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Extensive Dry Ball Milling of Wheat and Rye Bran Leads to *in Situ* Production of Arabinoxylan Oligosaccharides through Nanoscale Fragmentation

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This study investigated the potential of ball milling as a dry process for *in situ* production of arabinoxylan oligosaccharides (AXOS) in arabinoxylan (AX)-rich wheat and rye bran. An extensive lab-scale ball mill treatment (120 h, 50% jar volume capacity) increased the wheat bran water-extractable arabinoxylan (WE-AX) level from 4% (untreated bran) to 61% of the wheat bran AX. Extractable AX fragments had an arabinose/xylose ratio (A/X ratio) of 0.72 and a molecular mass (MM) of 15 kDa. Ball milling of rye bran gave rise to similar results, with the A/X ratio of the extractable AX being considerably lower (0.51). Optimization of the ball mill treatment by varying the degree of filling of the milling jar permitted us to obtain equally high WE-AX levels (>70%) in wheat and rye bran within a 24 h ball-milling period. Ball milling at optimal conditions (24 h, 16% jar volume capacity) yielded wheat bran AXOS, with an A/X ratio of 0.65 and a MM of 6 kDa. Ball milling (24 h, 50% jar volume capacity) of pericarp-enriched wheat bran increased the WE-AX level from 1% (untreated pericarp) to 63%. The extractable material had a high A/X ratio (0.97) and a low MM (5 kDa). Fluorescence microscopy revealed that the extensive ball mill treatment led to the almost complete disappearance of discernible tissue structures in the ball-milled material, indicating bran particle size reductions down to the nanoscale level. It further visualized the aggregation of the treated material. These results show that AXOS can be produced *in situ* from wheat or rye bran in a single-stage dry milling process, rendering a wet extraction step redundant. The higher A/X ratio of the obtained AXOS than of enzymically produced wheat bran-derived AXOS offers perspectives for the production of a wide range of AXOS structures. Moreover, ball milling makes upgrading of the low-value pericarp layer feasible.

KEYWORDS: Wheat bran; rye bran; ball milling; arabinoxylan; arabinoxylan oligosaccharides; extractability; aleurone; pericarp; particle size; nanoscale

INTRODUCTION

Wheat and rye bran are byproducts of conventional milling and commercially available in large quantities. Although they are mainly used as low-value ingredients in animal feed (*1*), their use as sources of dietary fiber is increasing. They indeed contain high levels of arabinoxylan (AX) (17−32%) and cellulose (9−11%) (*2*, *3*). Cereal bran AX consists of a backbone of β-(1→4)-linked D-xylopyranosyl residues (xylose), some of which are mono- or disubstituted with α-L-arabinofuranosyl residues (arabinose) (*4*). Typical for bran AX is the presence of uronic acids, mostly glucuronic acid and its 4-*O*-methylether, linked to some of the xylose residues (*5*). Hydroxycinnamic acids, such as ferulic and *p*-coumaric acids, are ester-linked to some of the arabinose residues (*4*). The average arabinose/xylose ratio (A/X ratio) of bran AX is approximately 0.5−0.6 (*2*, *3*, *6*). However, the actual fine structure of AX in different bran tissues is very diverse. Wheat and rye AX in pericarp (outer bran) are highly substituted (A/X ≥ 1.0), whereas AX in the aleurone layer (A/X = 0.4−0.5) and the nucellar epidermis (A/X = 0.1) have a lower A/X ratio (*7*−*9*).

Wheat bran-derived arabinoxylan oligosaccharides (AXOS), produced by enzymic or chemical cleavage of AX, have been shown to selectively stimulate *Bifidobacterium* species in *in vitro* pure cultures (*7*, *8*) and in *in vivo* trials with rats (*10*, *11*), mice (*10*), chickens (*12*, *13*), and humans (*14*) and are therefore considered to have potential prebiotic properties. Production of AXOS from wheat bran is generally performed through incubation with endo-β-(1,4)-xylanases (EC 3.2.1.8, xylanases), which attack the AX backbone in a random manner. These enzymes solubilize and degrade water-unextractable AX (*15*) and, hence, release AXOS. Although the process is sufficiently controllable and production on pilot scale is feasible (*16*), enzymic production of AXOS from

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wheat bran is faced with limitations. While the aleurone layer is largely degradable, the outer pericarp is not degraded at all by xylanases (*17*). The high level of substituents, the strong cross-linking through ferulic acid dimerization, and the embedding of AX in a lignin matrix all contribute to the enzymic resistance of the outer pericarp (*18*, *19*). Production of bran-derived AXOS upon xylanase treatment is therefore limited to solubilization of the aleurone AX, resulting in AXOS preparations with low A/X ratios. Solubilization of (part of) the pericarp AX would significantly increase the AXOS yield and provide AXOS with different average structures than those obtained by enzymic treatment. Irrespective of the poor fermentability of intact highly substituted pericarp AX (*8*), pericarp-derived AXOS may be promising substrates for selective bacterial fermentation. An additional difficulty of the enzymic production of wheat bran-derived AXOS is the wet incubation step, which leads to high-energy costs when drying the end products. Production of bran-derived AXOS in a dry process would avoid this drying step.

