PUBLICATION II

Treatments with xylanase at high (90%) and low (40%) water content have different impacts on physicochemical properties of wheat bran

In: Food and Bioprocess Technology 6(2013), pp. 3102–3112.

Food Bioprocess Technol (2013) 6:3102–3112
DOI 10.1007/s11947-012-0967-6

ORIGINAL PAPER

Treatments with Xylanase at High (90 %) and Low (40 %) Water Content Have Different Impacts on Physicochemical Properties of Wheat Bran

Outi K. Santala · Emilia A. Nordlund · Kaisa S. Poutanen

Received: 21 May 2012 / Accepted: 12 September 2012 / Published online: 10 October 2012

Abstract The aim of the work was to elucidate the impacts of treatment with xylanase at high (90 %) and low (40 %) water contents on the structural and physicochemical properties of wheat bran. The bran treatments at 40 % water content, both with and without added xylanase, resulted in a smaller average bran particle size, more changes in bran microstructure, and higher solubilization of polysaccharides than the corresponding treatments at 90 %. Also, the water holding capacity of bran (3.6±0.1 g water/g bran dm), determined by Baumann method, decreased more already after 4-h xylanase treatments at 40 % (2.4±0.1) than at 90 % (2.9±0.2). The solubility of salt-extractable bran proteins decreased during the treatments, especially at 40 %, also without added xylanase. Protein aggregation was detected in the SDS+DTT-extractable bran fraction, which also contained small proteins of 10–20 kDa not detectable in the untreated bran. The use of xylanase had only minor effect on bran proteins as compared to the treatments without added xylanase. The results indicate the large role of mechanical shear on the bran properties at 40 % water content. The low arabinose/xylose ratio (0.32) in the bran water extract after 24-h xylanase treatment at 40 %, however, suggests that the solubilization of arabinoxylan was caused by enzymatic action, and not by mechanical degradation. Arabinose/xylose ratio of the bran water extract decreased similarly during all the treatments, suggesting similar solubilization pattern of arabinoxylan at both water contents. The study showed that bran properties can be significantly modified by adjusting the water content and mechanical energy used in processing.

O. K. Santala (✉) · E. A. Nordlund · K. S. Poutanen
VTT Technical Research Centre of Finland,
P.O. Box 1000, FI-02044 VTT, Espoo, Finland
e-mail: outi.santala@vtt.fi

Keywords Wheat bran · Xylanase · Enzyme · Modification · Water content

Introduction

Cereal bran comprises the outer layers of grain which are separated in the milling process during the production of refined flours. Wheat bran is high in dietary fiber (DF) and a good source of phytochemicals, but its use in food applications is limited by technological and sensory challenges. The reasons behind the adverse effects of bran in food processes are not fully elucidated, but both physical and chemical mechanisms have been suggested (Gan et al. 1992; Lai et al. 1989; Noort et al. 2010). The well-documented evidence about the positive health effects of the consumption of DF and whole grain foods has increased the interest to study and improve the technological and nutritional properties of bran and other DF-rich plant materials.

The technological functionality as well as the physiological effects of bran is dependent on its physicochemical characteristics, which are influenced by particle size, cell wall architecture, chemical composition, and molecular structure of the DF polymers of bran (Noort et al. 2010; Auffret et al. 1994; Izydorczyk 2009). These properties affect, for example, the hydration characteristics of the material, including water binding capacity (WBC), water holding capacity (WHC), and solubility, which are important for both the technological applicability and the physiological function of DF (Thebaudin et al. 1997; Chaplin 2003). In wheat, the main DF component is arabinoxylan (AX), and especially AX solubilization is considered essential for the baking performance of bran and motivates the use of AX-degrading enzymes in breadmaking processes (Figueroa-Espinoza et al. 2004; Courtin and Delcour 2002). Desired

modifications of nutritional and technological properties can be achieved by the use of hydrolytic enzymes, such as endoxylanases (Figueroa-Espinoza et al. 2004; Katina et al. 2006; Anson et al. 2009). Endo-β-1,4-xylanases depolymerise and solubilize AX by cleaving the β-xylosidic bond between two D-xylopyranosyl residues linked in β-(1,4). Enzyme function and AX hydrolysis can also be facilitated by thermal or mechanical methods, such as extrusion, high shear treatment, and intensive ball milling (Figueroa-Espinoza et al. 2004; Gajula et al. 2008; Van Craeyveld et al. 2009). In addition to DF, bran also contains a relatively high amount of protein (Kamal-Eldin et al. 2009). Bran protein has been very little studied, but it is obvious that bran processing also influences the functional properties of the protein fraction.

In cereal processing, water content is an important parameter, influencing for example diffusion of components, rheological properties of the material as well as enzyme activity. Enzymatic treatment at reduced water content could be economically beneficial, especially when targeting dry end products. We have previously studied the impact of water content, ranging from 20 to 90 %, on the solubilization of AX during xylanase treatment of wheat bran (Santala et al. 2011). Interestingly, AX solubilization was detected to be highest at the water contents of 40 and 90 %. In the current study, the aim was to elucidate further the factors affecting the solubilization of AX at the low (40 %) and high (90 %) water content treatment of bran (with and without xylanase) by assessing the subsequent structural and physicochemical changes in bran properties, with focus on water-extractable polysaccharides and bran proteins.

Materials and Methods

Bran

The bran was obtained from mixed wheat varieties (Mühle Rüningen GmbH & Co. KG, Braunschweig, Germany). Before bran removal, the grains were peeled to remove 2–3 % of the grain outer layers in order to reduce the level of contaminating microbes and enzymes on the surface layers of the grains. The chemical composition was: (percentage of dm): DF 49.5 (including AX 22.8, fructan 3.6, and ß-glucan 2.8), protein 19.5, starch 11.6, fat 4.8, and ash 6.7. The bran was ground in batches of about 4 kg by passing each batch three times through a mill (Hosokawa Alpine, 100 UPZ, Retsch GmbH, Haan, Germany; mill sieve size, 0.3 mm). After grinding, the mean particle size was 110 μm, and 90 % of the particles were smaller than 450 μm as determined by Coulter LS320 particle size analyzer wet module (Coulter Corporation, Miami, FL, USA).

