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Structural features of two heteroxylan polysaccharide fractions from wheat bran with anti-complementary and antioxidant activities

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ABSTRACT

Wheat bran is a rich source of bioactive substances ascribed to its arabinoxylan component. Two water-soluble arabinoxylans were sequentially extracted from wheat bran. WB1, released during enzymatic digestion of starch and protein, contained medium-branched arabinoxylan (A/X = 0.88) consisting of 3-*O*-substituted (22%), di-substituted (19.8%) and 58% unsubstituted Xyl*p* residues. It was slightly contaminated with (1 → 3,1 → 4)-β-glucan and arabinogalactan, and free of protein. WB2 extracted with 0.5% NaOH contained ~95% arabinoxylan (A/X = 1.09). WB2 and two 5% NaOH-extracted arabinoxylans were rich in protein and phenolic compounds. All radical-scavenging assays indicated a relation with the protein and total phenolics contents. The protein-free WB1 displayed the highest hydroxyl radical scavenging effect indicating the distinct role of phenolic acids. The immunomodulatory activity of WB1 was somewhat lower, whereas, that of WB2 higher in comparison to the immunogenic polysaccharide PMII. The arabinoxylans have the potential as immuno-enhancing and antioxidant additives in functional foods.

1. Introduction

Dietary fiber intake provides many health benefits. Individuals with high intakes of dietary fiber appear to be at significantly lower risk for developing coronary heart disease, stroke, hypertension, diabetes, obesity, and certain gastrointestinal diseases (Anderson et al., 2009). The broad research activities in the field of immunologically active polysaccharides concerned not only those from medicinal plants, but also of from cereals and grasses (Hromádková & Ebringerová, 2003; Patel et al., 2007; Paulsen, 2001). Many of the civilization diseases are connected with oxidative stress which is combined with free radical formation. Therefore, antioxidants became of interest also in connection with the immune system as reflected by the increasing number of reports dealing with both the immunological and antioxidant activities of polysaccharides (Nergard et al., 2005; Rao & Muralikrishna, 2006).

Soluble dietary fiber such as mixed-linkage (1 → 3,1 → 4)-β-D-glucan and arabinoxylans (AX) may affect inflammatory processes and immune responses by several mechanisms (Hromádková, Košťálová, & Ebringerová, 2008; Samuelsen, Rieder, Grimmer, Michaelsen, & Knutsen, 2011).

Industrial wheat bran is one of the most representative available hemicellulose-rich by-products and, hence, a rich source of dietary fibers. Crude extracts from wheat bran contain a complex of acidic and neutral AX and co-extracted mixed-linkage β-glucan and arabinogalactans contaminated in various proportions with other cell wall components (protein, phenolics, lipids). Quantitatively, most of the acidic AX in grains is located in the outer grain layers, which contribute about 25% of the dry weight of the grain (Hollmann & Lindhauer, 2005), whereas, the neutral AX is accumulated in the aleurone and endosperm layers (Hoffmann, Kamerling, & Vliegenthart, 1992). Cereal AX xylans have the main chains composed of (1 → 4)-linked β-D-xylopyranosyl (β-Xyl*p*) residues which are mainly substituted with (1 → 2)- and/or (1 → 3)-linked α-L-arabinofuranosyl (α-Ara*f*) residues, and can also carry single α-D-glucopyranosyluronic acid or its 4-O-methyl ether (acidic AX), and acetyl substituents (Ebringerová, Hromádková, & Heinze, 2005). Ferulic and *p*-coumaric acids may be ester-linked to AX at position 5 of some of their α-Ara*f* side chains (Ishii, 1997).

Usually, the antioxidant activity of AX has been ascribed to contaminating and/or covalently bound phenolics, such as in case of feruloylated arabinoxylans or feruloylated xyloligosaccharides (Hromádková et al., 2008; Rao & Muralikrishna, 2006; Wang, Sun, Cao, & Wang, 2010). AXs from wheat bran showed activity in lymphocyte transformation and complement-fixing tests (Patel et al., 2007).

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The aim of the present study was to isolate AX from industrial wheat bran under mild conditions minimizing destructive effects of the used chemicals and other treatments which might cause loss of bioactivity. The composition, main structural features and molecular properties of the obtained AX will be characterized by chemical and spectral methods and the bioactivity evaluated by antioxidant and immunomodulating activity in *vitro tests*.

2. Experimental

2.1. Materials and chemicals

The wheat bran (*Triticum aestivum*) was a commercially available domestic product. The air-dried bran was grinded and passed through a 0.5 mm sieve. Crude AX fractions (D2-I and D5-I) were preparations from a previous study (Hromádková et al., 2008). They were isolated from wheat bran by extraction with 5% NaOH at 60 °C with and without assistance of ultrasound for 10 min. The extracts were exhaustively dialyzed using cellulose membrane (MWCO, 3.5 kg/mol, Serva) and lyophilized.

The stable free radical 1,1-diphenyl-2-picryl-hydrazyl (•DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Germany). Gallic acid and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Fluka (Germany), the Folin–Ciocalteu's phenol reagent and $K_2S_2O_8$ from Merck (Darmstadt, Germany) and 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) salt (ABTS$^{\bullet+}$) from Polysciences, Inc. (Warrington, PA). $NaBD_4$ and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were from Sigma Aldrich (Milwaukee, WI). All other used chemicals were of analytical grade.

2.2. Extraction and purification of polysaccharides

The air-dried wheat bran was exhaustively pre-extracted by Soxhlet with chloroform–ethanol (65:35, v/v) to remove lipophilic and colored extractive compounds. The resulting extractive-free wheat bran (WB) was treated with acetate buffer (pH 5.4) at 70 °C for 30 min under stirring, cooled and, then the enzymatic digestion of starch was performed with the α-amylase from *Aspergillus oryzae* (60.5 U/mg) and amyloglycosidase (50 U/mg) at 37 °C for 43 h under stirring. After inactivation of the enzymes (100 °C for 10 min), the mixture was centrifuged at 5000 rpm for 15 min and filtered. The extract was adjusted to pH 7.5 with NaOH, and Pronase (from *Streptomyces griseus*) was added to the sample solution. The solution was mixed at 27 °C for 48 h. The enzymes were inactivated by heating the mixture at 100 °C for 10 min. The insoluble part was removed by centrifugation and the supernatant was extensively dialyzed with deionized water (MWCO, 3.5 kg/mol, Serva) and lyophilized (yield 2.2%). The digesting step with Pronase was repeated two times yielding the purified polysaccharide fraction WB1 (yield 1.35%).