Ball milling has been shown to increase the water solubility of wheat starch (*20*, *21*), carrot insoluble fiber (*22*), and psyllium seed husk AX (*23*) and to decrease molecular sizes of pectin molecules (*24*) and AX from psyllium seed husk (*23*). The present study aimed to investigate the potential of ball milling for production of wheat and rye bran-derived AXOS with possible prebiotic properties and, thus, for contributing to the upgrading of cereal bran, in particular, the pericarp layer.

EXPERIMENTAL SECTION

Materials. Commercial wheat bran was obtained from Dossche Mills and Bakeries (Deinze, Belgium). Commercial rye bran was from Paniflower (Merksem, Belgium). Enzyme preparations used were heat-stable α-amylase (Termamyl 120 L, Novozymes, Bagsvaerd, Denmark), bacterial protease (Neutrase 0.8 L, Novozymes), a glycosyl hydrolase family 11 *Bacillus subtilis* xylanase (Grindamyl H640, Danisco, Copenhagen, Denmark), and a glycosyl hydrolase family 10 xylanase from *Aspergillus aculeatus* (Shearzyme 500 L, Novozymes). Xylanase activities were determined as described earlier (*16*). One unit of enzyme activity (U) was the amount of enzyme required to yield an absorbance of 1.0 at 590 nm under the assay conditions (40 °C, pH 4.7, 10 min). Amylase and protease were dosed on the basis of the activity units (U) defined by the suppliers. Chemicals and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) unless indicated otherwise and were of at least analytical grade.

Production of Pericarp-Enriched Wheat Bran. To investigate the impact of ball milling on pericarp AX, we produced pericarp-enriched wheat bran based on a procedure described by Swennen et al. (*16*). Wheat bran suspended in tap water (1:10, w/v) was treated with Termamyl 120 L (120 U/kg wheat bran, 90 min, 90 °C) and Neutrase 0.8 L (32 U/kg wheat bran, 4 h, 50 °C, pH 6.0) to hydrolyze starch and residual proteins, respectively. Thereafter, the suspension was boiled for 20 min and filtered. The destarched and deproteinized residue was washed with water and resuspended in deionized water (1:14, w/v). The suspension was incubated under continuous stirring with the *B. subtilis* (1.4 U/g bran) and *A. aculeatus* (21 U/g bran) xylanases for 16 h at 50 °C to solubilize as much wheat bran AX as possible. These xylanases solubilize the lowly substituted AX in the aleurone layer and nucellar epidermis and leave the highly substituted AX in the pericarp layer largely untouched (*16*, *17*). Their action thus results in a residue enriched in components mainly originating from the pericarp layer. After boiling (20 min), filtration of the suspension, and washing, the pericarp-enriched residue was freeze-dried.

Standard Analyses. Moisture and ash contents of wheat and rye bran and pericarp-enriched wheat bran were analyzed according to American Association of Cereal Chemists, Inc. (AACCI) methods 44-19 and 08-01, respectively (*25*). Protein contents were determined using the Dumas combustion method, an adaptation of the Association of Official Analytical Chemists (AOAC) Official Method (*26*) to an automated Dumas protein analysis system (EAS VarioMax N/CN, Elt, Gouda, The Netherlands), using 5.7 as a factor for conversion of nitrogen to protein content. Total noncellulosic neutral carbohydrate contents were determined with gas chromatography upon hydrolysis and derivatization of the resulting monosaccharides into alditol acetates (*27*). Uronic acid contents were determined colorimetrically as described earlier (*27*).

Ball Milling of Wheat Bran, Rye Bran, and Pericarp-Enriched Wheat Bran. Wheat bran and rye bran were ground (500 rpm) in a laboratory Retsch PM 100 planetary ball mill (Retsch, Haan, Germany), equipped with a 250 mL zirconium milling jar with six zirconium balls (20 mm in diameter). To investigate the influence of milling time, wheat bran [25 g, corresponding to a total filling degree (including the ball volumes) of 50% of the jar volume capacity] and rye bran (25 g or 36% filling degree) were ground for up to 120 h. Every 24 h, a sample (1.0 g) was withdrawn for analysis. Pericarp-enriched wheat bran was ground for 24 h (50% filling degree). In a second experiment, different amounts of wheat and rye bran (filling degree between 16 and 50%) were ground for 24 h.