Xylanase Enzyme Preparation

A commercial *Bacillus subtilis* xylanase preparation Depol 761P (Biocatalysts Ltd, Cardiff, UK) was used for the bran treatments. According to the manufacturer, it is especially suitable for extraction of soluble fiber from wheat bran. The activity profile of the enzyme preparation was as follows: xylanase (1 % birch glucurone xylan as substrate) 28,660 nkat/g (Bailey et al. 1992), polygalacturonase (0.4 % polygalacturone acid) 1,317 nkat/g (Bailey and Pessa 1990), β-glucanase (1 % barley β-glucan) 1,625 nkat/g (Zurbriggen et al. 1990), α-amylase (*p*-nitrophenyl maltoheptaoside) 44 nkat/g (Megazyme Ceralpha method), and β-xylosidase (5 mM *p*-nitrophenyl-β-D-xylopyranoside) 2 nkat/g (Poutanen and Puls 1988). The preparation was found to be free from cellulase (filter paper) (IUPAC 1987), endoglucanase (1 % hydroxyethyl cellulose) (IUPAC 1987), mannanase (0.5 % locust bean gum) (Stålbrand et al. 1993), β-glucosidase (1 mM *p*-nitrophenyl-β-D-glucopyranoside) (Bailey and Linko 1990), α-arabinosidase (2 mM *p*-nitrophenyl-α-L-arabinofuranoside) (Poutanen et al. 1987), endo-protease (azurine-crosslinked casein, Protazyme AK tablet method, Megazyme International Ireland), and ferulic acid esterase (4 mM ethyl ferulate) (Forssell et al. 2009) activities. All activity measurements were performed at pH 5, 50 °C.

Enzymatic Treatment of Wheat Bran

Bran was treated with or without added enzyme using two different mixing systems which have also been described previously (Santala et al. 2011). The enzyme preparation was dosed according to its xylanase activity at a level of 200 nkat/g bran, which corresponds the dosage used previously (970 EU/g bran) (Santala et al. 2011). The enzyme powder was mixed with bran before water addition. The treatments at 40 % water contents were performed in a farinograph mixing bowl (Brabender Farinograph, mixer type S300 with z-blades, Brabender, Duisburg, Germany). The mixer bowl was heated by water circulation (50 °C), and the mixing speed was 60–63 rpm. One hundred twenty-five grams of ground bran (with or without enzyme addition) was placed in the mixer, and preheated water was added by spraying for 1–3 min while the blades were rotating to obtain an even distribution of water. The mixing bowl was sealed tightly to avoid evaporation of moisture. At water contents of 90 %, due to the liquid form of the mixture, the reactions were performed in a covered steel container (0.5 l) with double-blade mixer (speed 160 rpm) placed in a water bath (50 °C). The water content of the treatment refers to the total water content, i.e., the water content of bran was taken into account.

The experiments were done in duplicate. The reaction time was 4 or 24 h, after which a sample was taken and

reaction stopped by cooling down and freezing the sample immediately. Subsequently, part of the frozen sample was freeze dried and ground with a laboratory mill (0.5 mm sieve) for later analyses.

Standard Analyses

The moisture content of the bran and the freeze dried bran samples was determined by oven drying (1 h at 130 °C). Analyses of the chemical composition of bran were made as follows: total protein content by the American Association of Cereal Chemists (AACC) method no. 46–11A (AACC 2003), total DF by AOAC method no. 985.29 (AOAC 1990), ß-glucan by AACC method no. 32–23 (AACC 2003), fructan by AOAC method no. 999.03 (AOAC International 2003), fat by AOAC method no. 922.06 (AOAC International 2003), digestible starch by Megazyme method (Mccleary et al. 1994), and ash gravimetrically by burning at 550 °C.

Particle Size Determination

The particle size distribution of bran samples was determined in duplicate by laser light (750 nm) diffraction using Coulter LS320 particle size analyzer wet module (Coulter Corporation, Miami, FL, USA). Seventy-five milligrams of freeze dried sample was dispersed in 1.5 mL of distilled water. The particle size measurement was performed after 15 min hydration including vortexing and incubation in an ultrasound bath (5 min) in order to prevent formation of aggregates. The results were calculated from the volumetric distribution of the particles using Fraunhofer optical model and geometric statistics.

Light Microscopy Analyses

Microscopy analyses were done using both freeze dried and fresh (frozen) samples. After defrosting at ambient temperature, the sample with 90 % water was first centrifuged to remove excess water. The bran samples were treated as described by Dornez et al. (2011) and sectioned to 2-μm sections in a rotary microtome HM 355 (Microm Laborgeräte GmbH, Walldorf, Germany). Sections were stained either with Light Green and Lugol's iodine solution, which stain protein green and starch purple, respectively, or with Acid Fuchsin and Calcofluor White, which stain protein red and β-glucan-rich cell walls light blue, respectively, as described by Andersson et al. (2011). The samples were imaged using exciting light (excitation 400–410 nm, emission >455 nm) and examined with an Olympus BX-50 microscope (Olympus, Tokyo, Japan). Micrographs were obtained using a PCO SensiCam CCD camera (PCO, Kelheim, Germany) and the Cell^P imaging software (Olympus).

Analysis of Monosaccharide Composition and Content

For the analysis of water-extractable monosaccharide composition, 0.25 g of freeze dried sample was mixed with 7 mL of 4 °C distilled water and shaken with glass pearls for 15 min at 4 °C. After centrifugation, the supernatant was boiled for 15 min and centrifuged again. The supernatant was hydrolysed with 3.75 M H_2SO_4. The hydrolysate and the monosaccharide standards (glucose, arabinose, xylose, galactose, and mannose) were analyzed as their alditol acetates as described by Blakeney et al. (1983) by gas chromatography (GC) using a Agilent 6890 GC equipped with a flame ionization detector (Agilent, Palo Alto, CA, USA). The column was DB-225 [30 m×0.32 mm; film thickness 0.15 μm (Agilent)]. Helium was used as carrier gas 1.2 mL/min. Split injection (1:3) was performed at 250 °C, and the detector was operated at 250 °C. The analytes were separated at 220 °C for 15 min. The monosaccharides were identified according to their retention times and quantitated with corresponding standard curves. Free hexose sugars were corrected by a factor of 0.9 to anhydro sugars, and pentose sugars by factor of 0.88. The fact that some of the arabinose residues might originate from arabinogalactan peptides (AGP) was not taken into account in the calculations of AX levels because the level of AGP in wheat bran is assumingly very low, as discussed previously (Santala et al. 2011). For the analysis of total monosaccharide composition, 50 mg of bran sample was mixed with 1.56 mL of 72 % (26 N) H_2SO_4 and incubated at room temperature for 30 min. After addition of 15.6 mL of water, the samples were boiled for 2 h and centrifuged. After that, the supernatants were acetylated and analyzed by GC as described above. All analyses were made in duplicate.

Protein Extraction and Analysis

Proteins of the bran samples were extracted using a sequential buffered extraction procedure modified from that of Loponen et al. (2007). Salt-soluble proteins were first extracted by mixing 50 mg of freeze dried sample with 1.5 mL of 4 °C 0.5 M Tris–HCl buffer (pH 8) containing 1 M NaCl, and shaken for 15 min at 4 °C. After centrifugation (21,100×*g*, 15 min, 4 °C), the extraction was repeated. The supernatants were combined and stored frozen until analysis. The residual protein fraction was extracted from the sediment at 50 °C water bath for 60 min, vortexing at 10-min intervals, using 1.5 mL of mixture containing 2 % sodium dodecyl sulphate (SDS), 10 % glycero1, 1.5 % dithiothreitol (DTT), and 0.05 M Tris–HCl pH 8 buffer. After centrifugation (21,100×*g*, 15 min, 21 °C), the extraction was repeated. The supernatants were combined and stored frozen until analysis.