The fiber residue of wheat bran was further extracted with 0.5% NaOH at 60 °C for 1 h. The insoluble material was separated by centrifugation at 7000 rpm for 15 min and the supernatant was neutralized to pH 6.8, dialyzed (MWCO, 3.5 kg/mol) and lyophilized (yield 4.3%). The separated fraction was dissolved in buffer (pH 7.5), digested three times with Pronase as in case of the former sample yielding fraction WB2 (yield 1.7%). All treatments after digestion with Pronase were performed under the same conditions as described in the extraction procedure with acetate buffer.

2.3. General methods

Sugar analysis of the polysaccharides was performed after hydrolysis with 2 M trifluoroacetic acid (reflux, 2 h). The neutral sugar composition of the hydrolysates was determined by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C/min) to 165 °C (20 °C/min) and flow rate of hydrogen 20 cm^3/min in form of the alditol trifluoroacetates. The uronic acid content was determined by the 3-hydroxydiphenyl assay using glucuronic acid as standard (Blumenkrantz & Asboe-Hansen, 1973) and identified by paper chromatography (p.c.) (Ebringerová, Hromádková, Petráková, & Hricovíni, 1990). The nitrogen content (%N) was obtained by elemental analysis (Elemental Analyser, Perkin-Elmer Model 240). The content of total of phenolic compounds (TP) was performed using the modified Folin–Ciocalteau method (Yu et al., 2002) using gallic acid as standard. The TP content was expressed as gallic acid equivalents. The measurements of TP, protein, uronic acids and neutral sugars were performed in triplicate.

The molecular characterizations of samples were investigated by HPSEC method (a high-performance size-exclusion chromatographic method). The samples (3 mg/mL) were dissolved in the eluent at room temperature for 1 day and centrifuged before analysis at 6000 rpm for 5 min. The HPLC system (Shimadzu, Wien, Austria) comprised a high pressure pump LC-10AD, membrane degasser (GT-104), an injector (Rheodyne 77251), an UV–vis detector (SPD-10AV) and a differential refractometer (RID-6A). Columns HEMA-BIO 300 (8 mm × 250 mm; 10 μm sorbent particle size; Tessek, Prague, Czech Republic) were used. The UV detector was used at λ_0 = 280 nm. The mobile phase 0.1 M $NaNO_3$ was used at a flow rate 0.4 mL/min, and weight molecular weight (M_w) obtained using a calibration from pullulan standards P10-P800 (Shodex Standard P-28, Macherey-Nagel, Germany). The elution profiles were recorded by Class-VP chromatography software.

2.4. FTIR and NMR spectroscopies

Fourier transform infrared spectra (FTIR) of polysaccharides were measured using the Nicolet-Magna 750 spectrophotometer (Nicolet, Madison, USA) with DTGS detector and OMNIC 3.2 software (Nicolet, Madison, USA) in the form of KBr pellets (2 mg sample/200 mg KBr). The NMR spectra were measured at 40 °C in D_2O (99.96% D) solutions using a Varian 400 MR spectrometer operating at 400 MHz for protons and 100 MHz for ^{13}C nuclei. Acetone was used as an internal standard (δ 2.225 for ^1H and δ 31.07 for ^{13}C). Homonuclear $^1H/^1H$ correlation spectroscopy (COSY), and heteronuclear ^{13}C–^1H correlation experiments (HSQC, HMBC) were run using the standard Varian pulse sequence. Experimental data were processed with the MestReNova 7.0 software.

2.5. Methylation analysis

Methylation analysis of WB1 was performed according to Ciucanu and Kerek (1984), modified for polysaccharides (unpublished results). After methylation with methyl iodide in the solid NaOH/DMSO medium, the isolated product was hydrolyzed by 4 M TFA under reflux for 6 h, reduced with $NaBD_4$, converted into alditol acetates and analyzed by GLC–MS on FINNIGAN SSQ 710 spectrometer equipped with SP 2330 column (0.25 mm × 30 m) at 80–240 °C, 70 eV, 250 mA, and ion-source temperature 150 °C.

2.6. Antioxidant capacity tests

2.6.1. Assay of DPPH radical-scavenging activity

Free radical scavenging ability (RSA) of the arabinoxylan fractions against the stable free DPPH radical (•DPPH) was determined spectrophotometrically (Rao & Muralikrishna, 2006); using the Spectronic 20 Genesys device. Briefly, 1 mL of polysaccharide sample (0.33–6.67 mg/mL in water) was mixed with 1 mL of a freshly

prepared solution of •DPPH (0.08 mg/mL in methanol) and reacted at laboratory temperature for 1 h at room temperature in dark. The absorbance at 517 nm of the tested solution was read against a suitable blank. Analyses were performed in duplicate. The RSA was calculated as follows:

$$\mathrm{RSA}(\%) = \frac{A_o - A_{\mathrm{test}}}{A_o - A_{\mathrm{ref}}} \times 100,$$

where A_o is the initial absorbance of •DPPH solution (•DPPH in methanol diluted with water 1:1), A_{test} is the absorbance of the tested sample in the •DPPH solution, and A_{ref} is the absorbance of gallic acid in the •DPPH solution. Gallic acid (0.7 mg/mL) was used as a reference corresponding to 100% activity. RSA was plotted as a function of the mass ratios (mg sample/mg •DPPH), and from the curve the mass ratio at RSA = 50% was obtained representing the value EC_{50}.

2.6.2. Assay of reduction potential

The reduction of ferric tripyridyltriazine complex (Fe^{3+}–TPTZ) to ferrous tripyridyltriazine (Fe^{2+}–TPTZ) form by a reductant at low pH was measured according to a slight modification of the ferric reducing antioxidant power (FRAP) method (Rao & Muralikrishna, 2006). The fresh FRAP reagent was prepared by mixing of 300 mM acetate buffer (pH 3.6), 20 mM $FeCl_3 \cdot 6H_2O$ and 10 mM TPTZ (in 40 mM HCl) in the ratio 10:1:1 (v/v). The reagent was warmed up to 37 °C and then an aliquot of each sample (0.2 mL) was added to 1.8 mL of the FRAP reagent. After the incubation period (up to 4 min), the absorbance at λ = 595 nm was read against water. Aqueous solution of $FeSO_4 \cdot 7H_2O$ (100–1000 μM) was used for the calibration. The FRAP value was expressed as μmol Fe^{2+}/1 g sample. All experiments were performed in triplicate.

2.6.3. Determination of antioxidant activity by EPR-spectroscopy

All measurements were performed using a portable X-band EPR spectrometer e-scan (Bruker BioSpin, GmbH, Karlsruhe (Germany). Three types of radical sources were used: •DPPH, $ABTS^{+\bullet}$ and $K_2S_2O_8$ in presence of DMPO. The initial concentration of the DPPH and ABTS radicals was determined via measurement of UV absorbance using the molar extinction coefficients (Staško, Polovka, Brezová, Biskupič, & Malík, 2006).

