinoxylan throughout the layers with the largest total amount of released xylose of all samples. Esterase treatment showed an enrichment of arabinoxylan in the very outer (10 min) as well as the very deepest layers (120 min) and reached overall values comparable to untreated wheat bran. Similar amounts of total xylose were released during peeling for the treatments with xylanase; the combination of esterase and xylanase; and *L. brevis* in combination with esterase (−8% to −13%). Xylanase treatment resulted in rather low concentration of arabinoxylan in the outer layers (up to 30 min) while *L. brevis* in combination with esterase and xylanase suggested high concentrations in the outer layers (10 min) and lower amounts in deeper layers (60–120 min). The combination of esterase and xylanase gave a rather even distribution throughout.

3.3. Immunolocalization

Fig. 3 shows all three channels recorded with confocal microscopy as an RGB overlay to provide a spatial context for arabinoxylan. Fig. 4 shows only the signal of the arabinoxylan label fluorescence in black on white.

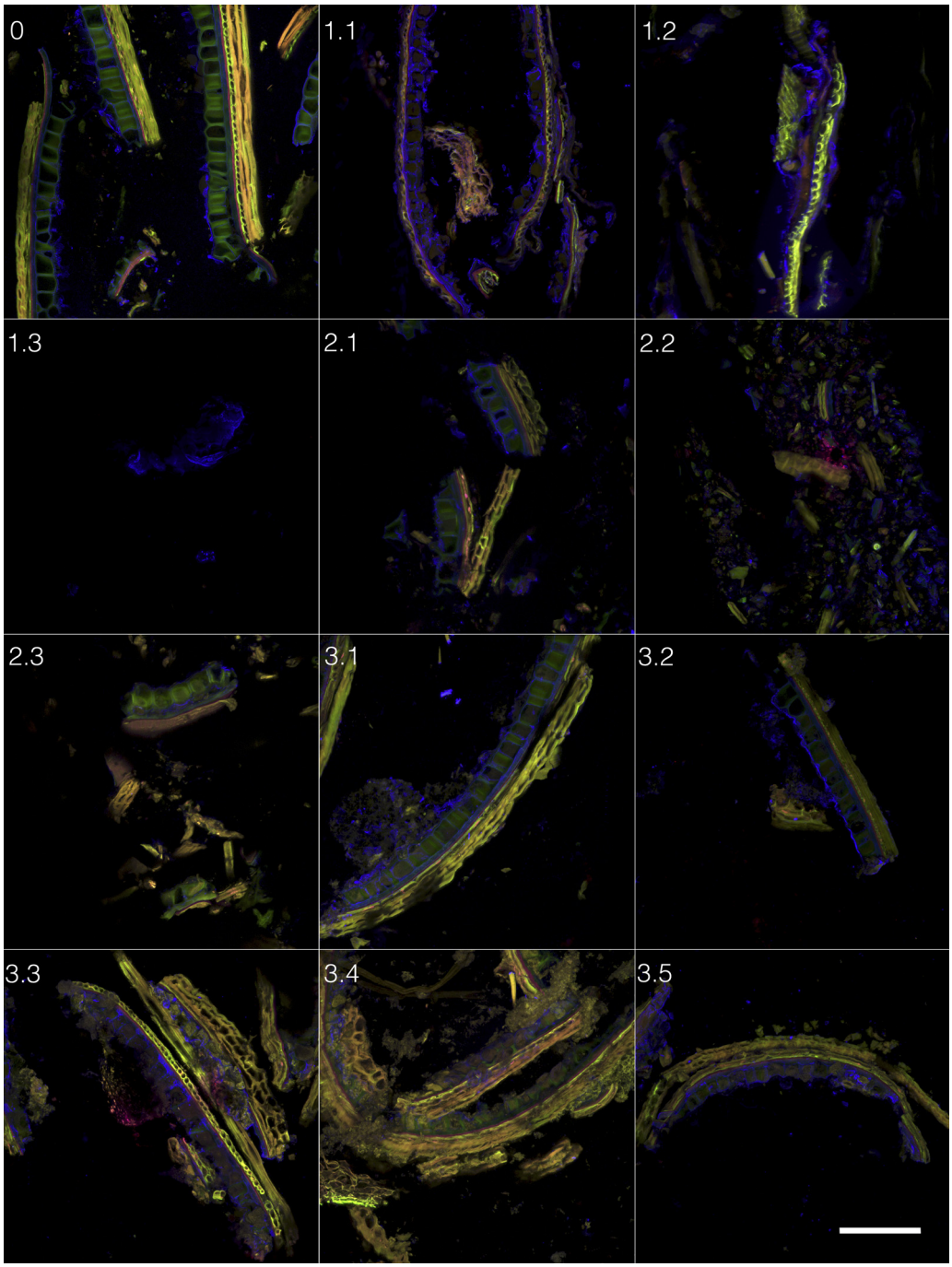

Fig. 3. Immunolocalization images of arabinoxylan (blue) and autofluorescence (red and green) as overlay images. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase. Bar = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.1. Sample preparation

The sample preparation employed proved drastically more time efficient and substantially less laborious than the commonly used fixation and subsequent embedding in acrylic resin (Fasseas, Roberts, & Murant, 1989). Ten samples could be prepared within about 2 h instead of several days. Furthermore, only a few drops of antibody solution were required to cover a block of wax as compared to several hundred μL for soaking samples, while washing could be performed by simple rinsing instead of having to pipette or filter off supernatant multiple times. Whether this technique is limited to softer samples such as cereals or whether it could as well be applied to wood samples remains to be tested.

No overlap of fluorescence from the antibody and autofluorescence from the sample were detected. Omission of the primary antibody accordingly resulted in the absence of fluorescence according to the specificity of the secondary antibody.

3.3.2. Untreated wheat bran

Wheat bran arabinoxylan is mainly located in the aleurone layer (ca. 25%), nucellar epidermis (ca. 25%) and pericarp (ca. 38%) (Benamrouche et al., 2002). Since its occurrence is limited to cell