The different protein fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–

PAGE) (Laemmli 1970). Two volumes of protein extracts were mixed with one volume of SDS-SB (20 % glycerol, 4 % SDS, 10 % β-mercaptoethanol, and 0.02 % bromophenol blue in 0.1 M Tris–HCl pH 6.8 buffer) and boiled for 10 min. Samples were run in Criterion TGX stain-free precast Tris–HCl gradient gels (4–20 %; Bio-Rad, Hercules, CA, USA) and visualized with a Criterion stain-free imaging system (Bio-Rad) where protein visualization is based on UV light-driven reaction of tryptophan residues in the presence of trichlorocompounds (Kazmin et al. 2002). The used protein standards were Precision Plus Unstained Protein Standards (Bio-Rad), ranging from 10 to 250 kD.

The protein content of all extracts was analyzed by a commercial kit (RC DC Protein Assay, Bio-Rad, Hercules, CA, USA) that is compatible for samples containing both reducing agents and detergents. In the assay procedure, sample proteins were precipitated and separated by centrifugation before the actual quantification, which is based on the Lowry protein assay (Lowry et al. 1951). Bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard protein. The analysis was made in duplicate.

Water Binding/Retention Capacity

WBC of bran samples was determined in duplicate by a simple centrifugation method modified from Sollars (1973). One hundred milligrams of freeze dried sample was weighed in an Eppendorf tube, and the tube with the contents was tarred. One milliliter of water was added and mixed by vortex. The bran suspension was shaken at room temperature (22–23 °C) for 30 min and centrifuged (12,100×*g*, 15 min). The supernatant was removed carefully, and the tube was weighed. The gained weight (plus the water contained initially in the sample) per gram of bran sample dry matter was the WBC of the sample.

Water Holding Capacity

WHC and its kinetics were determined using the Baumann apparatus (Baumann 1966). The Baumann apparatus consists of a small thermostated water tank that is connected to a horizontal graduated capillary tube filled with water. A sintered glass with Whatman grade 5 filter paper (Whatman, Little Chalfont, UK) was placed on top of the water tank, and after 5 min, the zero value was read from the graduated capillary tube. Seventy-five milligrams of dry sample was sprinkled for 30–40 s on the wetted filter paper, and the water uptake was recorded for 25 min at room temperature (22–23 °C). A glass lid was set on the sinter glass to minimize evaporative losses during the measurement. The results are expressed as milliliters of water uptake (plus the water contained initially in the sample) per 1 g of sample dry matter. Each sample was analyzed at least in duplicate.

Statistical Analysis

The results were calculated as means of at least four analysis results (duplicate analyses for each bran sample). Data were subjected to analysis of variance using the IBM SPSS Statistics 19 (IBM Corporation, Somers, NY, USA), and significant differences ($P<0.05$) between individual means were identified by the Tukey's test.

Results and Discussion

Impact of Processing on the Particle Size and Microstructure of Bran

Treatments at water content of 40 % resulted in notably smaller average bran particle size (upon grinding the freeze dried processed sample) than the corresponding treatments at 90 % water content (Table 1). The initial particle size of bran (mean diameter, 113 μm) did not significantly change during 4 h of treatment without added xylanase at water content of 90 %, while at 40 %, water content the average particle size decreased to 56 μm. After 24 h of treatment at 40 %, the average particle size was further reduced to 37 μm (35 % reduction as compared to the 4-h sample), while at 90 %, the particle size reduced to 94 μm (14 % reduction as

Table 1 Mean particle size (micrometer), water binding capacity (grams of water per gram of bran dm) and water holding capacity (grams of water per gram of bran dm) of untreated bran and bran samples treated at 40 and 90 % water content (determined from freeze dried and ground samples)

		4 h treatment				24 h treatment			
		No added enzyme		With xylanase		No added enzyme		With xylanase	
	Untreated	90 %	40 %	90 %	40 %	90 %	40 %	90 %	40 %
Mean particle size (μm)	113±9a	110±7a	56±3c	65±3c	37±3d	94±2b	37±4d	60±4c	26±2e
Water binding capacity (g water/g bran dm)	2.8±0.2a	3.0±0.1a	2.5±0.2b	2.1±0.1c	1.7±0.1d	3.0±0.2a	2.3±0.2bc	2.3±0.1bc	1.3±0.1e
Water holding capacity (g water/g bran dm)	3.6±0.1b	3.9±0.2a	3.2±0.2c	2.9±0.2d	2.4±0.1e	3.6±0.1ab	3.0±0.1d	2.9±0.2d	2.0±0.2f

The results are expressed as mean ($n=4$)±standard deviation. Values marked with different letters within the same row are significantly different ($P<0.05$)

compared to the 4-h sample). The use of xylanase reduced the particle size at both water contents, and already after 4-h treatment with xylanase, the mean particle decreased to 65 μm at 90 %, and to 37 μm at 40 % water content (Table 1).

Bran micrographs stained with Acid Fuchsin and Calcofluor White are presented in Fig. 1. The microscopy analyses were done using both freeze dried, ground samples and fresh (frozen) samples, but no differences were detected, and thus, only the images of fresh samples are shown. Light microscopy examination showed that processing of bran caused a significant release of proteins from the cells of the bran particles (Fig. 1). The change was most obvious after the treatments at 40 % (Fig. 1c, e), but detectable also at 90 % water content (Fig. 1b, d). The release of protein during endoxylanase treatment of wheat bran has also been noted by others (Benamrouche et al. 2002). Furthermore, the layered cell wall structure of the bran treated at 40 % water content was much more degraded, also when treated without added xylanase (Fig. 1c), as compared to the bran treated at 90 % (Fig. 1b). When bran was treated with xylanase at 90 %, the cell walls of the aleurone layer became thinner and lost their color, while seed coat and nucellar epidermis still remained attached to aleurone (Fig. 1d). In the bran treated at 40 % with no added enzymes, the structure of aleurone cells had broken, and the seed coat with nucellar epidermis had separated from aleurone cells (Fig. 1c). The use of xylanase at 40 % caused further degradation of the bran structures and the separation of seed coat and nucellar epidermis from each other (Fig. 1e).