In the DPPH and ABTS tests, 1 mL of polysaccharide solution was placed into one syringe and into the second one, 1 mL of the solution of •DPPH in ethanol ($c_{0(\bullet\mathrm{DPPH})}$ = 0.1 mM), or the solution of $ABTS^{\bullet+}$ in water ($c_{0(\mathrm{ABTS}\bullet+)}$ = 72 μM) was placed. As a reference, deionized water was used. The respective radical concentration was monitored in time domain for 12 min. A set of 15 EPR spectra was recorded; each spectrum represents an average of 30 individual scans. The experimental EPR spectra processing as previously described (Staško et al., 2006) using WIN EPR and SimFonia programs (Bruker). The radical-scavenging ability of the fractions was expressed as the Trolox equivalent antioxidant capacities (TEAC) using the following equation:

$$\mathrm{TEAC}_{\bullet\mathrm{DPPH/ABTS}^{\bullet+}} = \frac{(c_{0(\bullet\mathrm{DPPH/ABTS}^{\bullet+})} - c_{t(\bullet\mathrm{DPPH/ABTS}^{\bullet+})}) \times V_{(\bullet\mathrm{DPPH/ABTS}^{\bullet+})}}{V_{(\mathrm{sample})}} \times \nu \times Z,$$

where c_0 is the initial concentration of •DPPH ($ABTS^{\bullet+}$) solutions; c_t(•DPPH)/$ABTS^{\bullet+}$) is the concentration of •DPPH ($ABTS^{\bullet+}$) after the addition into sample extract determined in chosen time t; $V_{(\bullet\mathrm{DPPH})/\mathrm{ABTS}^{\bullet+})}$ is the volume of •DPPH ($ABTS^{\bullet+}$) solutions added to the system; $V_{(\mathrm{Sample})}$ is the volume of the sample added to the system; ν is the stoichiometric coefficient of the reaction between •DPPH ($ABTS^{\bullet+}$) and Trolox (in both cases, ν = 1/2); Z is the dilution factor.

The formation of short-lived radical species (primarily •OH) was generated immediately before EPR measurements by mixing the prepared sample and phosphate buffer with $K_2S_2O_8$ in presence of DMPO (Staško et al., 2006). Each measurement started exactly 3 min after $K_2S_2O_8$ addition into the system and was monitored for 27 min. Every EPR spectrum represents an accumulation of 30 individual scans. The difference between the double integrals of the reference (deionized water) and the samples characterizes the amount of radicals scavenged (RS, %) by the antioxidants present in the sample calculated according to equation:

$$\mathrm{RS}(\%) = \left(1 - \frac{I_{\mathrm{sp15}} - I_{\mathrm{sp1}}}{I_{\mathrm{ref15}} - I_{\mathrm{ref1}}} \times 100\right),$$

where I_{sp} is the double integral of the first (1) and last (15) EPR spectrum recorded for system containing sample (sp) and deionized water (ref). The RS (%) directly reflects and quantifies the antioxidant properties of the tested sample. The experiments with DPPH and ABTS radicals were performed in triplicates and with $K_2S_2O_8$ in duplicates.

2.7. Complement-fixing assay

The complement-fixing activity of samples (15–500 μg/mL) was studied as previously described by Michaelsen, Gilje, Samuelsen, Hogaêsen, and Paulsen (2000). The activity is expressed as ICH_{50} which is the concentration of the fraction needed to inhibit 50% of the lysis of sensitized red blood cells from sheep by comparison with the non-stimulated control. Percentage of inhibition of lysis was calculated as:

$$\%\mathrm{inhibition} = \left[\frac{A_{\mathrm{control}} - A_{\mathrm{test}}}{A_{\mathrm{control}}}\right] \times 100,$$

where A is the absorbance read at 405 nm. Samples were run in quadruplicates. The ICH_{50} values of the tested xylan samples were related to $ICH_{50\,(\mathrm{PMII})}$ of the positive control PMII – an immunogenic polysaccharide isolated from *Plantago major* L. (Samuelsen, Paulsen, & Wold, 1996) yielding relative values Rel-ICH_{50}. The relative value of ICH_{50} was calculated as: Rel-ICH_{50} = $ICH_{50\,(\mathrm{sample})}$/$ICH_{50\,(\mathrm{PMII})}$. Low Rel-$ICH_{50}$ values mean a high complement fixing activity.

2.8. Statistics

Data were expressed as mean ± SD. Student's t-test and linear regression were used to analyze experimental data (statistical program MS Excel; Microsoft Office 2007 Professional). P value < 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Chemical composition and molecular properties of water soluble polysaccharide fractions

The extractive-free wheat bran (WB) contained ~74% carbohydrates and considerable amounts of non-carbohydrate components – protein (13.2%), ash (5.3%) and lignin (7.4%). Neutral sugar analysis revealed that 64.4 mol% comprised xylosyl and arabinosyl residues (Hromádková et al., 2008). Similar data were reported for bran of the wheat cultivar Bussard (Hollmann & Lindhauer, 2005). In order to isolate arabinoxylans from wheat bran at mild extraction conditions and not contaminated with non polar extractives, the pre-extracted bran (WB) was subjected at first to enzymatic digestion of the starch and protein components in acetate buffer, whereby, the most accessible polysaccharides were released from the cell walls. They were recovered from the extract as fraction WB1. Subsequent treatment of the bran residue with 0.5%

Table 1
Compositional data of polysaccharide fractions isolated from the wheat bran.

Sample	TP (wt%) [a]	N[b] (wt%) [a]	UA (wt%) [a]	Ara/Xyl[c,d]	X_m/X_d[d]	Neutral sugars (mol %)				
						Rha	Ara	Xyl	Glc	Gal
WB1	2.2 ± 0.3	0	2.9 ± 0.3	0.88 (0.72)	0.70	–	38	43	10	9
WB2	2.7 ± 0.3	1.5 ± 0.1	2.3 ± 0.3	1.09 (0.74)	0.85	tr	49	45	2	3
D2-I [e]	4.3 ± 0.2	3.7 ± 0.2	2.2 ± 0.2	0.85 (nd)	nd	0	39	46	11	4
D5-I [e]	4.5 ± 0.5	4.5 ± 0.2	2.0 ± 0.3	0.79 (nd)	nd	0	39	49	10	2

TP, total phenolic content as gallic acid equivalents; UA, uronic acid content expressed as glucuronic acid equivalents; ±, standard deviation; tr, trace, nd, not determined.
[a] Based on the polysaccharide fraction.
[b] Protein = %$N \times 6.25$.
[c] Mole ratio from sugar analysis, values in parenthesis from ^{1}H NMR experiments.
[d] Mole ratio of mono- and disubstituted Xyl*p* residues from ^{1}H NMR experiments.
[e] Samples published (Hromádková et al., 2008).