Determination of Water Extractability of AX and A/X Ratio of Extracted AX. Untreated and ball-milled bran samples were suspended in water (0.4%, w/v) and shaken for 60 min at 6 °C to extract water-extractable AX (WE-AX). After centrifugation (24000*g*, 10 min, 6 °C), total and monomeric (free) carbohydrate contents of the extracts were determined with gas chromatography following derivatization as described earlier (*27*). WE-AX levels were defined as the sum of the contents of the monosaccharides arabinose and xylose times 0.88 to correct for water uptake in the hydrolysis procedure (*16*). A combination of data for total and monomeric saccharides allowed us to calculate the arabinose/xylose ratio (A/X ratio)

$$\text{A/X ratio} = \frac{\text{total arabinose level} - \text{monomeric arabinose level}}{\text{total xylose level} - \text{monomeric xylose level}}$$

Measurements were performed in duplicate. Standard deviations of WE-AX levels were smaller than 1.0%. Standard deviations for A/X ratios were below 0.01.

Determination of Apparent Molecular Mass (MM) Distributions. Apparent MM distributions of WE-AX in extracts from untreated and treated bran samples were studied by high-performance size-exclusion chromatography on a Shodex SB-804 HQ column (300 × 8.0 mm inner diameter) with a Shodex SB-G guard column (50 × 6 mm inner diameter) from Showa Denko K.K. (Kawasaki, Japan). Samples were prepared and analyzed as described earlier (*23*). MM markers (1.5 mg/mL) were Shodex (Showa Denko K.K.) standard P-82 pullulans with MMs of 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kDa and glucose (0.18 kDa).

Particle Size Distribution Analyses of Wheat and Rye Bran. The particle size distributions of untreated wheat and rye bran were determined by mechanical sieving (Endecotts Ltd., London, U.K.) into six fractions: $a < 125\,\mu$m, $125 < b < 250\,\mu$m, $250 < c < 400\,\mu$m, $400 < d < 710\,\mu$m, $710 < e < 1000\,\mu$m, and $f > 1000\,\mu$m. The d_{50} values were estimated as the particle sizes below which 50% by mass of the particles fall. Particle size distributions of ball-milled samples were determined by laser diffraction in a Malvern 2000 Mastersizer (Goffin/Meyvis, Hoeilaart, Belgium). Volume percentages of particles with diameter sizes between 0.49 and 840.90 μm were obtained. For the milled samples, d_{50} values were determined on a volume basis.

Microscopy Analyses. Untreated and ball-milled wheat bran samples were embedded in 2.0% agar and fixed in 1.0% glutaraldehyde in 0.1 M sodium potassium phosphate buffer (pH 7.0), dehydrated in a graded ethanol series, and embedded using the Leica historesin embedding kit (Heidelberg, Germany). Polymerized samples were sectioned (2.0 μm sections) in a rotary microtome HM 355 (Microm Laborgeräte GmbH, Walldorf, Germany) using a steel knife, and the sections were transferred onto glass slides. Protein was stained with aqueous 0.1% (w/v) acid fuchsin (BDH Chemicals, Poole, Dorset, U.K.) in 1.0% acetic acid for 1 min, and β-glucan was stained with aqueous 0.01% (w/v) Calcofluor White (Fluorescent Brightener 28) for 1 min as outlined by Wood et al. (*28*) and Parkkonen et al. (*29*). In exciting light (epifluorescence, 330−385 nm; fluorescence, >420 nm), intact cell walls stained with Calcofluor appear blue and proteins stained with acid fuchsin appear red. Starch is unstained and appears black. The samples were examined with an Olympus BX50 microscope (Olympus, Tokyo, Japan). Micrographs were obtained using a PCO SensiCam CCD camera (PCO, Kelheim, Germany) and the Cell^P imaging software (Olympus).

Table 1. Chemical Composition of Wheat Bran, Rye Bran, and Pericarp-Enriched Wheat Bran[a]

	wheat bran	rye bran	pericarp-enriched wheat bran
ash (% dm)	6.0 ± 0.2	5.7 ± 0.2	2.9 ± 0.1
protein (% dm)	14.9 ± 0.5	15.6 ± 0.3	6.0 ± 0.4
total noncellulosic neutral carbohydrate (% dm)[b]	45.9 ± 0.1	52.4 ± 0.2	36.5 ± 0.4
arabinose (% dm)	9.9 ± 0.5	7.6 ± 0.1	19.6 ± 0.3
xylose (% dm)	16.2 ± 0.2	16.0 ± 0.3	19.2 ± 0.2
AX (% dm)[c]	23.0 ± 0.6	20.8 ± 0.3	34.1 ± 0.4
A/X ratio[d]	0.61 ± 0.02	0.48 ± 0.01	1.02 ± 0.01
mannose (% dm)	0.8 ± 0.1	2.0 ± 0.1	0.2 ± 0.1
galactose (% dm)	1.3 ± 0.1	1.1 ± 0.1	1.4 ± 0.1
glucose (% dm)	23.4 ± 0.7	32.0 ± 0.6	1.0 ± 0.1
uronic acid (% dm)	1.4 ± 0.1	0.8 ± 0.1	2.4 ± 0.1

[a] Values are expressed as means ± standard deviations. [b] Total noncellulosic neutral carbohydrate = 0.88(% arabinose + % xylose) + 0.9(% mannose + % galactose + % glucose), with the factors 0.88 and 0.9 to correct for water uptake in the hydrolysis procedure. [c] AX = 0.88(% arabinose + % xylose), with the factor 0.88 to correct for water uptake in the hydrolysis procedure. [d] Arabinose/xylose ratio.

