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New enzyme-based method for analysis of water-soluble wheat arabinoxylans

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Abstract—Arabinoxylans (AX) are the predominant cell-wall polysaccharides in wheat flour. Water-extractable AX are essential for dough and bread properties and performance. However, there is no specific and accurate way of determining the content and structure of AX. An enzyme-assisted method employing an efficient enzyme mixture for the total hydrolysis of AX was developed in the present work. Enzymatic hydrolysis (EH) is a gentle method during which no unwanted sugar destruction occurs. Following EH, liberated monosaccharides were analysed by gas chromatography (GC) and liquid chromatography using HPAEC–PAD. The results were compared with acid methanolysis (AM) and acid hydrolysis (AH). EH performed better on commercially isolated AX samples than the reference method AM. Its action in the water extract from wheat flour was also more efficient than that of AM and comparable to the efficiency of AH. HPAEC–PAD revealed a significant amount of fructose in the water extract following EH, originating from fructans in wheat flour not detected in the GC analysis. The wheat flour examined contained 0.29% water-extractable AX. The arabinose/xylose ratio was 0.32. The enzyme-based method developed is applicable for comparison of different wheat flours and can be used in evaluating the effect of processing on the content and structure of water-extractable AX.

Keywords: Enzymatic hydrolysis; Xylanase; Arabinoxylan; Fructan; Wheat flour; Water extract

1. Introduction

Arabinoxylans (AX), which are often referred to as pentosans, are the major nonstarch polysaccharides in wheat (*Triticum aestivum*) grains, constituting 1.5–2.5% of the endosperm. They are the primary cell-wall polysaccharides in wheat endosperm; 25–30% of the AX in wheat flour are water-extractable.[1] Minor cell-wall components include (1→3)(1→4)-β-D-glucans, arabinogalactans and cellulose.[2,3] Water-extractable AXs are important because they increase viscosity and may thus lower the glycemic index of bread.[4] In bread they stabilize the foam structure that prolongs oven rise, leading to higher loaf volume and finer and more homogeneous crumb structure.[1]

Cereal AX consist of a β-(1→4)-D-xylopyranosyl backbone with α-L-arabinofuranosyl substituents. In water-soluble wheat AX, the xylan backbone can be singly or doubly substituted with α-L-arabinofuranosyl groups at positions C-2 or C-3.[5] About one third of the α-L-arabinofuranosyl groups are located on singly substituted and two thirds on doubly substituted β-D-xylopyranosyl units.[6] The side groups are not evenly distributed along the main chain, and there are regions of substituted and unsubstituted β-D-xylopyranosyl residues.[5,7] α-L-Arabinofuranosyl units may further carry a feruloyl group at the C-5 position through an ester linkage. In addition, the xylan backbone can be substituted with α-D-glucopyranosyl uronic acid or its 4-*O*-methyl ether and with acetyl groups. The structure of AX varies in different parts of the grain. Water-soluble AX from wheat endosperm are mainly substituted with α-L-arabinofuranosyl units. The arabinose:xylose (Ara:Xyl) ratio in purified water-soluble wheat AX is about 0.5–0.6,[1]

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but wide natural variations are observed in different cultivars and subfractions.[8,9]

Currently there is no reliable and easy method available for analysis of the content of water-soluble AX in flour suspensions or dough. The most commonly used methods are colorimetric assays.[10,11] However, these methods are not specific enough since starch and arabinogalactans, which are also present in wheat flour, likewise react in the assay.[12] Furthermore, these methods do not provide information on the composition of AX (such as the Ara:Xyl ratio), which affects the behaviour and characteristics of AX, for example, water solubility and viscosity of AX solutions. Therefore, there is a need for better analytical method(s) for quantification of water-soluble AX.