NaOH yielded a water-soluble polysaccharide fraction WB2. Chemical analysis data of polysaccharide fractions WB1 and WB2 were summarized in Table 1. For comparison, included were also two arabinoxylan samples (D2-I and D5-I) isolated under harsher conditions (5% NaOH, with and without short ultasonication) in a previous study (Hromádková et al., 2008). As seen, all fractions contain xylose and arabinose as the major neutral sugars. The high Ara/Xyl mole ratios of the fractions ranged between 0.79 and 1.09, which are similar to those published by Hollmann and Lindhauer (2005) for arabinoxylan fractions extracted with NaOH/H_2O_2 and then purified by enzymes. The hemicellulose fractions D2-I and D5-I contained a low amount of starch detected by the KI/I_2 test and were rich in proteins. All xylan fractions showed a very low content of uronic acids (1.9–2.8%), identified (by p.c.) as 4-O-methylglucuronic acid. Fraction WB1 was free-of protein, whereas, WB2 contained a low level of Pronase-resistant proteins. However, they might be contaminants from the enzyme-treatments of the polysaccharide fraction. Such possibility was suggested also by Zhang et al. (2011) in case of the enzymically treated wheat bran AXs rich in protein. Phenolics were present in all fractions. Their content was, similarly as in case of proteins, higher in both fractions (D2-I and D5-I) isolated under harsher extraction conditions from the bran without a preceding removal of extractives and proteins. The analytical data indicate WB2 to be an arabinoxylan–phenolic complex only slightly contaminated with proteins.

HPSEC was used to characterize the molecular properties of the arabinoxylan fractions using pullulan standards for molecular weight calibration. The RI- and UV_{280}-detected chromatograms were illustrated in Fig. 1A. The elution profiles indicate the presence of different macromolecular populations. WB1 showed a broad peak only at higher elution volumes (5.5–7.8 mL) with peak molecular weight (MW) of 62.7 kDa belonging to the major population III. However, overlapped were minor populations II (MW at 89 kDa) and IV (MW at 10 kDa). The UV_{280}-elution curve followed the RI-detected one and indicated the presence of phenolics accumulated in populations II and IV. On the contrary, WB2 contained a large population I eluting at the lowest elution volume (4.5–5.5 mL) with MW of 389 kDa and minor proportions of populations II and IV. However, the corresponding UV_{280}-elution curve indicated the presence of phenolic compounds and proteins, predominating in population IV. Fractions D2-I and D5-I showed two populations I and IV with MW of 430 and 415 kDa, respectively. Such high MW values were suggested to origin from aggregates formed by intermolecular association of AX chains (Patel et al., 2007; Zhang et al., 2011), carbohydrate–protein associations (Ebringerová, Hromádková, & Berth, 1994) or by cross-linking

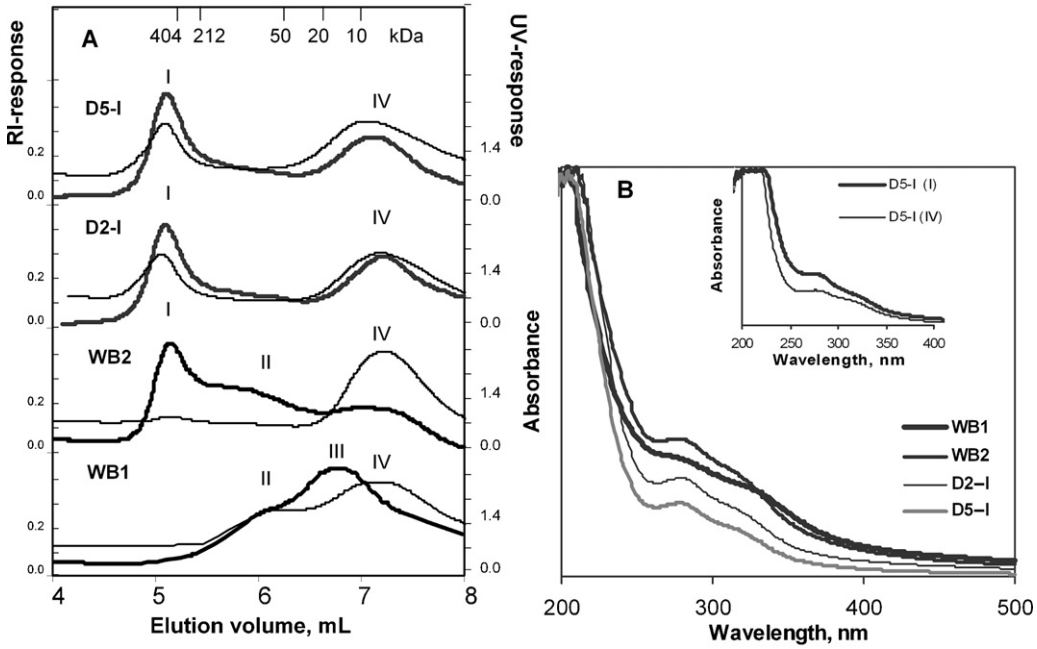

Fig. 1. (A) HPSEC chromatograms of arabinoxylan fractions from RI-(thick line) and UV_{280}-(thin line) detectors. Pullulan molecular weight markers (kDa) used as a calibration scale are shown at the top. Numbers I–IV indicate different molecular populations. (B) UV spectra of arabinoxylan fractions. Inserted are the UV spectra of the high-molecular weight population I and low-molecular weight population IV of fractions D2-I.

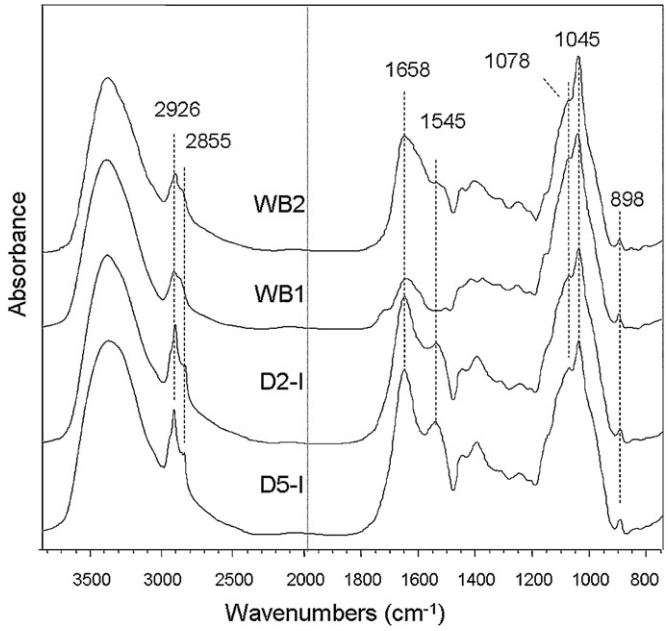

Fig. 2. FT-IR spectra of arabinoxylan fractions WB1, WB2 and crude xylan fractions D2-I, D5-I isolated from wheat bran.

through the ferulic acid substituents (Lapierre, Pollet, Ralet, & Saulnier, 2001). As reported by the authors, some diferulates (mainly the 8-8′-diferulate) survived at dilute alkali extraction of AX. The presence of phenolic acids in fractions D1- and D5-I isolated under harsher alkaline conditions can be explained by the existence of ether linkages between ferulic acid (and dehydrodiferulic acid) esterified to the arabinoxylan and lignin structures (mainly syringyl units) as suggested in case of maize bran heteroxylan (Lapierre et al., 2001).