RESULTS

Composition of Wheat Bran, Rye Bran, and Pericarp-Enriched Wheat Bran. Wheat bran and rye bran both contained a high level of noncellulosic carbohydrates (45.9 and 52.4%, respectively) (**Table 1**). The most abundant monosaccharide moieties in wheat and rye bran were glucose (23.4 and 32.0%, mainly starch-derived), xylose (16.2 and 16.0%), and arabinose (9.9 and 7.6%), respectively. Calculated AX levels were 23.0 and 20.8% for wheat and rye bran, respectively, while their A/X ratios were 0.61 and 0.48. These results agree well with the findings of other authors, who reported AX levels of 17−32% and A/X ratios between 0.5 and 0.6 for wheat and rye bran (*2*, *3*, *6*, *17*). Wheat and rye bran also contained fairly high levels of proteins (15%) and ash (6%) and minor levels of uronic acids (1%).

Pericarp-enriched wheat bran contained a high level of noncellulosic carbohydrates (36.5%), with arabinose and xylose being the major monosaccharides (**Table 1**). The AX level was 34.1%, and the A/X ratio was 1.02. Such a high degree of arabinose substitution is typical for pericarp AX (*7*, *9*) and indicates that the produced wheat bran fraction is indeed enriched in pericarp material.

Changes in Physicochemical and Structural Properties of Bran AX upon Milling. Wheat and rye bran were ball-milled for up to 120 h (50 and 36% filling degree for wheat and rye bran, respectively), and samples were withdrawn at several time points. Because the degree of filling of the milling jar influences the milling process (*23*), withdrawal of material for sampling can be assumed to have affected the further size reduction process. However, the difference in sample amount because of withdrawal of sample aliquots during the consecutive stages of the ball-milling process was limited, and only a slight overestimation can be expected.

The AX content of aqueous wheat and rye bran extracts increased with treatment time, implying that AX gradually became extractable in water. The average A/X ratio of the extracted AX slightly decreased with milling time (**Table 2**). Under the dilute conditions used, only 4 and 7% of all wheat and rye bran AX, respectively, could be extracted from the untreated sample (A/X ratio = 0.74 and 0.65 for wheat and rye bran, respectively). This level increased to 21% (wheat) and 39% (rye) for fractions ball-milled for 24 h and to 42% (wheat) and 63% (rye) after 48 h of treatment, respectively. Prolonged treatment (120 h) brought 61% of all wheat bran AX (A/X ratio = 0.72) and 73% of all rye bran AX (A/X ratio = 0.51) in solution. Even more wheat bran AX (79%) could be extracted in water upon even more prolonged treatment (240 h).

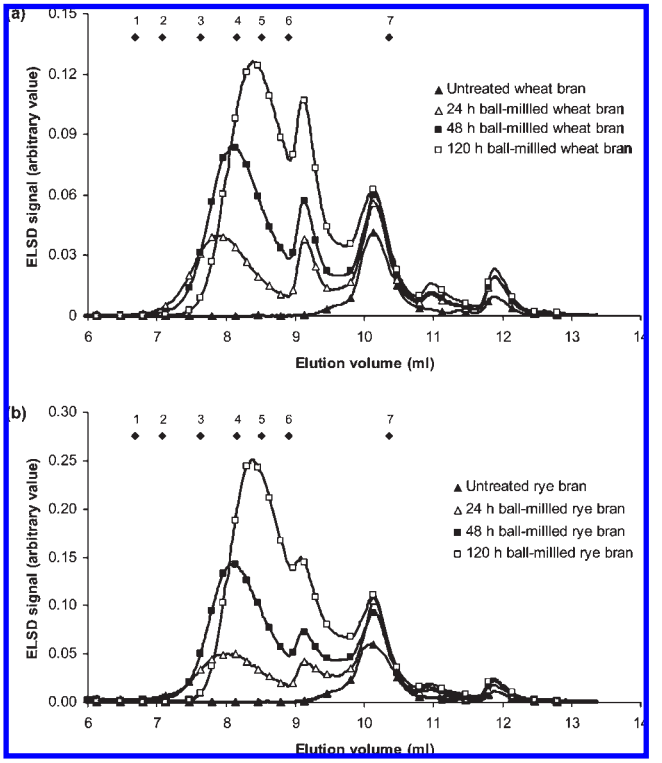

Figure 1. Influence of ball milling on MM of extracted polysaccharides. Curves show MM distributions for (a) untreated and ball-milled (BM) wheat bran (24, 48, and 120 h; 500 rpm; 50% filling degree) and (b) untreated and ball-milled rye bran (24, 48, and 120 h; 500 rpm; 36% filling degree). MM markers (◆) from the left to the right are 212 (1), 112 (2), 47.3 (3), 22.8 (4), 11.8 (5), 5.9 (6), and 0.18 (7) kDa.