More specific analysis of AX can be achieved by complete depolymerization of the sample to monosaccharides. This can be done by acid hydrolysis (AH), acid methanolysis (AM) or enzymatic hydrolysis (EH). AH is often a compromise between complete hydrolysis with possible sugar destruction and incomplete hydrolysis.[13] Analysis of the monosaccharide composition liberated after hydrolysis can be achieved by the use of high-performance liquid chromatography (HPLC),[14] gas chromatography (GC)[15] or colorimetric methods. AM is a milder alternative to AH of polysaccharides. The method is based on the depolymerization of polysaccharides by addition of hydrochloric acid in anhydrous methanol rather than acid in water. In this way, methoxyl groups are formed at the anomeric carbon instead of hydroxyl groups during cleavage of the glycosidic bond. Liberated methyl glycosides are derivatized by trimethylsilylation for GC analysis.[16,17] EH is carried out at ambient temperature and near-neutral pH, thus no unwanted sugar destruction occurs. However, complete hydrolysis of complex polysaccharides requires a mixture of all necessary enzymes. Methods based on specific enzymes are used in the analysis of cereal (1→3)(1→4)-β-D-glucans,[18,19] for analysis of total starch in food products[20] and for fructooligosaccharides and fructan polysaccharides in plant materials and food products.[21] Additionally enzyme mixtures containing commercially purified cellulases and hemicellulases were used for total hydrolysis of chemical pulp[22,23] or pulp-derived oligosaccharides[24] prior to quantification of carbohydrates. Enzymatic fingerprinting with pure *endo*-(1→4)-β-D-xylanase was also applied to evaluate variations of AX structures in different wheat varieties.[25]

Due to the complex composition of AX, their complete EH requires the action of a mixture of several depolymerising and debranching enzymes. The xylan backbone is degraded by *endo*-(1→4)-β-D-xylanases (EC 3.2.1.8) that release xylose, xylobiose and longer xylooligosaccharides. β-D-Xylosidases (EC 3.2.1.37) are essential for converting xylooligosaccharides to xylose by liberating nonsubstituted xylopyranosyl residues from the nonreducing end of xylooligosaccharides. Enzymes cleaving the side groups include α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.131), feruloyl esterases (EC 3.1.1.73), and acetylxylan esterases (EC 3.1.1.72).[26] α-L-Arabinofuranosidases differ further in their substrate specificities. Some of them act strictly on α-L-(1→2)- and (1→3)-linked arabinofuranosyl units on monosubstituted xylopyranosyl residues, whereas others release only α-L-(1→3)-linked arabinofuranosyl units from doubly substituted xylopyranosyl residues.[6,27–29] Both types of α-L-arabinofuranosidases are thus needed in the complete hydrolysis of water-soluble wheat AX.

The most sophisticated procedure would be to construct an enzyme cocktail for selective total hydrolysis of water-soluble AX. In this way the monosaccharides released would only originate from AX. Xylose is mainly present in AX, but arabinose also exists in water-soluble arabinogalactan. Unfortunately, not all the enzymes required for complete hydrolysis of water-soluble wheat AX currently are commercially available in pure form and at reasonable prices. Another option is to use an enzyme cocktail consisting of several commercial enzyme preparations. Commercial enzyme preparations are mixtures containing enzymes that may hydrolyse several polysaccharides; in the present study they were used for hydrolysis of wheat AX.

The aim of this work was to develop a reliable, easy and specific method for analysis of water-soluble AX of wheat flour and dough, using a mixture of commercially available enzyme preparations. The effectiveness of the mixture was first tested with pure, isolated, water-soluble wheat AX preparations and secondly with water extracts of wheat flour. The hydrolysis results were compared with those of AH and AM.

2. Results and discussion

2.1. Enzyme mixture

Four commercial enzyme preparations (Celluclast 1.5 L, Depol 740 L, Shearzyme 500 L and Novozyme 188) were chosen for further characterisation according to the preliminary tests. Celluclast 1.5 L is a multicomponent enzyme preparation produced by *Trichoderma reesei* that contains several cellulolytic and xylanolytic enzymes. Depol 740 L is a cellulolytic and pectinolytic enzyme mixture produced by *Humicola* sp. and also contains feruloyl esterase. Shearzyme 500 L is a monocomponent endoxylanase preparation produced by *Aspergillus oryzae* harbouring an endoxylanase-encoding gene from *Aspergillus aculeatus*. Even though Shearzyme 500 L is not an enzyme mixture it was chosen to complement other enzyme preparations because of its

endo-(1→4)-β-D-xylanase, which is very efficient towards highly substituted AX.[30] Novozyme 188 is a β-D-glucosidase preparation produced by *Aspergillus niger* and also contains other glycosidases. All enzyme preparations were of fungal origin and work optimally under slightly acidic conditions.