Because WB2, D2-I and D5-I contained protein and phenolic compounds both absorbing at 280 nm, the UV spectra of the xylan fractions (Fig. 1B) were used to discriminate between these components. As seen, the spectra confirmed the presence of phenolic acids in all fractions, indicated by the absorption maxima at 320–340 and 280 nm ascribed to esterified and free ferulic acid, respectively (Saulnier, Marot, Elgottiaga, Bonnin, & Thibault, 2008). Evidently, the hump at 280 nm in WB1 can originate only from phenolic acids as the sample was free of protein (Table 1). The same UV spectral pattern of populations I and IV for D5-I, inserted in Fig. 1B, confirmed the presence of protein and phenolic acids in both molecular populations.

3.2. Structural properties of the polysaccharide fractions

3.2.1. FT-IR spectroscopy

FT-IR spectroscopy was used to provide identification of polysaccharides and proteins, based on reported typical spectral patterns and bands (Kačuráková et al., 1999). The FT-IR spectra of all fractions were depicted in Fig. 2. They contained absorption bands of protein at 1658 cm^{-1} (amide I) and 1548 cm^{-1} (amide II) and lipids at 2926 and 2855 cm^{-1}. As the amide I band was overlapped by the water absorption band, only the intensity of amide II was shown to correlate with the protein content of the fractions. It was absent in WB1 and strong in the other fractions. The spectral pattern in the region 1200–900 cm^{-1} is typical arabinoxylan-type polysaccharides as indicated by the presence of the bands at 1045 and 898 cm^{-1}. High arabinose substitution at C-3 of xylose residues was indicated by low intensity shoulder at 990 and 1162 cm^{-1}. The loss of peak multiplicity in 1120–1000 cm^{-1} is a typical characteristic of highly substituted arabinoxylans. The bands at 1078 cm^{-1} varying in intensity corresponded to vibrations of galactans (Kačuráková et al., 1999).

Table 2
Sugar linkage analysis of fraction WB1.

Sugar derivative	Mode of linkage	mol%
2,3,5-O-Me_3-Ara*f*	Ara*f*-(1 →[a]	32.4
2,3-O-Me_2-Ara*f*	→ 5)-Ara*f*-(1 →	3.3
Pentaacetate Ara*f*	→ 2,3,5)-Ara*f*-(1 →	0.3
2,3,4-O-Me_3-Xyl*p*	Xyl*p*-(1 →	0.1
2,3-O-Me_2-Xyl*p*	→ 4)-Xyl*p*-(1 →	23.2
2-O-Me-Xyl*p*	→ 3,4)-Xyl*p*-(1 →	8.8
Pentaacetate-Xyl*p*	→ 2,3,4)-Xyl*p*-(1 →	7.9
2,3,6-O-Me_3-Glc*p*	→ 4)-Glc*p*-(1 →	8.4
2,4,6-O-Me_3-Glc*p*	→ 3)-Glc*p*-(1 →	1.6
2,4,6-O-Me_3- Gal*p*	→ 3)-Gal*p*-(1 →	6.9
2,3,4-O-Me_3-Gal*p*	→ 6)-Gal*p*-(1 →	7.1

[a] 2,3,5-Me_3-Ara = 1,4-di-O-acetyl-2,3,5-tri-O-methyl-arabinitol, etc.

3.2.2. Glycosidic linkage analysis of fraction WB1

Fraction WB1 was subjected to methylation analysis (Table 2). The results revealed that WB1 comprised mainly a relatively medium-branched arabinoxylan. The 1,4-β-D-xylan backbone contained 58.2% of un-substituted Xyl*p*, 22% of mono-substituted Xyl*p* at position 3, and 19.8% of Xyl*p* di-substituted at both positions 2 and 3. Most of the large proportion (32.4 mol%) of non-reducing Ara*f* end-units % were attached to the xylan backbone as single side chains. Few Ara*f* residues were *O*-substituted at position 5 and positions 2, 3 and 5. Present was also a considerable amount of Gal*p* units linked at positions 3 and 6. Very probably, the rest of the non-reducing Ara*f* units might terminate some carbohydrate parts of arabinogalactan peptides known to occur in wheat grains (Ingelbrecht et al., 2002). The sample contained also Glc*p* units linked at positions 4 or 3 in the mole ratio ~1:5 indicative of mixed-linkage β-glucans (Roubroeks, Andersson, & Aman, 2000).

3.2.3. NMR spectrosocpy

Combination of 1D and 2D NMR spectra was used to determine the primary structural features of the AX component of WB1 and WB2 and identify contaminating glycans indicated by the presence of galactose and glucose (Table 1). The HSQC and HMBC NMR spectra of WB1 were depicted in Fig. 3a and b, respectively. The chemical shifts in the anomeric region of the ^1H- and ^{13}C NMR spectra (top and side of the figure), typical of the structural elements **u** (the unsubstituted β-Xyl*p* (X_u) residues of the 1,4-β-D-xylan backbone), **d** (di-*O*-2,3-substituted β-Xyl*p* residues (X_d) with single α-Ara*f* residues (A_{3d} and A_{2d})) and **m** (mono-*O*-3-substituted β-Xyl*p* (X_{3m}) and the corresponding α-Ara*f* residue (A_{3m})) were assigned in accord with already reported data (Ebringerová et al., 1990; Hoffmann, Kamerling, et al., 1992; Skendi, Biliaderis, Izydorczyk, Zervou, & Zoumpoulakis, 2011). The anomeric protons used as starting points for the assignment of protons H-2 and H-3 by the COSY experiments (not shown) and the further cross-peaks in the HSQC spectrum enabled to recognize and identify all chemical shifts of the sugar residues in elements (u, d and m). The chemical shifts were summarized in Table 3. Splitting of H-1 signals from A_{3d} and A_{2d} was obviously observed also in the spectra of other arabinoxylans and derived oligosaccharide fragments (Hoffmann, Geijtenbeek, Kamerling, & Vliegenthart, 1992). It was ascribed to the presence of two consecutive X_d units. Affected by such blockwise distribution of the α-Ara*f* branches were also the anomeric cross-peaks of X_u and X_m residues which were also overlapped.