Apparent MM distribution profiles of untreated and ball-milled bran extracts showed a shift to decreased MM with increasing ball mill pretreatment time (**Figure 1**). The WE-AX level in the untreated samples was too low to allow for determination of their apparent peak MM. AX from material subjected to ball milling for 24 h had an apparent peak MM of 32.4 kDa (wheat) and 25.6 kDa (rye) (**Table 2**). After 120 h, extracted AX fragments had an apparent peak MM of 14.6 kDa (wheat) and 15.7 kDa (rye).

Optimization of the Ball-Milling Process through Variation of the Jar Filling Degree. Ball milling increased the extractability of wheat and rye bran AX and gave rise to AX degradation products. However, high WE-AX levels and low MM AX were only obtained after a sufficiently long treatment (120 h). The ball-milling process was optimized through variation of the jar filling degree, because this parameter has been shown previously to influence the milling process (*23*). For both wheat and rye bran, decreasing the filling degree of the milling jar resulted in increased WE-AX levels within the same milling time (24 h). Upon milling at the lowest filling degree (16% filling degree), 77 and 75% of the wheat and rye bran AX, respectively, were extractable (**Table 3**) compared to 21% upon milling at the highest filling degree (50% filling degree). A 24 h treatment at the lowest filling degree made even more AX extractable (≥75%) than a 120 h treatment at a higher filling degree (< 74%).

With decreasing filling degree of the milling jar, A/X ratios of the extracted wheat and rye AX fragments slightly decreased, while their MM more strongly decreased (**Table 3**). Upon milling

Table 2. Characteristics of WE-AX upon Extraction of Untreated and Ball-Milled (500 rpm; 24, 48, and 120 h) Wheat Bran (50% Filling Degree), Rye Bran (36% Filling Degree) and Pericarp-Enriched Wheat Bran (50% Filling Degree)

	wheat bran				rye bran				pericarp-enriched wheat bran	
		ball milled				ball milled				ball milled
	untreated	24 h	48 h	120 h	untreated	24 h	48 h	120 h	untreated	24 h
WE-AX level (% of bran AX)	3.7	21.2	41.8	61.3	6.9	38.5	62.8	73.3	0.8	63.3
A/X of WE-AX[a]	0.74	0.77	0.76	0.72	0.65	0.55	0.55	0.51	0.18	0.97
apparent peak MM of WE-AX (kDa)[b]	nm[c]	32.4	24.3	14.6	nm	25.6	21.9	15.7	nm	5.2

[a] Arabinose/xylose ratio. [b] Apparent peak molecular mass, determined by high-performance size-exclusion chromatography. [c] nm = not measurable.

Table 3. Characteristics of WE-AX upon Extraction of Ball-Milled Wheat and Rye Bran (500 rpm, 24 h, 16−50% Filling Degree)

	ball-milled wheat bran				ball-milled rye bran			
	filling degree (%)				filling degree (%)			
	50.0%	34.0%	22.8%	16.4%	49.5%	36.0%	22.5%	16.2%
WE-AX level (% of bran AX)	21.2	37.2	62.3	76.8	20.8	38.5	60.4	75.0
A/X of WE-AX[a]	0.77	0.72	0.68	0.65	0.64	0.55	0.53	0.52
apparent peak MM of WE-AX (kDa)[b]	32.4	16.9	7.6	6.0	37.9	25.6	18.8	9.7

[a] Arabinose/xylose ratio. [b] Apparent peak molecular mass, determined by high-performance size-exclusion chromatography.

at the lowest filling degree (16%), AXOS with an A/X ratio of 0.65 and a MM of 6.0 kDa were obtained from wheat bran, while AXOS with a somewhat lower A/X ratio (0.52) and a higher MM (9.7 kDa) were obtained from rye bran.

Changes in Pericarp-Enriched Wheat Bran AX Extractability and Molecular Properties upon Milling. Wheat aleurone and outer pericarp AX make up 30 and 38%, respectively, of the wheat bran AX, with the remaining AX being present in the nucellar epidermis, the seed coat, and the inner pericarp (*9*). Because wheat bran ball milling at optimal conditions gave rise to 77% WE-AX (**Table 3**), at least part of the pericarp AX must be present in the extract. To assess the impact of ball milling on the pericarp layer as such, pericarp-enriched wheat bran was produced and ball-milled.