The enzyme activities of the main xylanolytic enzymes (*endo*-(1→4)-β-D-xylanase, β-D-xylosidase and α-L-arabinofuranosidase) required for degradation of AX were assayed for each commercial enzyme preparation, *endo*-(1→3)(1→4)-β-D-glucanase and β-D-glucosidase activities were also determined. Shearzyme 500 L had the highest *endo*-(1→4)-β-D-xylanase activity (77,200 nkat/mL) and β-D-xylosidase activity (860 nkat/mL) (Table 1). Celluclast 1.5 L and Depol 740 L also possessed significant xylanase and β-D-xylosidase activities. Novozyme 188 and Celluclast 1.5 L had the highest α-L-arabinofuranosidase activity (229 and 193 nkat/mL, respectively). β-D-Xylosidase, β-D-glucosidase and α-L-arabinofuranosidase activities were assayed using artificial *p*-nitrophenyl glycosides as substrates. It was recently reported that activity towards them does not necessarily correlate with the activity of natural oligomeric and polymeric substrates. Even though Celluclast 1.5 L exhibits clear activity on *p*-nitrophenyl α-L-arabinofuranoside, it is inefficient in liberating arabinose from wheat AX;[31] however, it contains an efficient β-D-xylosidase. On the other hand, the β-D-xylosidase activity on *p*-nitrophenyl β-D-xylopyranoside in Shearzyme 500 L is mostly due to the *A. aculeatus* xylanase, which is able to hydrolyse this artificial substrate, but not xylobiose.[30] Thus, in addition to enzyme activity assays, hydrolysis experiments with pure water-soluble wheat AX were performed to determine the most efficient enzyme mixture. The best combination tested was an equal volumetric mixture of all four enzymes. The enzyme mixture was purified by gel filtration to remove small-molecular-weight impurities. The enzyme activities measured were preserved well during the gel filtration. Interestingly the analysed *endo*-(1→4)-β-D-xylanase, *endo*-(1→3)(1→4)-β-D-glucanase and β-D-glucosidase activities of the mixture were clearly higher (24%, 11% and 42%, respectively) than that calculated based on results from individual enzyme preparations. This may have been due to the synergistic action of enzymes from different preparations or removal of inhibiting compounds during gel filtration.

2.2. Hydrolysis of commercial wheat xylans

The efficiency of the enzyme mixture at hydrolysing water-soluble wheat AX was tested with three commercially available wheat AX preparations (high-, medium- and low-viscosity wheat AX; WAX-HV, WAX-MV and WAX-LV, respectively), in which the AX content was 94–95% of the carbohydrates according to the manufacturer. The monosaccharide yield and content of Ara, Xyl and glucose, and also the Ara:Xyl ratio following EH were compared with those obtained by AM. The total organic carbon (TOC) analysis was also carried out to determine the amount of TOC and to further estimate the maximum AX (pentosan) content in each AX preparation.

The enzyme mixture worked efficiently, giving higher monosaccharide yields in all cases than AM (Table 2). TOC analysis assuming that all TOC originated from AX revealed that WAX-HV, WAX-MV and WAX-LV each contained a maximum of 83%, 89% and 79% AX, respectively. This was slightly higher than the hydrolysis yields obtained following EH. However, this is expected because some carbon originates from protein impurities. Recently these preparations were also reported to contain low percentages of fructose,[32] which is not detectable by the GC method used here. Hydrolysis of WAX-MV with a cocktail of pure xylanolytic enzymes was recently studied by Sørenssen et al.[33] In the present study, the AX yields obtained after AH (0.4 N HCl, 2 h, 100 °C) and the best enzyme cocktail were 66% and 77% (dry matter), respectively.

In all cases, the arabinose yields were higher after EH than after AM (WAX-HV: 25% and 22%, WAX-MV: 30% and 25%, WAX-LV: 16% and 13%, respectively). This may indicate some degradation of arabinose or incomplete hydrolysis during AM. The xylose yields were, on the other hand, only marginally higher after EH than after AM. Interestingly the glucose yield was also clearly higher after EH than after AM. The source of the glucose impurity is not known, although it may

Table 1. Enzyme activities of four commercial enzyme preparations and of the enzyme mixture after gel filtration

Enzyme preparation	Xylanase (nkat/mL)	β-Glucanase (nkat/mL)	β-Xyl (nkat/mL)	β-Glu (nkat/mL)	α-Ara (nkat/mL)	Xylanase/ β-Xyl	Xylanase/ α-Ara
Celluclast 1.5 L	22,160	95,590	569	663	193	39	115
Depol 740 L	17,780	16,970	335	678	75	53	237
Shearzyme 500 L	77,200	658	860	54	41	90	1883
Novozyme 188	2230	2000	119	6330	229	19	10
Mixture	10,544	9148	136	784	39	78	270

Xylanase = *endo*-(1→4)-β-D-xylanase, β-glucanase = *endo*-(1→3)(1→4)-β-D-glucanase, β-Xyl = β-D-xylosidase, β-Glu = β-D-glucosidase, α-Ara = α-L-arabinofuranosidase.