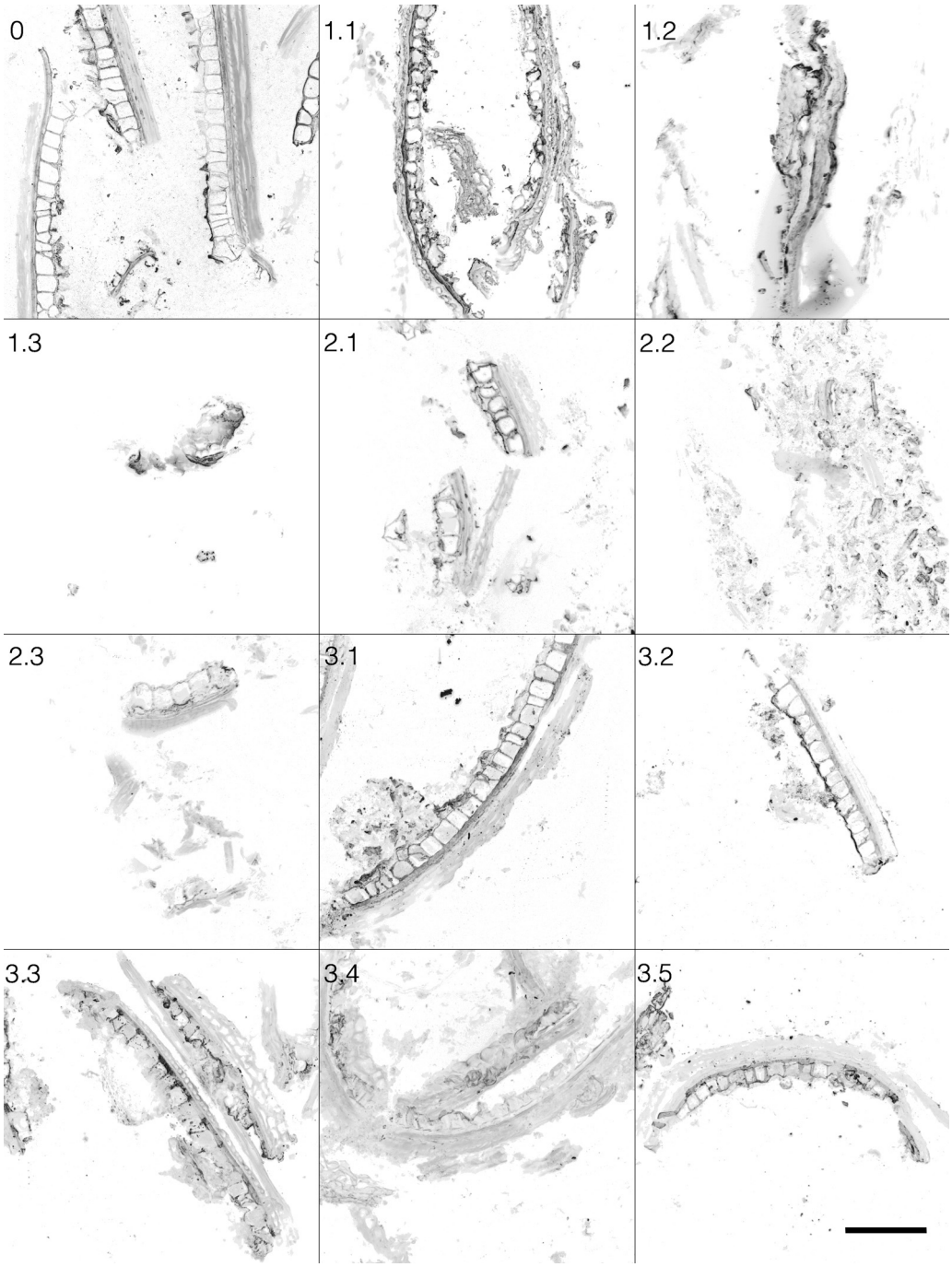

Fig. 4. Immunolocalization images of arabinoxylan shown as single channel recordings. Colors have been inverted and rendered black and white for better discernibility. **0**: Untreated wheat bran; **1.1**: acid treatment; **1.2**: lye; **1.3**: hydrogen peroxide; **2.1**: 5 min ball-milling; **2.2**: 60 min ball milling; **2.3**: extrusion; **3.1**: *Lactobacillus plantarum*; **3.2** esterase; **3.3** xylanase; **3.4**: esterase + xylanase; **3.5**: *Lactobacillus brevis* + esterase + xylanase. Bar = 200.

walls, arabinoxylan is more concentrated in the aleurone layer due to its comparatively thin cell walls, as can be deduced from the distribution of fluorescence from the immunolabel in Figs. 3 and 4.

3.3.3. Chemical treatments

Acid treatment caused the cell walls of the aleurone layer to swell so that the distribution of arabinoxylan was accordingly broader. Only little arabinoxylan seems to have been mobilized which is in agreement with the findings from methanolysis (see Section 3.1.1). Given that the enzymatic peeling could digest merely an outer layer of the sample, the smaller release of xylose as compared to untreated wheat bran is probably due to the wider distribution of arabinoxylan within the cell walls of the aleurone layer.