Untreated pericarp-enriched wheat bran contained only 1% WE-AX, with an A/X ratio of 0.18. The WE-AX level increased up to 63% upon ball milling (24 h). The now extracted AX had a much higher A/X ratio (0.97) and a low apparent peak MM (5.2 kDa) (**Table 2**).

Changes in Bran Particle Size and Microscopic Structure upon Milling. To gain insight in the mechanism behind the observed effects, the particle size and microscopic structure of the untreated and treated bran material was studied. The majority of the particles of untreated wheat and rye bran were larger than 250 μm, with a mass-based d_{50} of about 1000 and 500 μm for wheat and rye bran, respectively (**Figure 2a**). A 24 h ball-mill treatment of wheat and rye bran led to materials with volume-based d_{50} values of 32 and 40 μm, respectively. Whether the bran material was reduced to this average size or reduced to much smaller particle sizes and formed aggregates could not be determined. The fact that particle sizes did not decrease to smaller values upon further treatment but, on the contrary, even seemed to become somewhat larger upon ball milling for 48 or 120 h (**Figure 2b**) would, however, suggest the latter.

Microstructural analysis showed that untreated wheat bran consisted mostly of large fragments of pericarp and aleurone layers still attached to each other or as separate structures (**Figure 3a**). Some cell walls and protein originating from subaleurone and endosperm and some structures of embryo were also present in the sample. In contrast to this, ball-milled samples appeared under fluorescence microscopy as aggregates of material in which only very few recognizable structures, including small fragments of aleurone cell walls and some protein aggregates,

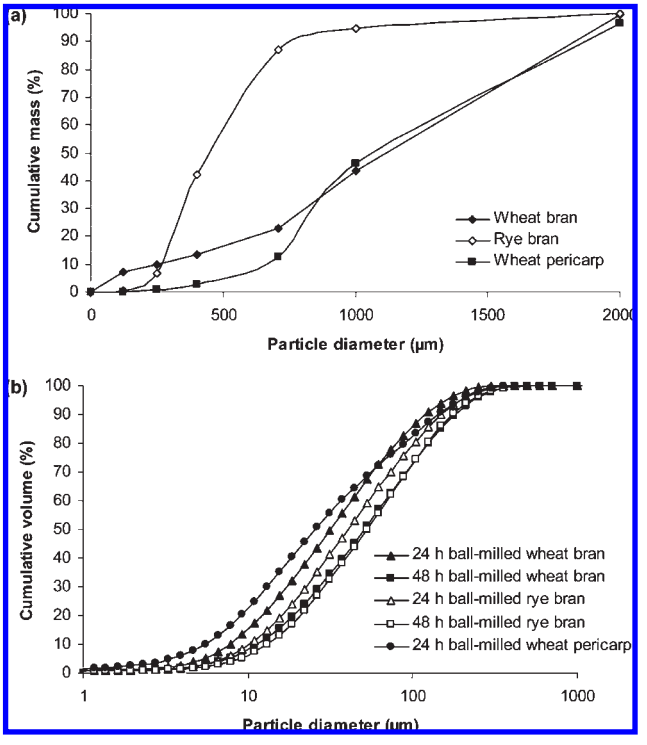

Figure 2. Influence of milling on particle size distribution of wheat and rye bran. (a) Cumulative amount of particles (mass %) as a function of the particle diameter (μm) for untreated wheat bran, rye bran, and pericarp-enriched wheat bran. (b) Cumulative amount of particles (volume %) as a function of the particle diameter (μm) for ball-milled wheat bran (24 and 48 h; 50% filling degree), rye bran (24 and 48 h; 36% filling degree), and pericarp-enriched wheat bran (24 h; 50% filling degree).

were observed (panels **b**−**d** of **Figure 3**). The sizes of the aggregated particles were consistent with those observed with laser diffraction. Somewhat more and larger aleurone cell-wall fragments were observed in the 24 h ball-milled sample (50% filling degree; **Figure 3b**) than in the 48 h ball-milled sample (50% filling degree; **Figure 3c**), which contained only very few and small-sized stained cell-wall pieces. Wheat bran that was ball-milled for 24 h at a lower filling degree (16%) contained only very few structures that could be recognized (**Figure 3d**).