Table 2. Carbohydrate content (total monosaccharide yield as weight %), monosaccharide composition (%) and Ara:Xyl ratio of commercial wheat arabinoxylans analysed by GC after degradation by acid methanolysis (AM) and enzymatic hydrolysis (EH)

Sample	Method	Carbohydrate content (%)	Carbohydrate composition			Ara:Xyl ratio
			Ara (%)	Xyl (%)	Glc (%)	
WAX-HV[a]	AM	74	30	65	5	0.46
	EH	80	31	61	8	0.51
WAX-MV[b]	AM	78	32	68	n.d.	0.47
	EH	84	36	64	n.d.	0.56
WAX-LV[c]	AM	64	21	75	3	0.28
	EH	68	23	72	5	0.32

$n = 2$. Ara = arabinose, Xyl = xylose, Glc = glucose, n.d. = not detected.
[a] WAX-HV = wheat AX high viscosity.
[b] WAX-MV = wheat AX medium viscosity.
[c] WAX-LV = wheat AX low-viscosity.

have originated from starch or (1→3)(1→4)-β-glucan. Higher increase in arabinose yield compared with that of xylose was also observed by Sørenssen et al.[33] when AH and EH were compared.

The Ara:Xyl ratios were slightly higher after EH than AM (Table 2); the largest difference was in the results from WAX-MV. The Ara:Xyl ratio reported by the manufacturer for all three wheat AXs was 0.61. The closest value to this (0.56) was obtained from WAX-MV using EH, while WAX-LV had a much lower Ara:Xyl ratio (0.32) according to our analysis. The value from WAX-HV was also clearly lower (0.51) than that reported by the manufacturer. The other values reported for the Ara:Xyl ratio of WAX-MV were 0.58 and 0.70, which were analysed after AH (HCl) and EH, respectively.[33] Thus there is wide variation between the results from the same AX. However, in comparing our results with those by Sørenssen et al.,[33] we concluded that the difference in the Ara:Xyl ratio is mainly due to the difference in xylose yield. The xylose yield was higher after our EH (54%) than Sørenssen's EH (45%), whereas there was no as significant a difference between arabinose yields (30% and 32%, respectively). Recently, Ara:Xyl ratios of 0.61, 0.61 and 0.40 for WAX-HV, WAX-MV and WAX-LV, respectively, analysed with GC after the trifluoroacetic acid hydrolysis (2 N TFA, 2 h, 120 °C), were reported.[32] The monosaccharide yields of dry matter were, unfortunately, not reported in the work of Hughes et al.[32]

2.3. Hydrolysis of water extract of wheat flour

The effectiveness of the enzyme mixture was also evaluated, using water-soluble AX extracted from wheat flour, by the method of Rouau and Surget.[11] The flour extract was degraded by EH, AM and AH after which the monosaccharides released were analysed by GC as alditol acetates (AH and EH) or trimethylsilyl derivatives (AM).

The results from the water-soluble extract of wheat flour are shown in Table 3. The amounts of arabinose, xylose and galactose analysed by GC were similar after AH and EH; AH resulted in slightly higher values than EH. With AM the amount of xylose and especially galactose was lower than after the other two degradation methods. After AH and EH the amount of galactose released, mainly from arabinogalactan, was similar to that of xylose (0.24% and 0.22%, respectively). These results were comparable to those of Loosveld et al.[3] for two wheat varieties for which they reported 0.23% and 0.26% for galactose. Since arabinose is also released from arabinogalactan, the arabinose value needs to be corrected prior to estimation of the AX content. The amount of total arabinose (Ara_{tot}) was corrected by subtracting from Ara_{tot} the amount of arabinose released from arabinogalactan (Ara_{Gal}). The amount of arabinose from arabinogalactan was calculated as 0.7 times the amount of galactose.[3] The content of water-soluble AX in wheat flour was 0.29–0.31% after arabinogalactan correction (Table 3). The presence of disaccharides and oligosaccharides after EH was checked with a high-performance anion-exchange chromatograph instrument equipped with pulse amperometric detection (HPAEC–PAD),[30] and no oligosaccharides were found in the EH samples.