The HMBC spectrum of WB1 (Fig. 3a) enabled to identify the well-separated inter-residue H-1/C-4 cross-peak (**A**) at δ 4.47/77.7 corresponding to 1,4-β-xylosidic bonds of contiguous X_u residues of the xylan backbone and further intra-residue cross-peaks of this

Table 3
^{1}H and ^{13}C chemical shifts for structural moieties of WB1 and WB2 as determined by ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMBC and ^{1}H–^{13}C HSQC NMR spectroscopy analyses.

Unit	Chemical shifts δ (ppm)[a]					
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
Arabinoxylan: **u**, → 4)-β-D-Xyl*p*-(1 →; **m**, → 4)-[α-L-Ara*f*-(1 → 3)]-β-D-Xyl*p*-(1 →; **d**, → 4)-[α-L-Ara*f*-(1 → 3)] [α-L-Ara*f*-(1 → 2)]-β-D-Xyl*p*-(1 →						
X_u	4.47/102.5	3.30/73.6	3.55/74.5	3.95/77.7	4.10_e, 3.30_a/63.8	
X_m	4.43/102.2	3.54/74.9	3.76/78.0	3.84/74.5	4.12_e, 3.40_a/63.8	
A_{3m}	5.39/108.5	4.15/82.1	3.95/77.7	4.28/85.4	3.80_e, 3.78_a/62.1	
X_d	4.65/100.6	3.58/74.6	3.87/77.8	3.82/74.4	4.14_e, 3.40_a/63.8	
A_{3d}	5.28/109.1	4.15/82.1	3.95/77.7	4.28/84.8	3.79_e, 3.73_a/62.1	
A_{2d}	5.22/109.5	4.15/82.1	3.95/77.7	4.12/85.2	3.82_e, 3.73_a/62.1	
(1→3), (1 → 4)-β-glucan: $_4G_3$, → 4)-β-Glc*p*-(1 → 3; $_3G_4$, → 3)-β-Glc*p*-(1 → 4; $_4G_4$, → 4)-β-Glc*p*-(1 → 4						
$_4G_3$	4.78/104.0	3.39/–[b]	3.66/74.7	–/79.5	–	–/60.9
$_3G_4$	4.54/104.0	3.58/–	3.76/84.7	–/69.5	–	–/60.9
$_4G_4$	4.54/103.6	–	3.67/–	3.54/79.3	–	–/60.9
Galactan: A_g, Ara*f*-(1 → 3; A_5, → 5)-α-Ara*f*-(1 →; Ga_6, → 6)-β-Gal*p*-(1 →; Ga_3, → 3)-β-Gal*p*-(1→; Ga_{36}, → 3,6)-β-Gal*p*-(1 →						
A_g	5.26/110.1	4.22/82.3	–	–	–	–
A_5	5.09/108.5	–	–	–/85.0	–/67.9	–
Ga_6	4.49/104.3	3.60/–	–	3.92/–	3.91/74.4	4.05_e;3.93_a/70.3
Ga_3	4.56/104.7	3.75/–	3.87/81.9	–	–	–/61.5
Ga_{36}	4.55/104.4	3.68/–	3.90/80.8		–	3.91_a/70.5

[a] In D_2O at 30 °C and acetone as an internal standard.
[b] Not determined; A, α-Ara*f*; X, β-Xyl*p*; G, β-Glc*p*; Ga, β-Gal*p*.

moiety at δ 3.30/102.5 (H-2/C-1) and δ 3.30/74.5 (H-2/C-3). The X_d residues of the arabinoxylan backbone bearing Ara*f* side chains were unequivocally identified by the inter-residue cross-peak (**B**) H-1/C3 at δ 5.28/77.8 between A_{3d} and X_d, and (**C**) H-2/C-1 at δ 3.58/109.5 between X_d and A_{2d}. The anomeric protons of Ara*f* units A_{3m}, A_{3d}, A_{2d} and A_g gave intra-residue connectivities with C-4 yielding three non-well resolved H-1/C-4 cross-peaks.

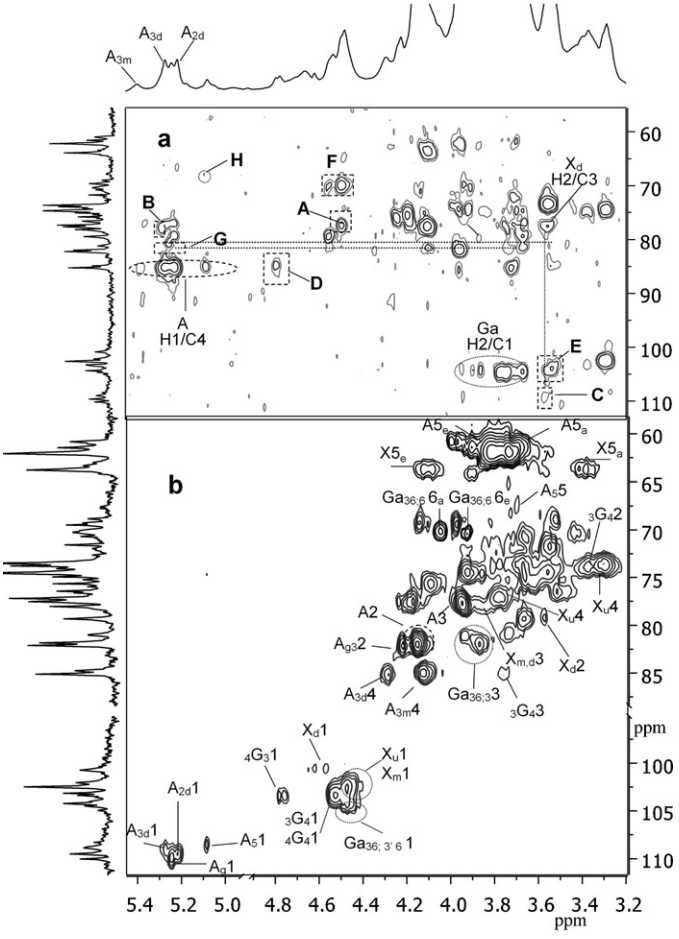

Fig. 3. HMBC (a) and HSQC (b) NMR spectra of WB1 in D_2O at 40 °C. The abbreviations of the sugar residues are explained in Table 3. Letters A–H correspond to inter-residue cross-peaks.