The experiments at 40 and 90 % water content were performed in two different kinds of mixing devices because of the very different consistency of the bran–water mixtures. The difference in the consistency and the use of two different mixing systems brought about different shear forces, which obviously had an impact on the resulting bran properties. We have previously demonstrated that during the treatment at 40 % water content, the bran–water mixture transformed into a compact, plastic-like mass (Santala et al. 2011), which probably enhanced the impact of the mechanical shear during the treatment in the farinograph mixer. In the current study, the particle size measurement and

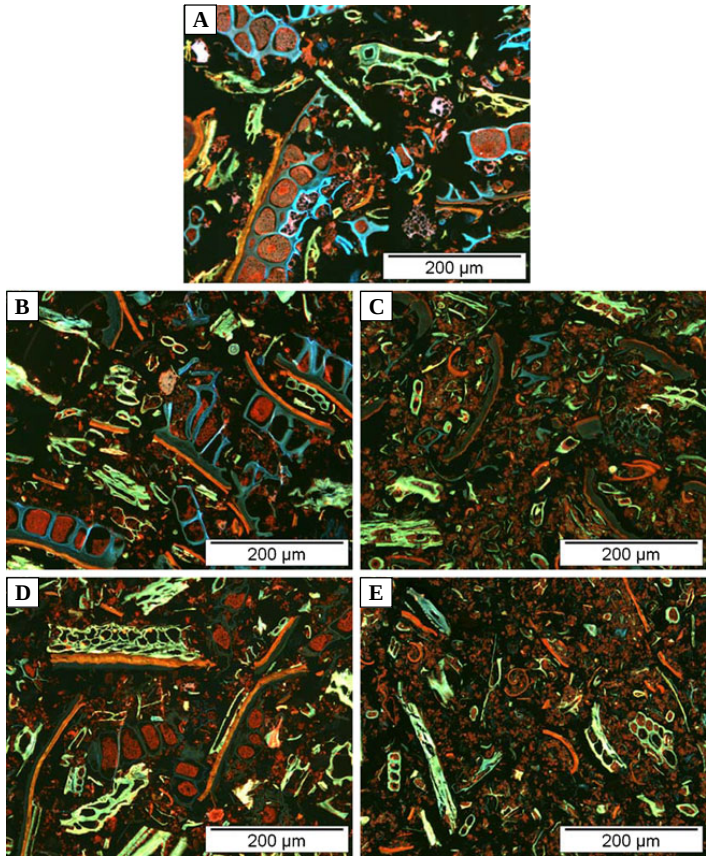

Fig. 1 Microstructure of untreated bran (**a**) and bran samples treated for 24 h without added enzymes at 90 % (**b**) and without added enzymes at 40 % (**c**), and bran samples treated for 24 h with xylanase at 90 % (**d**) and with xylanase at 40 % (**e**). Micrographs, prepared from fresh (frozen) bran samples, were stained with Acid Fuchsin and Calcofluor White. ß-glucan-rich endosperm and aleurone cell walls appear in *blue*, pigment strand (between pericarp and aleurone layer) in *orange*, pericarp layer in *light green and yellowish*, and proteins in *red and reddish brown*

microscopy analyses confirmed that the physical structure of the bran was significantly broken down by the treatments at 40 % water content, also without added enzymes, while smaller changes were detected in the bran treated at 90 %. The microscopy results also indicated that the bran components were broken down already during the treatments and not during the grinding of the freeze dried samples as no differences were detected between the micrographs prepared form fresh and frozen samples. The bran micrographs (prepared from fresh samples) stained with Light Green and Lugol's iodine solution further showed that starch granules were not significantly affected by any of the treatments (data not shown).

Total and Water-Extractable Monosaccharide Composition

The impact of treatment of bran at high (90 %) and low (40 %) water content on the solubilization of carbohydrates was followed by analyzing the monosaccharide composition of the water extracts of freeze dried bran samples after acid hydrolysis (Table 2). The total water-extractable monosaccharide content was higher after treatments at 40 % water content than at 90 %, especially after 24-h treatment without added enzymes, as the content of water soluble monosaccharides was 18.97 and 10.03 % of bran dm after treatments at 40 and 90 %, respectively (Table 2). This is mainly due to the glucose content, which increased at 40 % but did not significantly change at 90 % from 4 to 24 h. (Table 2). An increase in the soluble glucose content at 40 % refers to the degradation of starch, cellulose, or β-glucan. There was a trend of increase in the content of water-extractable mannose at 40 % and decrease at 90 % after 24-h treatments (Table 2). Mannose in the GC chromatograms originated probably from reduced fructose (fructan) because fructose as such cannot be detected by GC since fructose as a ketose produces mannitol and glucitol in the reduction step that was applied in the preparation of GC samples. Thus, fructose cannot be distinguished in GC analysis from glucose or mannose, which, respectively, forms glucitol and mannitol on reduction (Virkki et al. 2008). The increase in mannitol content at 40 % may thus be due to degradation of fructose, which bran contained 3.6 %.

The solubilization of AX, analyzed by the content of arabinose and xylose in the bran water extract, was rather similar at 40 and 90 % water content after 24-h treatment with xylanase (12.09 and 11.11 % of the bran dm, respectively; Table 2). However, without added enzyme, somewhat more solubilization was detected after the 24-h treatment at 40 than at 90 % water content (4.25 vs. 2.64 %). This indicates that the solubilization of AX at 40 % might not have been caused only by the action of the added xylanase alone, but also by other factors, such as the physical breakdown of bran cell walls by shear forces. The analysis of A/X ratio

Table 2 Monosaccharide composition (percentage bran dm) and A/X (arabinose/xylose) ratios of the water extracts of untreated bran and freeze dried bran samples after acid hydrolysis

		4 h treatment				24 h treatment			
		No added enzyme		With xylanase		No added enzyme		With xylanase	
	Untreated	90 %	40 %	90 %	40 %	90 %	40 %	90 %	40 %
Glucose (% bran dm)	3.64±0.17a	6.82±0.33b	8.43±0.38c	8.86±0.50c	10.69±1.23d	6.55±0.32b	13.58±0.45e	7.95±0.67bc	12.72±0.42e
Galactose (% bran dm)	0.48±0.03a	0.54±0.04ab	0.62±0.04bcd	0.63±0.04bcd	0.65±0.08 cd	0.57±0.03bc	0.60±0.02bcd	0.67±0.03d	0.65±0.02 cd
Mannose (% bran dm)	0.47±0.02bcd	0.37±0.03ab	0.41±0.03bcd	0.40±0.03abc	0.43±0.03bcd	0.27±0.11a	0.54±0.02d	0.39±0.10abc	0.53±0.03 cd
Ara+xyl (% bran dm)	0.66±0.03a	1.64±0.23ab	2.76±0.29b	9.12±0.56d	8.51±1.09d	2.64±0.16b	4.25±0.16c	11.11±0.43e	12.09±0.48e
A/X	0.99±0.02a	0.89±0.06b	0.72±0.02c	0.40±0.02e	0.41±0.02e	0.75±0.09c	0.59±0.01d	0.36±0.01e	0.32±0.01e
Sum[a]	5.24±0.24a	9.37±0.61b	12.22±0.66c	19.01±1.08d	20.28±2.39d	10.03±0.33bc	18.97±0.35d	20.12±1.02d	25.99±0.92d

The results are expressed as mean (n=4)±standard deviation. Values marked with different letters within the same row are significantly different (P<0.05). Free hexose sugars were corrected by a factor of 0.9 to anhydro sugars, and pentose sugars by a factor of 0.88