The water-soluble pentosan content of the wheat flour was also measured with a colorimetric assay.[10] The value obtained was 0.41%, which is similar to the sum of total arabinose and xylose ($Ara_{tot} + Xyl$) from the water extract of wheat flour measured using AH, AM and EH (0.47%, 0.41% and 0.44%, respectively) (Table 3). The colorimetric method measures the total amount of pentosans, whereas with chromatographic quantification of individually released monosaccharides after degradation of water-soluble polysaccharides, the actual amount of AX as well as the Ara:Xyl ratio can be analysed.

The content of glucose in the extract varied with the hydrolysis method used and was high in all methods (1.13–1.76%) compared with other carbohydrates (results not shown). Glucose mainly originates from soluble starch and (1→3)(1→4)-β-glucan. Amylase activities in the enzyme mixture were not measured, and their efficiency in degradation of starch or

Table 3. Monosaccharide content (percentage of wheat flour) of the water extract of flour analysed by GC after acid hydrolysis (AH), acid methanolysis (AM) and enzymatic hydrolysis (EH)[a]

	AH	AM	EH	
			GC	HPAEC–PAD
	% ± s.d.	% ± s.d.	% ± s.d.	% ± s.d.
Ara_{tot}	0.24 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.17 ± 0.01
Xyl	0.23 ± 0.01	0.19 ± 0.01	0.22 ± 0.01	0.15 ± 0.04
Gal	0.24 ± 0.01	0.15 ± 0.00	0.22 ± 0.01	0.17 ± 0.02
Ara_{tot} + Xyl	0.47	0.41	0.44	0.32
Ara_{tot}/Xyl	1.0	1.2	1.0	1.1
Ara_{corr}	0.08 ± 0.01	0.11 ± 0.00	0.07 ± 0.01	0.06 ± 0.00
Ara_{corr} + Xyl	0.31	0.30	0.29	0.21
Ara_{corr}/Xyl	0.35	0.58	0.32	0.40

[a] The HPAEC–PAD results were obtained only after EH. Ara_{tot} is the total amount of arabinose. $Ara_{corr} = Ara_{tot} - Ara_{Gal}$ where Ara_{Gal} is the amount of arabinose originating from arabinogalactan calculated as $0.7 \times Gal$[3] $n = 3$.

(1→3)(1→4)-β-glucan was not evaluated. Thus glucose values were not followed systematically. Mannose (as mannitol after the reduction step prior to GC analysis) was also found in the extract of wheat flour. Interestingly, the content varied with the hydrolysis method very much being 0.57% with EH and 0.14% with AH. With AM no mannose was found (results not shown).

The amounts of water-extractable AX of wheat flour analysed by GC after AH and corrected for Ara_{Gal} were reported previously by Loosveld et al.[3] for two wheat cultivars (0.35% and 0.54%) and by Ordaz-Ortiz and Saulnier[25] for 20 wheat cultivars (0.26–0.73%). Saulnier et al.[34] analysed 22 varieties of wheat grains for their water-soluble AX content by GC after AH without taking arabinogalactan into account. The water-soluble AX contents varied between 0.38% and 0.83% of the grain. Izydorczyk et al.[35] reported the amount of water-soluble pentosans analysed by HPLC after AH from eight wheat varieties. The amounts of AX (corrected for arabinogalactan) ranged from 0.50% to 0.68%. Thus there is significant variation in the amounts of water-soluble AX between wheat varieties. Comparison is, however, difficult due to the different extraction temperatures and times, which affect the amount of AX extracted since more AX is expected to solubilize at higher temperatures and longer extractions. Izydorczyk et al.[35] used a 5 min extraction time, Ordaz-Ortiz and Saulnier[25] 30 min and Loosveld et al.[3] 60 min all at room temperature. In the present study the extraction was carried out at 4 °C for 15 min. Low temperature was used by Rouau and Surget[11] to restrict the degradation of AX by endogenous enzymes. The content of water-soluble AX in the wheat flour studied was rather low. However, taking into account the low extraction temperature and short extraction time used in the present study, our results are in agreement with those previously published.