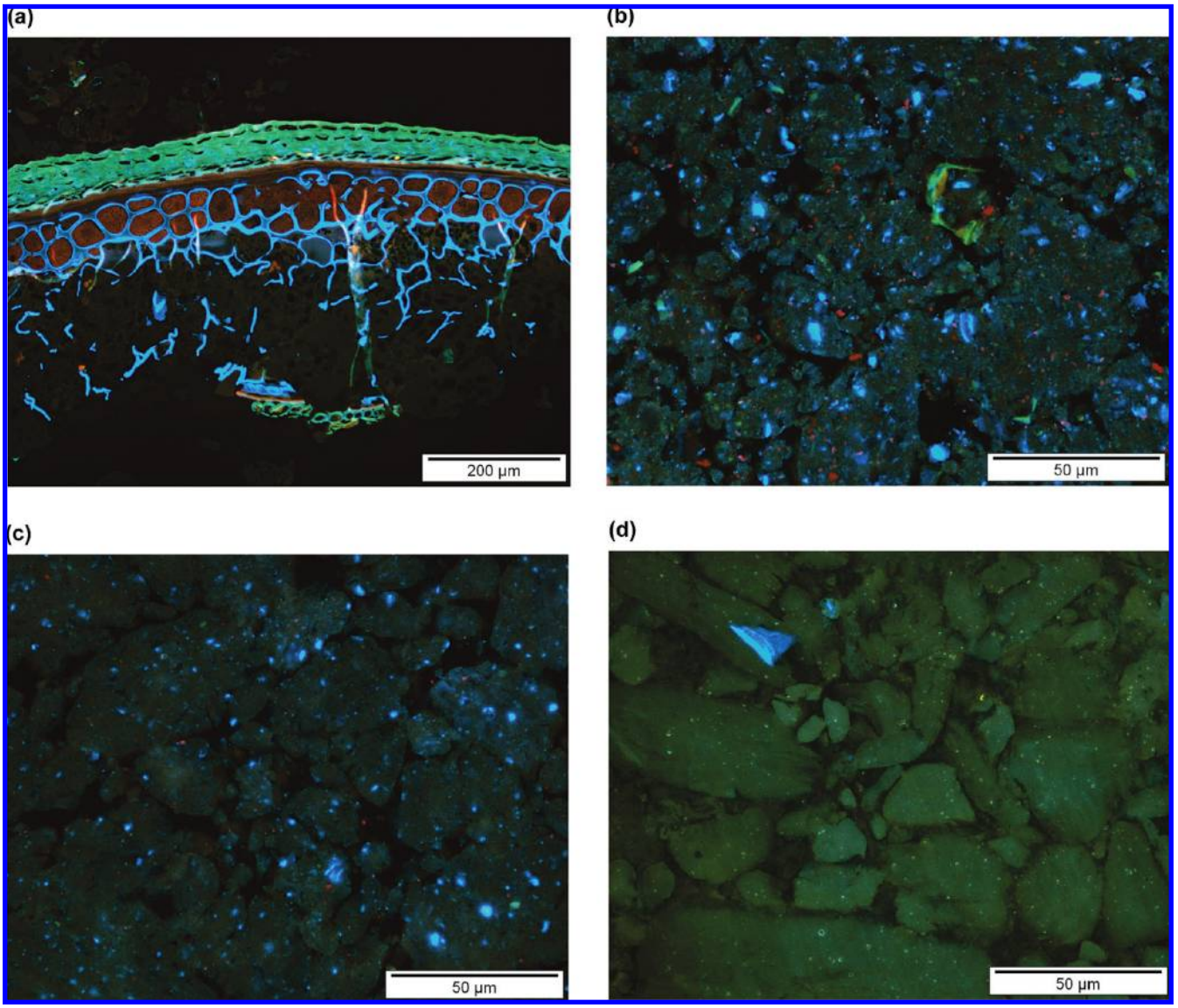

Figure 3. Micrographs of (a) untreated wheat bran, (b) 24 h ball-milled wheat bran (50% filling degree), (c) 48 h ball-milled wheat bran (50% filling degree), and (d) 24 h ball-milled wheat bran (16% filling degree). Intact cell walls stained with Calcofluor appear blue, and proteins stained with acid fuchsin appear red. Starch is unstained and appears black.

The observations on the protein in the samples were similar; more severe ball-mill treatments led to less intensely stained protein fragments (**Figure 3**).

The micrographs thus confirm the particle size measurements using laser diffraction but, at the same time, show that macromolecular structures and tissues are almost completely destroyed and that the size of the particles is the result of agglomeration of much finer material. Sizes of the few still discernible individual recognizable cell-wall fragments are in the low micrometer to high nanometer range, implying a smaller size of the material, which cannot be distinguished anymore.

DISCUSSION

The present study investigated the potential of ball milling, a dry milling process, for *in situ* production of AXOS in wheat and rye bran. Changes in the microscopic structure of wheat and rye bran upon milling were explored, and the structural and physicochemical properties of the obtained bran-derived AXOS preparations were studied.