[a] The sum of the monosaccharides (percentage bran dm)

provided new information about the role of the enzymes in the solubilization of AX during the treatment at 40 % water content. The A/X ratio of the water extract of untreated bran was 0.99, and the ratio decreased with increasing treatment time and by the use of xylanase at both water contents (Table 2). It is known that xylanases preferably attack and solubilize AX fragments with low arabinose substitution (Benamrouche et al. 2002). This kind of AX occurs mostly in the aleurone (A/X 0.3-0.5) and in the nucellar epidermis (A/X 0.1), while the AX of outer tissues are more substituted (A/X≥1.0), and thus more resistant to enzymatic attack, as also indicated before (Van Craeyveld et al. 2010; Antoine et al. 2003; Barron et al. 2007). Interestingly, during the treatments at both water contents, the A/X ratio of the bran water extract decreased rather identically with increasing arabinose+xylose content (Table 2), suggesting that AX was solubilized from the same bran tissues regardless of the processing conditions studied. Furthermore, the A/X ratio of the water extract of bran treated for 24 h at 40 % was 0.32 (Table 2), which was in the range of the values reported in the literature for enzymatically solubilized wheat bran AX (0.27–0.32) (Beaugrand et al. 2004; Swennen et al. 2006). On the contrary, AX oligosaccharides produced by ball milling treatment have been reported to have much higher A/X ratio (0.65–0.72) (Van Craeyveld et al. 2009), suggesting that also some pericarp AX was rendered water-extractable by the ball milling treatment (Van Craeyveld et al. 2009). Thus, although the mechanical shear obviously enhanced the solubilization of AX during the treatments at 40 % water content, the mechanical energy input was probably not high enough to cause similar mechanical solubilization of AX as in the ball milling treatment (Van Craeyveld et al. 2009), and the solubilization of AX was also caused by enzymatic action at the 40 % treatments. The solubilization of AX during the treatments without added xylanase could have been due to the action of bran-associated (endogenous or microbial) enzymes. Instead of direct mechanical solubilization, the shear forces and the physical breakdown of bran particles have probably enhanced the action of endo- and exogenous enzymes by improving the availability of the substrate. The decrease of bran particle size during the treatment at 40 % (Table 1) increased the surface area of the substrate, which probably made the cell wall components more easily accessible to enzymes. It has been shown that the decrease of particle size may impact the hydrolysis of the substrate by enzymes (Mahasukhonthachat et al. 2010; Hemery et al. 2010).

The total monosaccharide composition of bran (glucose 24.0±0.8 %, galactose 1.16±0.04 %, mannose 0.63±0.04 %, AX 22.8±0.6 % with A/X ratio of 0.60±0.03) was not affected by any of the treatments, except for the glucose content, which decreased to 21.3±1.3 % after 24-h treatment at 90 % both with and without added xylanase (data not shown).

Extractability and Electrophoretic Patterns of Proteins

The physicochemical characteristics of bran proteins are not yet well established, although the protein content of bran is relatively high. In the current study, the impact of bran treatments on the solubility and molecular weight of bran proteins was analyzed by sequential extraction and electrophoretic analysis of salt-extractable and SDS+DTT-extractable (residual) proteins (Fig. 2) and by the spectrophotometrical quantification of protein in the extracted fractions (Fig. 3). The concentration of salt-extractable proteins decreased during the bran treatments, especially during the treatments at 40 % water content. This was detectable by both SDS–PAGE and protein quantification analysis. In the electrophoretic patterns

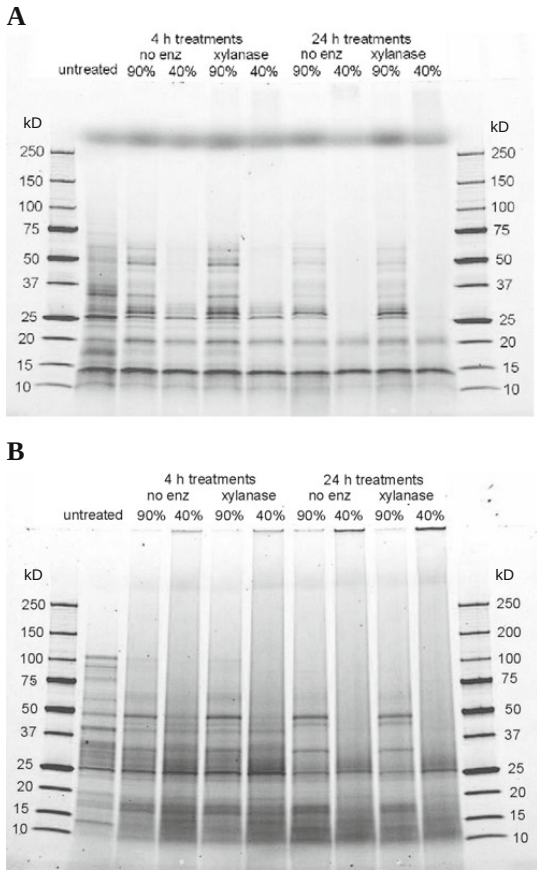

Fig. 2 SDS–PAGE patterns of salt extractable (**a**) and SDS+DTT-extractable (**b**) (residual) proteins of untreated bran and bran samples treated at 40 and 90 % water content

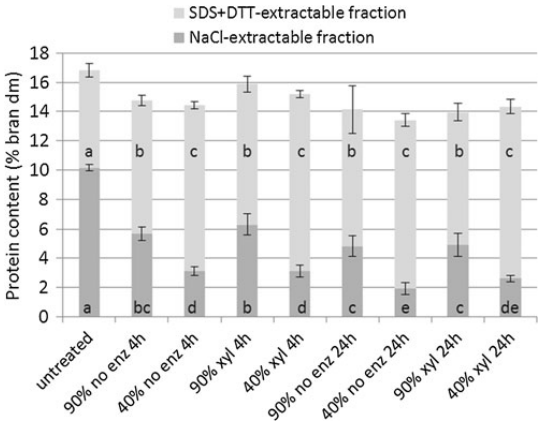

Fig. 3 Protein content (percentage bran dm) of the NaCl and SDS+DTT extracts of untreated bran and bran samples treated at 40 and 90 % water content. The results are expressed as mean (n=4), and the standard deviations are indicated by *error bars*. Columns marked with *different letters* within the same fraction are significantly different (P<0.05)

of the salt-soluble fraction, the proteins above 25 kDa had disappeared from the bran treated at 40 % water content (Fig. 2a). These protein bands also appeared lighter in the sample treated at 90 % for 24 h than in the untreated bran. The initial content of salt-extractable protein in the untreated bran was 10.2 % of bran dm, while after 4-h treatments without added enzyme at 90 and 40 % water, the content of salt-extractable proteins dropped to 5.7 and 3.1 % of bran dm, respectively (Fig. 3). After 24-h treatment without added enzyme, the content of salt-extractable protein decreased further at 40 % (to 1.9 % of bran dm).