Lye treatment effected a complete disintegration of the aleurone layer, which accounts for most of the decrease in arabinoxylan content, and a swelling of the nucellar epidermis. Since the aleurone layer is by far the most accessible for enzymatic hydrolysis (Benamrouche et al., 2002), removal of the layer should lead to poor solubilization of additional arabinoxylan as has been observed during enzymatic peeling.

Peroxide treatment caused the strongest disruption of wheat bran cells as expected after 74% of total mass loss. Since peroxide is able to degrade lignin (Sun, Sun, & Wen, 2001), treatment resulted

in a near obliteration of auto-fluorescence. The fragments that are found with fluorescent microscopy are difficult to assign to distinct cell layers due to their deformation, but the presence of arabinoxylan, especially in the outer layers, is evident, as has been confirmed by sugar analysis and enzymatic peeling.

3.3.4. Mechanical treatments

Five minutes of ball-milling only exhibited a reduction in particle size affecting neither cellular structures nor the distribution of arabinoxylan. This increase in surface area is in accordance with the increase in overall solubility and the similarity in residual sugar composition with untreated wheat bran.

After 60 min of ball-milling, however, cells are broken up beyond recognition and arabinoxylan is scattered loosely. The rather selective extractability of arabinoxylan observed by methanolysis (see Section 3.1.2) can be deduced from the image. The relatively low release of xylose during enzymatic peeling is probably due to depletion of arabinoxylan after washing.