In accord with the high purity of WB2, the pattern of its HSQC NMR spectrum (not displayed) was simpler than that of WB1 showing well-separated anomeric protons of residues A_{3m}, A_{3d} and A_d. Their splitting indicated blockwise distribution of the **m** and **d** elements in the xylan chains. The additional small anomeric cross-peak in the region of α-Ara*f* residues was assigned to A_g linked to a β-galactan and the weak H-1 signal at δ 5.09 to a further α-Ara*f* residue. The signal at δ 3.30 was assigned to the H-2 proton of the X_u residues. It was used to calculate the Ara/Xyl ratio and the ratio X_m/X_d by integrating the H-1 areas of the Ara*f* residues A_{3m}, A_{3d} and A_{2d} belonging to AX relative to H-2 of the X_u residue (Hoffmann, Geijtenbeek, et al., 1992). The Ara/Xyl ratio of WB1 (Table 1) from sugar analysis was about the same as derived from the methylation analyis (~0.90). The A/X ratios calculated from the ^{1}H NMR spectrum were lower (mainly for WB2) than those from sugar analysis. The calculated X_m/X_d ratio indicated a lower degree of mono-substitution in WB1 than in WB2.

Based on the results from the methylation analysis (Table 2) mixed-linkage β-glucan was suggested to be a contaminating polysaccharide component of WB1. Mixed-linkage β-glucan was detected in the HSQC spectrum by the broad anomeric ^{1}H/^{13}C cross-peak at δ 4.79/103.4 (Roubroeks et al., 2000). The (1 → 4)-linked and (1 → 3)-linked Glc*p* residues referred to $_4G_4$ and $_3G_4$, respectively, gave the corresponding anomeric cross-peaks at δ 4.54/103.3 and 4.54/103.4. The intense H-3/C-3 cross-peak at δ 3.76/84.7 was assigned to of $_3G_4$ residue as suggested by Roubroeks et al. (2000). HMBC experiment gave inter-residue cross-peak **D** between H-1 of $_4G_3$ at δ 4.78 and C-3 of $_3G_4$ at δ 84.7. It confirmed the 1,3-glycosidic bonding between both residues, whereas, the inter/residue H-4/C-1 cross-peak **E** at δ 3.54/104.0 corresponded to the 1,4-glycosidic linkage between two 4-linked Glc*p* residues $_4G_4$ and $_3G_4$.

The second suggested minor polysaccharide component of WB1 was an arabinogalactan. The presence of 1,6-linked galactan chains was derived from the inter-residue cross-peaks **F** between H-1 at δ 4.55 and 4.49 and C6 at δ 70.5 and 70.3, and the intra-residue H-5/C-6 cross-peaks at δ 3.91/70.5 and 3.93/70.1. The HSQC cross-peaks at ~δ 4.05/70.3 and ~3.93.70.3 are typical signals of axial and equatorial H-6/C-6 of 6- and 3,6-linked Gal*p* units. Due to the low amount of the galactan component and its

Table 4
Antioxidant capacity of water-soluble polysaccharide fractions.

Sample	Antioxidant methods				
	Colorimetric		EPR		
	DPPH EC_{50}	FRAP[a] μmol/g	$TEAC_{/ABTS}{}^{\bullet+}$ μmol/g	$TEAC_{\cdot DPPH}$ μmol/g	RS %
WB1	9.6 ± 0.3	58.4 ± 4.7	6.5 ± 0.4	2.6 ± 0.2	37.4 ± 1.2
WB2	4.2 ± 0.3	89.1 ± 5.5	nd	nd	nd
D2-I	3.8 ± 0.2	83.8 ± 2.1	9.4 ± 0.6	4.3 ± 0.3	10.3 ± 0.9
D5-I	2.8 ± 0.4	82.1 ± 1.9	8.8 ± 0.6	5.1 ± 0.3	13.7 ± 0.8

nd, Not determined. ±, standard deviation $n = 3$ for TEAC and colorimetric methods, the relative standard deviation was less than 5%.
[a] Antioxidant activity in μmol $FeSO_4$ equivalents/1 g sample.
[b] TEAC, Trolox equivalent antioxidant capacity in μmol/g. EC_{50}, Amount of sample needed to reach 50% of the radical scavenging capacity expressed as mg sample/mg •DPPH.

branched structure, the assignment was not complete. The H-1 of Ara*f* residue (A_g) gave a weak inter-residue (H-1/C-3) cross-peak **G** at δ 5.26/80.8 with C-3 of a 3,6-linked Gal*p* residue, which might be branching points of 1,3-galactan or 1,6-galactan chains, both evidenced by methylation analysis. The H-1 of the terminal Ara*f* at δ 5.09 (A_5) showed connectivity with C-5 at δ 67.9 of another Ara*f* residue (inter-residue cross-peak **H**. The results indicate the mentioned Ara*f* and Gal*p* units might belong to the carbohydrate part of arabinogalactan–proteins (Capek, Matulová, Navarini, & Suggi-Liverani, 2010; Ingelbrecht et al., 2002) which were probably released during Pronase treatment.

3.3. Antioxidant activity

The antioxidant capacity of the xylan fractions was tested by different colorimetric assays (DPPH and FRAP) measuring the antioxidant activity and reducing power, respectively (Table 4). Low EC_{50} values from DPPH assay and high values from the FRAP assay corresponded to high antioxidant activities/reducing effects. As can be seen, the dilute alkali-extracted fraction WB2 and the crude fractions D2-I and D5-I (both non-purified from protein) displayed high antioxidant capacity in both tests. The EC_{50} values increased in the order D5-I < D2-I < WB2 < WB1 indicating decreasing antioxidant effects. The observed differences were assumed to be connected with the presence of non-carbohydrate components – phenolic compounds (TP) and protein (Table 1; Fig. 1B). Evidently, fractions D5-I, D2-I and WB2 displayed activities which were significantly ($p < 0.0001$) higher in comparrison to the protein-free fraction WB1 containing a low amount of TP. Phenolic components, particularly phenolic acids, have been reported to play an important role in the overall radical scavenging capacity of xylans and xylooligosaccharides from the wheat bran (Hromádková et al., 2008; Veenashri & Muralikrishna, 2011). As previously documented (Hromádková et al., 2005), WB1 and WB2 contained several phenolic compounds, including phenolic acids. The correlation between both the TP and protein contents and the measured EC_{50} values from the colorimetric DPPH measurements was higher ($R = 0.805$ and $R = 0.876$, respectively) than that from the FRAP measurements ($R = 0.542$ and $R = 0.633$, respectively). Fraction D2-I and D5-I with similar high TP contents and rich in protein showed about three times lower EC_{50} values than WB2. However, D2-I and D5-I exhibited almost the same reducing effects as WB2 ($p = 0.216$ and $p = 0.172$, respectively). As seen, the FRAP method was less sensitive to the protein content. It can be concluded that, except in case of the protein-free WB1, both proteins and phenolic acids contribute to the radical scavenging effects of the xylan fractions.