Contrary to the decrease in the salt-extractable protein content, the concentration of SDS+DTT-extractable, "residual" protein fraction increased during the bran treatments. Protein aggregation was also detected in this residual fraction. This is evident from the electrophoretic patterns of SDS+DTT-extractable fraction, where no protein above 100 kDa appeared in the untreated bran, while in the treated bran samples, protein "dust" was observed in this region (Fig. 2b). Furthermore, especially in the case of samples treated at 40 %, a lot of protein had remained in the wells of the electrophoresis gel, which also indicates the formation of large protein-containing aggregates. The SDS+DTT-extractable fractions also contained small proteins of molecular weight of 10–20 kDa not detectable in the initial bran (Fig. 2b). The increase in the content of SDS+DTT-extractable proteins was also confirmed by the spectrophotometrical quantification of protein in the fractions. The initial content of SDS+DTT extractable protein in the untreated bran was 6.7 % of bran dm, and after 4-h treatments without added enzyme at 90 and 40 %, the content of SDS+DTT-extractable proteins increased to 9.1 and 11.3 % of bran dm, respectively. The use of xylanase had no visible effect on the electrophoretic patterns of proteins at either water contents. According to the spectrophotometrical quantification, the use of xylanase slightly increased the content of SDS+DTT-extractable protein after 4-h treatment (9.6 % at 90 % and 12.1 % at 40 %), but the increase was not statistically significant.

The formation of the high molecular weight protein aggregates in the SDS+DTT-extractable fraction was most probably due to the formation of covalent bonds during the bran treatments. Because electrophoresis was performed in denaturing conditions, which leads to the reduction of disulfide bonds during preparation of samples for SDS–PAGE, the protein aggregation could not be only due to the formation of disulfide bonds between the proteins. Protein aggregation is probably due to the high shear forces especially during the treatment at water content of 40 %. It is known that heating and shearing may cause degradation or aggregation of proteins by disulfide or covalent bond formation, for example in extrusion processing, and several authors have reported a decrease in protein extractabilities after extrusion, as reviewed by Anderson and Ng (2000). Then again, the small proteins in the SDS+DTT-extractable fractions which were not detectable in the initial bran might have formed by nonenzymatic depolymerization of the larger proteins, which has also been observed to occur in extrusion processing of wheat flour (Anderson and Ng 2000). Another possibility is the action of endogenous proteases known to be present in bran (Galleschi and Felicioli 1994; Umetsu et al. 1981).

The spectrophotometrical quantification of salt-soluble and SDS+DTT-extractable proteins showed that the sum of proteins in these two extracts of processed brans was somewhat lower than the sum of protein in the extracts of untreated bran. It may be that some protein remained in the bran residue (from which the proteins were extracted) due to the possible formation of covalent bonds between proteins and insoluble polysaccharides, thus preventing the extraction of residual protein even with SDS+DTT. It is also possible that strong aggregation and conformational changes in the protein structure had inhibited the access of the colorimetric reagent to the proteins, thus leading to lower color development and lower result.

Impact of Processing on the Hydration Properties of Bran

Treatments at water content of 40 % resulted in lower WBC of the freeze dried sample than the corresponding treatments at 90 % water content (Table 1). With no added enzymes, the initial WBC of bran (2.8±0.2 g/g) was not significantly affected by the 4 or 24 h treatments at 90 % water content (WBC 3.0 g/g after both 4 and 24 h treatments), while after

the treatments at 40 % water content, WBC reduced to 2.5 g/g and 2.3 g/g after 4 h and 24 h, respectively. The use of xylanase decreased WBC at both water contents as compared to the corresponding treatments with no added enzymes (Table 1). The lowest WBC was measured for the sample treated at 40 % with xylanase for 24 h (1.3 g/g).

WHC curves of bran samples, determined by the Baumann apparatus, are shown in Fig. 4. The WHC values (the max value of the curve, Table 1) of the bran changed during the treatments in a similar manner as the WBC (with minor exceptions), but WHC was always 0.6–0.9 units higher than the WBC of the same sample (Table 1). The highest WHC was measured for the sample treated at 90 % water content with no added enzymes for 4 h (3.9 g/g) and the lowest for the sample treated at 40 % water content with xylanase for 24 h (2.0 g/g). The samples did not significantly differ in the kinetics of water holding, as observed from the estimated slopes of the WHC curves (Fig. 4).

Hydration characteristics are one of the most important physicochemical properties concerning the technological applicability of DF preparations (Thebaudin et al. 1997). Water binding is also particularly relevant for the physiological actions of DF (Chaplin 2003). In a simplified way, water bound to insoluble polysaccharides can be presented in two forms: (1) water bound by surface tension in the pores of the matrix and (2) water bound by hydrogen bonds, ionic bonds, and/or hydrophobic interactions. The distribution of water between these two states depends on the chemical structure of the components, the associations between molecules, the size of the particles, the porosity of the material, and the effects of solvents and temperature (Thebaudin et al. 1997). In this study, the use of xylanase decreased WBC and WHC at both water contents, which was expected as water unextractable AX is known to bind more water than WEAX (Courtin and Delcour 2002). Also, the degradation and solubilization of other fiber components, such as β-glucan, have probably affected the WBC in the same way as the solubilization of AX by binding less water. The impact of time on the reduction of WBC and WHC during the treatments was more obvious at 40 than at 90 %. This may be due to the significant decrease of the bran particle size during the treatment at 40 %. In general, particle size reduction of bran and other DF preparations decreases water binding capacity (Noort et al. 2010; Auffret et al. 1994; Zhu et al. 2010). When particle size increases, so does the trapped volume due to imperfect packing and consequential apparent water binding (Thebaudin et al. 1997). The effect of grinding on WBC is attributable not only to particle size reduction but also to the altering of the physical structure of the fiber matrix (Auffret et al. 1994).

Hydration properties were measured using two different methods, WBC by a centrifugation method and WHC by the Baumann method. The WBC can be defined as the quantity of water that remains bound to the hydrated fibers following the application of an external force (pressure, or most commonly, centrifugation), while the WHC is defined by the quantity of water that is bound to the fibers without the application of an external force (except for gravity and atmospheric pressure) (Thebaudin et al. 1997). In the Baumann method, the measurement is based on the principle of the diffusion of a liquid by capillary action. Both methods gave similar results about the impact of the different treatments, but the WBC was always lower than the WHC of the same sample measured by the Baumann apparatus. This is probably due to the fact that in the centrifugation method, the water-soluble components of the sample are lost in the supernatant, thus reducing the mass of the sample that is holding water (Rasper and DeMan 1980). Furthermore, the water retained by centrifugation depends on the g-force used (Chaplin 2003), while in the Baumann method, no external force is used.