Ball-milling obviously increased the water extractability of wheat and rye bran AX and lowered the apparent peak MM of the extracted bran AX. Enhanced solubility by ball-mill treatment has been observed for carrot insoluble fiber (*22*), psyllium seed husk AX (*23*), and wheat starch (*20*, *21*). Ball milling has also been reported to decrease the MM of maize (*30*) and wheat starches (*21*), pectin (*24*), and psyllium seed husk AX (*23*). The high WE-AX levels (> 75% of the total AX) and the low MM of the AX (6 kDa) obtained upon ball milling of wheat bran clearly show that this dry milling process is able to produce wheat and rye bran-derived AXOS components *in situ*.

Ball milling of wheat bran at optimal conditions (24 h, 16% jar volume capacity) gave rise to a high WE-AX level (77%) and AXOS with a high A/X ratio (0.65). The features of this wheat bran-derived AXOS preparation are clearly different from those of AXOS obtained upon xylanase treatment of wheat bran, because the latter are characterized by much lower AXOS yields (17−35% of the bran AX) and lower A/X ratios (0.26−0.30) (*16*, *31*). Because the action of xylanases is hindered by the presence of high levels of arabinose substituents (*32*, *33*) and lignin (*31*), xylanases generally act on the lowly substituted nucellar epider-

mis (A/X ratio = 0.1) and aleurone layers (A/X ratio = 0.4−0.5) (*9*, *17*) and leave the more inaccessible pericarp layer largely untouched. Xylanase treatment of wheat bran thus results in limited AXOS yields and AXOS preparations with low A/X ratios.

The high-energy impact to which particles are subjected during ball-mill treatment affects wheat bran AX in a totally different way than xylanases. AX originating from both aleurone and nucellar epidermis on the one hand and pericarp on the other hand are rendered water-extractable upon ball milling.

Antoine et al. (*34*) previously reported that bran particles as a whole were reduced more rapidly upon ball milling than isolated aleurone layer particles. They ascribed this to the fragility and the low extensibility of the pericarp layer (*34*, *35*). AXOS released from ball-milled wheat bran had an A/X ratio of at least 0.65. Because this value is between the A/X ratios of aleurone (0.4−0.5) and pericarp AX (≥1.0) (*9*, *17*), one may conclude that AX from both layers is equally rendered water-extractable upon ball-mill treatment. Treatment of pericarp-enriched wheat bran confirmed the assumption that ball milling made at least part of the pericarp AX extractable.

Microscopic analysis of ball-milled wheat bran showed that the cell-wall structures were completely destroyed by the ball-mill treatment. Almost no stained cell-wall particles could be observed anymore. Low-MM β-glucan (MM < 10000) cannot be detected with calcofluor. Fragmentation was more pronounced with increasing milling times or decreasing jar filling degrees. The micrographs of the ball-milled wheat bran samples suggest that the particle sizes observed by laser diffraction result from aggregation of very finely ground bran material. The obtained d_{50} values thus have to be considered as apparent values. Sizes of individual recognizable cell-wall structures observed upon ball milling were in the low micrometer to high nanometer range. This implies that ball milling is able to reduce the size of the majority of the (no longer discernible) bran material to the nanoscale level. This seems consistent with the observation that ball milling breaks covalent bonds inside AX molecules. Indeed, given the approximate size of a xylose molecule of 5.6 Å, a 15 kDa AXOS molecule would be 65 nm in size at full length. Breaking of covalent bonds is probably the combined result of the high-energy impact in the ball-milling process and the considerable development of heat in the milling jar and may also explain why such extended milling times are necessary to significantly increase the WE-AX level.

In conclusion, we showed that an extensive single-stage dry ball-milling process can reduce the size of wheat or rye bran to the nanoscale level and produce AXOS in the process. Ball-mill treatment renders AX from the aleurone layer as well as the pericarp layer water-extractable, resulting in higher AXOS yields and the production of AXOS with a higher A/X ratio compared to enzymically produced wheat bran-derived AXOS. In addition, treatment renders a wet extraction step redundant. Ball milling thus contributes to more efficient use of the cereal bran and to the production of a wider range of AXOS structures. Ball milling of wheat bran can be performed either separately or in combination with enzyme treatment. The former gives rise to AXOS preparations, which contain material from both the aleurone and pericarp layers, and has the advantage that the end product can be obtained without a wet incubation step. Besides, ball-mill treatment of xylanase-treated wheat bran results in AXOS preparations containing mainly pericarp-derived material. Ball milling thus offers perspectives for upgrading the low-value pericarp layer in particular. At the moment, however, the phenomena observed upon ball milling ask for further investigation. Insight into the ball-milling mechanism would also be useful to upscale the laboratory ball-milling process to pilot or even industrial scale.

ABBREVIATIONS USED

AX, arabinoxylan; AXOS, arabinoxylan oligosaccharides; A/X ratio, arabinose/xylose ratio; MM, molecular mass; WE-AX, water-extractable arabinoxylan.

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