The results of the DPPH and ABTS assays evaluated by EPR (Table 4) clearly demonstrated the high radical-scavenging activity expressed by the Trolox-equivalent antioxidant capacity (TEAC) of all the samples under study. The ability of the xylan fractions to terminate $ABTS^{\bullet+}$ radicals are several times higher than those calculated from •DPPH assay. This trend can be explained by differences in the redox potentials of •DPPH and $ABTS^{\bullet+}$ (Staško et al., 2006). Comparison of $TEAC_{ABTS}$-values of samples D2-I and D5-I differing markedly in protein content ($p = 0.008$) confirmed no significant difference ($p = 0.288$). Similar values were obtained due to negligible difference in the TP content ($p = 0.5658$).

In the EPR studies with DMPO as spin-trapping agent, the free hydroxyl radicals were generated by thermal decomposition of $K_2S_2O_8$. Time-courses of EPR spectra from the reference sample and the xylan fractions are depicted in Fig. 4. Obviously, the intensity of spectra, reflecting the concentration of spin adducts, reached the maximum for reference sample whereas in case of the xylan fractions, decay of the spin-adducts concentration was observed. This resulted from competitive reactions between the formed OH radicals and the antioxidants present in the xylan fractions. From all measured xylans fractions, WB1 showed the highest RS value (37.4%). Fractions D2-I and D5-I providing remarkable antioxidant activity in the former mentioned tests (10.3% and 13.7%, respectively) reached only one-third of the value determined for the protein-free WB1. The evaluation of the relationship between the composition of the xylan fractions (Table 1) and their antioxidant capacity revealed that there is a strong negative correlation ($R = 0.956$) between the RS values and protein content. The results

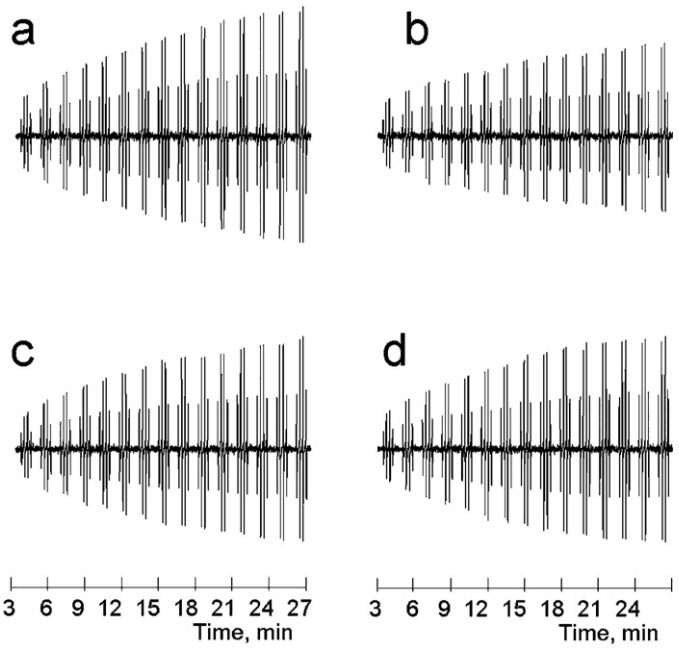

Fig. 4. Time course of EPR spectra from (a) reference, (b) WB1, (c) D2-I and (d) D5-I; samples ($c = 0.08$ g/100 mL) monitored for 27 min at 39 ± 1 °C in the presence of $K_2S_2O_8$ radical initiator, DMPO spin trap and phosphate buffer (pH 7).

of this test confirmed the distinct role of phenolic compounds on the scavenging of OH radicals which are known for their damaging effect in human body (Lipinski, 2011).

3.4. Immunomodulatory activity

Based on the purity of the wheat bran polysaccharide fractions (Table 1) and amount of material available, only xylan fractions WB1 and WB2 were tested for the immunomodulatory activity using the *in vitro* complement-fixing assay (Michaelsen et al., 2000). The inhibition of hemolysis by both xylan fractions expressed by the ICH_{50} values (57.2 μg/mL and 17.6 μg/mL, respectively) was compared to the value (44 μg/mL) achieved by the immunogenic polysaccharide PMII (Samuelsen et al., 1996) used as positive control. The results indicated an about 60% higher activity for WB2 and about 30% lower one for WB1 than displayed by PMII. In order to compare these complement-fixing activities with ICH_{50} values already published for other xylans, we used Rel-ICH_{50} values (interpreted in Section 2.7) comprising 1.3 for WB1 and 0.4 for WB2. Various wheat arabinoxylans prepared by different extraction procedures were reported (Patel et al., 2007) to display complement-fixing activity with ICH_{50} values ranging from 118 to 185 μg/mL what corresponded to Rel-ICH_{50} values of 2.6–4.1. These values confirmed a substantially lower immunomodulatory activity than that of the AX fractions tested in the present work. Also the alkali-extracted high-molecular weight barley arabinoxylans (Samuelsen et al., 2011) were shown to display only up to 45% activity of the positive control PMII. Recently, Zhou et al. (2010) reported on the immunoregulatory activities of arabinoxylans, obtained from wheat bran with alkali and xylanase-aided extractions, using splenocyte proliferation assay *in vivo* and *in vitro* as well as phagocytosis of peritoneal macrophages. The authors related the higher activity of the enzyme-extracted AX to the lower M_w, a low substituted population and to higher content of protein and ferulic acid. Evidently, there are many factors (composition, diversity in structure and molecular properties, presence of different polysaccharide types, protein and phenolic compounds) which might affect the complement-fixing activity.

4. Conclusions

Chemical and spectroscopic analyses of the two water-soluble polysaccharide fractions isolated from wheat bran during the enzymatic removal of starch and protein (WB1) and subsequent extraction with 0.5% NaOH (WB2) revealed that both fractions comprised medium-branched arabinoxylans with A/X ratios of 0.88 and 1.09, respectively. WB1 had 58% of the xylan backbone unsubstituted, 22% was 3-*O*-substituted and 19.8% was 2,3-*O*-di-substituted with single α-Araf side chains. The ratio of mono- to di-substituted Xyl*p* residues was in WB1 higher (0.85) than in WB2 (70). WB1 contained also small amounts of mixed-linkage β-glucan and arabinogalactans, probably released from arabinogalactan-proteins during the Pronase treatment. The arabinoxylans WB1, WB2 and the crude xylan fractions D2-I and D5-I (extracted with 5% NaOH in a former study) were to different extents contaminated with phenolic compounds and, except of WB1, also with protein. All xylans exhibited antioxidant activities. The antioxidant activity testing by different assays revealed that it depends on the content of the protein and phenolic compounds. In the experiment generating reactive spin adducts (mainly •OH), the potential of phenolic acids to reduce the concentration of OH radicals was determined. In this test, the protein-free WB1 exhibited the highest radical scavenging effect. The complement-fixing activity of WB1 was lower than that of WB2. The last displayed, even, a higher response than the immunogenic polysaccharide (PM II) used as positive control. The results suggested the water-soluble arabinoxylans to have the potential as immuno-enhancing and free-radical scavenging additives in functional foods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.05.021.

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