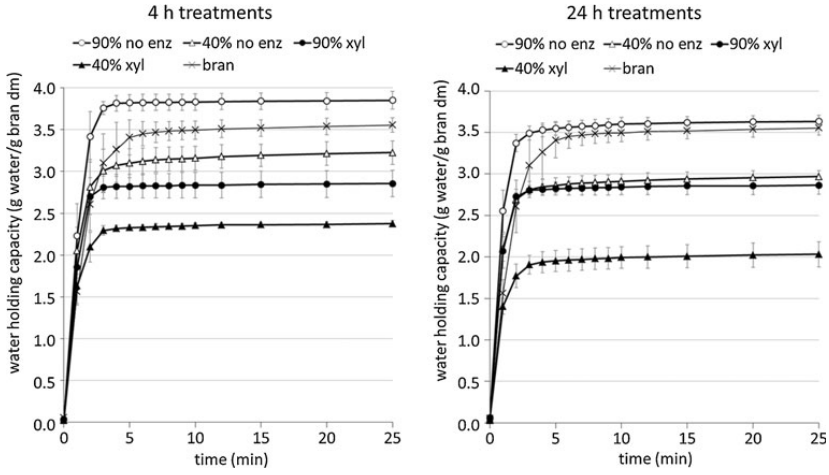

Fig. 4 Water holding capacity as a function of measurement time of untreated bran and bran samples treated at 40 and 90 % water content (determined from freeze dried and ground samples). The results are expressed as mean (n=4) and the standard deviations are indicated by *error bars*

Conclusions

The processing at 40 % water content brought about larger changes to bran properties than processing at 90 % water content. The physical structure of the bran changed more by the treatments at 40 % water content, also without added enzymes. The results indicated the strong impact of the shear forces at the 40 % treatment, which also enhanced the solubilization of polysaccharides. However, the A/X ratio results suggest that the solubilization of AX was caused by enzymatic action, and not by mechanical degradation, probably due to improved availability of the substrates. The observed changes in bran proteins were more obvious at 40 % treatment, and caused either by themomechanical mechanisms or by the action of bran-associated enzymes. Water binding capacity of bran was lower after the treatment at 40 than at 90 %, probably due to the smaller particle size of bran treated at 40 %, and higher content of water-extractable carbohydrates. The study showed that both structural and physicochemical properties of bran were affected by the water content and the way of processing, probably mostly through mechanisms related to the consistency of the bran–water mixture. This opens up new possibilities for hybrid processing aiming at improved bran applicability.

Acknowledgments We thank Kaarina Viljanen for the GC analyses and Ulla Holopainen for the help with interpreting the microscopy results. Ninon Piacere is thanked for help with bran treatments. Technical assistance of Ritva Heinonen in microscopy and Eeva Manninen in GC analyses is acknowledged.

Funding The work was financially supported by Raisio plc's Research Foundation (grant to Outi Santala) and by the Academy of Finland (Kaisa Poutanen).

References

AACC. (2003). *Approved methods of the American Association of Cereal Chemists. In Approved methods of the American Association of Cereal Chemists (10th edition)*. St. Paul: The Association.

Anderson, A. K., & Ng, P. K. W. (2000). Changes in disulfide and sulfhydryl contents and electrophoretic patterns and extruded wheat flour proteins. *Cereal Chemistry, 77*(3), 354–359.

Andersson, A. A. M., Landberg, R., Söderman, T., Hedkvist, S., Katina, K., Juvonen, R., Holopainen, U., Lehtinen, P., & Åman, P. (2011). Effects of alkylresorcinols on volume and structure of yeast-leavened bread. *Journal of the Science of Food and Agriculture, 91*(2), 226–232.

Anson, N. M., Selinheimo, E., Havenaar, R., Aura, A. M., Mattila, I., Lehtinen, P., Bast, A., Poutanen, K., & Haenen, G. R. M. M. (2009). Bioprocessing of wheat bran improves in vitro bioaccessibility and colonic metabolism of phenolic compounds. *Journal of Agricultural and Food Chemistry, 57*(14), 6148–6155.

Antoine, C., Peyron, S., Mabille, F., Lapierre, C., Bouchet, B., Abecassis, J., & Rouau, X. (2003). Individual contribution of grain outer layers and their cell wall structure to the mechanical properties of wheat bran. *Journal of Agricultural and Food Chemistry, 51*(7), 2026–2033.

AOAC. (1990). *Official methods of analysis of the Association of Official Analytical Chemists. In Official methods of analysis of the Association of Official Analytical Chemists (15th edition)*. Washington, DC: Association of Official Analytical Chemists.

Auffret, A., Ralet, M., Guillon, F., Barry, J., & Thibault, J. (1994). Effect of grinding and experimental conditions on the measurement of hydration properties of dietary fibres. *LWT-Food Science and Technology, 27*(2), 166–172.

Bailey, M. J., & Linko, M. (1990). Production of β-galactosidase by *Aspergillus oryzae* in submerged bioreactor cultivation. *Journal of Biotechnology, 16*(1–2), 57–66.

Bailey, M. J., & Pessa, E. (1990). Strain and process for production of polygalacturonase. *Enzyme and Microbial Technology, 12*(4), 266–271.

Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology, 23*(3), 257–270.

Barron, C., Surget, A., & Rouau, X. (2007). Relative amounts of tissues in mature wheat (*Triticum aestivum* L.) grain and their carbohydrate and phenolic acid composition. *Journal of Cereal Science, 45*(1), 88–96.

Baumann, H. (1966). Apparatur nach Baumann zur Bestimmung der Flüssigkeitsaufnahme von pulvrigen Substanzen. *Fette, Seifen, Anstrichmittel, 68*(9), 741–743.

Beaugrand, J., Crônier, D., Debeire, P., & Chabbert, B. (2004). Arabinoxylan and hydroxycinnamate content of wheat bran in relation to endoxylanase susceptibility. *Journal of Cereal Science, 40*(3), 223–230.

Benamrouche, S., Crônier, D., Debeire, P., & Chabbert, B. (2002). A chemical and histological study on the effect of (1→4)-β-endoxylanase treatment on wheat bran. *Journal of Cereal Science, 36* (2), 253–260.

Blakeney, A. B., Harris, P. J., Henry, R. J., & Stone, B. A. (1983). A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydrate Research, 113*(2), 291–299.

Chaplin, M. (2003). Fibre and water binding. *Proceedings of the Nutrition Society, 62*(1), 223–227.

Courtin, C. M., & Delcour, J. A. (2002). Arabinoxylans and endoxylanases in wheat flour bread-making. *Journal of Cereal Science, 35*(3), 225–243.

Dornez, E., Cuyvers, S., Holopainen, U., Nordlund, E., Poutanen, K., Delcour, J. A., & Courtin, C. M. (2011). Inactive fluorescently labeled xylanase as a novel probe for microscopic analysis of arabinoxylan containing cereal cell walls. *Journal of Agricultural and Food Chemistry, 59*(12), 6369–6375.

Figueroa-Espinoza, M. C., Poulsen, C., Søe, J. B., Zargahi, M. R., & Rouau, X. (2004). Enzymatic solubilization of arabinoxylans from native, extruded, and high-shear-treated rye bran by different endo-xylanases and other hydrolyzing enzymes. *Journal of Agricultural and Food Chemistry, 52*(13), 4240–4249.