Extrusion showed a small increase in residual material with a slight decrease in arabinose and xylose moieties compared to untreated wheat bran, which implies selectivity towards arabinoxylan. Enzymatic peeling released twice the amounts of xylose compared to native wheat bran and indicated enrichment of arabinoxylan in the outer layers (see **2.3** in Fig. 2). The immunolocalization images are in accordance with these findings, revealing a cell structure that is intact but slightly depleted of arabinoxylan.

3.3.5. Fermentation and enzymatic treatment

Fermentation and especially enzymatic treatments showed a surprisingly low selectivity.

Fermentation with *L. plantarum* did not affect the cell wall integrity and the distribution of arabinoxylan appeared virtually identical to that of untreated wheat bran. This supports the results of methanolysis, namely that *L. plantarum* mainly solubilized protein. The removal of protein might have increased the enzymatic accessibility to arabinoxylan which is reflected in the slightly increased release of xylose during enzymatic peeling.

Esterase treatment caused a light swelling of aleurone cell walls, the only layer effectively accessible to enzymatic treatment (Benamrouche et al., 2002), shifting the arabinoxylan distribution more towards the very border of cell walls. This observation is substantiated by the profile obtained during enzymatic peeling.

Xylanase treatment effected an overall more pronounced swelling of aleurone cell walls as well as damage to inward-facing cell walls so that the distribution of arabinoxylan was increasing towards the nucellar epidermis. This change can be expected to render arabinoxylan less accessible to further enzymatic attack as was shown by the lowered release of xylose during enzymatic peeling.

The combination of esterase and xylanase showed the strongest impact on aleurone cells. Cell walls were swollen, perforated or even partially disintegrated. Arabinoxylan was distributed in broader zones of smaller intensity across the aleurone layer. Keeping in mind that the increased overall solubilization gave a residual sugar ratio comparable to other less effective enzymatic treatments, extraction of further cell compounds must be concommitant to arabinoxylan extraction. As a consequence of the broader distribution of arabinoxylan, enzymatic peeling gave a profile with lower arabinoxylan concentrations.

Fermentation with *L. brevis* in combination with esterase and xylanase lead to swollen cell walls in the aleurone layer. The overall reduced amount of arabinoxylan was found to be highest in the outer layer where it could be detected by enzymatic peeling. Since the cells appeared to be less damaged compared to the treatment without *L. brevis*, the further increase in total mass loss must have come at the cost of compounds not made visible by fluorescence microscopy.

4. Conclusion

Little is known about the association of arabinoxylan and cellulose in wheat bran cells. The data obtained from enzymatic peeling and methanolysis after diverse treatments suggest a rather loose affiliation allowing for the manipulation of one polysaccharide without affecting the other.

Yet, defining a selective pretreatment for the isolation of arabinoxylan has proven difficult. Chemical methods, especially lye and peroxide treatment, solubilized by far the biggest percentage of wheat bran but did so at the cost of substantial coextraction of other compounds. Given their severe conditions, degradation of other valuables as well as formation of inhibitory and hazardous substances take place. Lye treatment has displayed the best selectivity among chemical treatments.

Extensive ball-milling resulted in the most promising results with a strong specificity for xylan-based polysaccharides. Since it applies mere mechanical force, the results point towards a less rigid integration of arabinoxylan into the cell wall or a greater disposition for depolymerization compared to cellulose. Extrusion, which combines heat and mechanical energy input, resulted in a slightly more selective extraction of arabinoxylan, but overall extractability was low so that the return is unlikely to justify the costs for this application.

Enzymatic extraction with or without fermentation showed little selectivity for arabinoxylan and mostly influenced degrees of overall solubilization. Ostensibly, enzymatic removal of arabinoxylan is always concomitant with the extraction of other cell compounds. Treatment with *L. plantarum* even showed a strong preference for non-carbohydrate material.

The application of immunolocalization to visualize the cell-structural changes perpetrated by pretreatments on arabinoxylan was helpful to interpret the findings of the chemical analyses. As a complementary method, it is very helpful in taking stock and pointing towards optimization strategies. For example, the depletion of arabinoxylan from the aleurone layer, which would mark a ceiling for enzymatic extraction, is difficult to measure in chemical analysis but easily observed by immunolocalization. Our simplified sample preparation for confocal laser fluorescence microscopy further facilitates rapid measurement.

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