Forssell, P., Kontkanen, H., Schols, H., Hinz, S., Eijsink, V., Treimo, J., Robertson, J., Waldron, K., Faulds, C., & Buchert, J. (2009). Hydrolysis of brewers' spent grain by carbohydrate degrading enzymes. *Journal of the Institute of Brewing, 114*(4), 306–314.

Gajula, H., Alavi, S., Adhikari, K., & Herald, T. (2008). Precooked bran-enriched wheat flour using extrusion: dietary fiber profile and sensory characteristics. *Journal of Food Science, 73*(4), S173–S179.

Galleschi, L., & Felicioli, F. (1994). Purification, characterization and activation by anions of an aspartic proteinase isolated from bran of soft wheat. *Plant Science, 98*(1), 15–24.

Gan, Z., Galliard, T., Ellis, P. R., Angold, R. E., & Vaughan, J. G. (1992). Effect of the outer bran layers on the loaf volume of wheat bread. *Journal of Cereal Science, 15*(2), 151–163.

Hemery, Y. M., Anson, N. M., Havenaar, R., Haenen, G. R. M. M., Noort, M. W. J., & Rouau, X. (2010). Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Research International, 43*(5), 1429–1438.

AOAC International. (2003). *Official methods of analysis of AOAC International. In Official methods of analysis of AOAC International (17th edition)*. Gaithersburg: Association of Analytical Communities.

IUPAC. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry, 59*, 257–268.

Izydorczyk, M. S. (2009). Arabinoxylans. In G. O. Phillips & P. A. Williams (Eds.), *Handbook of hydrocolloids (2nd edition)* (pp. 653–692). Cambridge: Woodhead Publishing.

Kamal-Eldin, A., Lærke, H. N., Knudsen, K. B., Lampi, A. M., Piironen, V., Adlercreutz, H., Katina, K., Poutanen, K., & Åman, P. (2009). Physical, microscopic and chemical characterisation of industrial rye and wheat brans from the Nordic countries. *Food and Nutrition Research, 53*(1), 1–11.

Katina, K., Salmenkallio-Marttila, M., Partanen, R., Forssell, P., & Autio, K. (2006). Effects of sourdough and enzymes on staling of high-fibre wheat bread. *Food Science and Technology, 39*(5), 479–491.

Kazmin, D., Edwards, R. A., Turner, R. J., Larson, E., & Starkey, J. (2002). Visualization of proteins in acrylamide gels using ultraviolet illumination. *Analytical Biochemistry, 301*(1), 91–96.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.

Lai, C., Hoseney, R., & Davis, A. (1989). Effects of wheat bran in breadmaking. *Cereal Chemistry, 66*(3), 217–219.

Loponen, J., Laine, P., Sontag-Strohm, T., & Salovaara, H. (2007). Behaviour of oat globulins in lactic acid fermentation of oat bran. *European Food Research and Technology, 225*(1), 105–110.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry, 193*(1), 265–275.

Mahasukhonthachat, K., Sopade, P. A., & Gidley, M. J. (2010). Kinetics of starch digestion in sorghum as affected by particle size. *Journal of Food Engineering, 96*(1), 18–28.

Mccleary, B. V., Solah, V., & Gibson, T. S. (1994). Quantitative measurement of total starch in cereal flours and products. *Journal of Cereal Science, 20*(1), 51–58.

Noort, M. W. J., van Haaster, D., Hemery, Y., Schols, H. A., & Hamer, R. J. (2010). The effect of particle size of wheat bran fractions on bread quality—evidence for fibre–protein interactions. *Journal of Cereal Science, 52*(1), 59–64.

Poutanen, K., & Puls, J. (1988). Characteristics of *Trichoderma reesei* β-xylosidase and its use in the hydrolysis of solubilized xylans. *Applied Microbiology and Biotechnology, 28*(4), 425–432.

Poutanen, K., Rättö, M., Puls, J., & Viikari, L. (1987). Evaluation of different microbial xylanolytic systems. *Journal of Biotechnology, 6*(1), 49–60.

Rasper, V. F., & DeMan, J. M. (1980). Measurement of hydration capacity of wheat flour/starch mixtures. *Cereal Chemistry, 57*, 27–31.

Santala, O., Lehtinen, P., Nordlund, E., Suortti, T., & Poutanen, K. (2011). Impact of water content on the solubilisation of arabinoxylan during xylanase treatment of wheat bran. *Journal of Cereal Science, 54*(2), 187–194.

Sollars, F. W. (1973). Fractionation and reconstitution techniques for studying water-retention properties of wheat flours. *Cereal Chemistry, 50*, 708–716.

Stålbrand, H., Siika-aho, M., Tenkanen, M., & Viikari, L. (1993). Purification and characterization of two β-mannanases from *Trichoderma reesei*. *Journal of Biotechnology, 29*(3), 229–242.

Swennen, K., Courtin, C. M., Lindemans, G. C. J. E., & Delcour, J. A. (2006). Large-scale production and characterisation of wheat bran arabinoxylooligosaccharides. *Journal of the Science of Food and Agriculture, 86*(11), 1722–1731.

Thebaudin, J., Lefebvre, A., Harrington, M., & Bourgeois, C. (1997). Dietary fibres: nutritional and technological interest. *Trends in Food Science & Technology, 8*(2), 41–48.

Umetsu, H., Abe, M., Sugawara, Y., Nakai, T., Watanabe, S., & Ichishima, E. (1981). Purification, crystallisation and characterisation of carboxypeptidase from wheat bran. *Food Chemistry, 7*(2), 125–138.

Van Craeyveld, V., Holopainen, U., Selinheimo, E., Poutanen, K., Delcour, J. A., & Courtin, C. M. (2009). Extensive dry ball milling of wheat and rye bran leads to in situ production of arabinoxylan oligosaccharides through nanoscale fragmentation. *Journal of Agricultural and Food Chemistry, 57*(18), 8467–8473.

Van Craeyveld, V., Dornez, E., Holopainen, U., Selinheimo, E., Poutanen, K., Delcour, J. A., & Courtin, C. M. (2010). Wheat bran AX properties and choice of xylanase affect enzymic production of wheat bran-derived arabinoxylan-oligosaccharides. *Cereal Chemistry, 87*(4), 283–291.

Virkki, L., Maina, H. N., Johansson, L., & Tenkanen, M. (2008). New enzyme-based method for analysis of water-soluble wheat arabinoxylans. *Carbohydrate Research, 343*(3), 521–529.

Zhu, K., Huang, S., Peng, W., Qian, H., & Zhou, H. (2010). Effect of ultrafine grinding on hydration and antioxidant properties of wheat bran dietary fiber. *Food Research International, 43*(4), 943–948.

Zurbriggen, B., Bailey, M. J., Penttilä, M. E., Poutanen, K., & Linko, M. (1990). Pilot scale production of a heterologous *Trichoderma reesei* cellulase by *Saccharomyces cerevisiae*. *Journal of Biotechnology, 13*(4), 267–278.