The Ara:Xyl ratio found in the present study for water-soluble wheat AX agrees well with that reported earlier by Loosveld et al.[3] (1.0 without arabinogalactan correction) but not with those of Ordaz-Ortiz and Saulnier[25] (0.47–0.58, corrected for arabinogalactan) or Izydorczyk et al.[35] (0.53–0.71, corrected for arabinogalactan). The Ara:Xyl ratio after arabinogalactan correction was 0.35 and 0.32 after AH and EH, respectively. This was similar to that obtained by EH for pure WAX-LV (0.32). Different extraction conditions as well as various cultivars and growing conditions may explain the differences in the Ara:Xyl ratio.[36]

2.4. Fructans in wheat flour

Monosaccharides released by EH of flour extract were also analysed with HPAEC–PAD. With this method no derivatization of monosaccharides prior to chromatographic analysis is needed. The results of the analysis for arabinose, xylose and galactose were somewhat lower than those obtained with GC, probably due to a lower resolution in HPLC than in GC (Table 3). Interestingly a peak corresponding to fructose was identified in the HPAEC–PAD chromatograms. Fructose as such cannot be detected by GC, since fructose as a ketose produces mannitol and glucitol in the reduction step, which was applied after hydrolysis and prior to derivatization and GC analysis. Thus, fructose cannot be distinguished in GC analysis from glucose or mannose, which, respectively, forms glucitol and mannitol on reduction. Since reduction was not carried out prior the HPAEC–PAD analysis, fructose could be analysed.

No mannose was identified in the HPAEC–PAD chromatograms, indicating that the mannitol in the GC chromatograms originated from reduced fructose. Furthermore, mannose was not detected after AM. In our experience, fructan and fructose are completely degraded under the conditions used for AM. Thus fructose detected in the HPAEC–PAD probably originated from fructans in the water extracts. The amount of fructose obtained after EH was about 0.3% of the flour. The quantification of fructose was, however, not very

accurate due to a broad peak and low response factor in the HPAEC–PAD analysis.

After clear identification of fructose in EH, the fructan content in wheat flour and water extract from wheat flour was analysed using the commercial kit. The fructan content was high, 1.0% in wheat flour and 0.6% in the water extract. Thus, the amount of water-soluble fructan was more than that of water-soluble AX (0.3%) or arabinogalactan (0.4%). The value obtained with the enzyme kit was also higher than that quantified after EH, further indicating that the fructose detected most probably originated from fructans. The fructan content of wheat flour ranges from 1% to 4%.[37,38] The fructans are either of the inulin, a β-(2→1)-fructan, or levan, a β-(2→6)-fructan, type.[37] Therefore, the action of the commercial kit towards isolated inulin from chickory and levan from *Erwinia herbicol* was tested. The fructan contents detected for the inulin and levan preparations were 87.4% and 12.6%, respectively; thus, the kit analysis does not work well with levan-type fructans, and the results for flour may therefore even be underestimated. The action of the enzyme mixture was also tested on inulin and levan. Both fructans were partially degraded by the mixture.

3. Conclusions

An efficient enzyme hydrolysis method for the total hydrolysis of water-soluble wheat AX was developed in the present investigation. Enzymatic hydrolysis is a gentle method during which no unwanted sugar destruction occurs, which is often a problem in acid hydrolysis. The enzymic hydrolysis was almost as efficient as the reference acid hydrolysis and slightly better than acid methanolysis. The monosaccharides formed were analysed by GC after reduction and derivatization. The enzyme hydrolysates were also analysed with liquid chromatography using HPAEC–PAD. The latter method is simpler to perform, since it does not require sample derivatization prior to chromatography. Combination of enzyme hydrolysis with GC or HPAEC–PAD results in a simpler method than acid hydrolysis because the degradation can be carried out under milder conditions, and no neutralization step prior to analysis is needed. The enzyme-based method developed is applicable to comparison of different wheat flours and can be used to evaluate the effect of processing on the content and structure of water-extractable AX.

4. Experimental

4.1. Materials

Wheat flour was kindly provided by Fazer Bakeries Ltd (Lahti, Finland). The flour contained 13.2% moisture, 0.77% ash and 13.2% protein as analysed using standard methods at the Technical Research Centre of Finland (VTT). The contents of total pentosan and water-soluble pentosans analysed with the colorimetric method[10] were 1.39% and 0.41%, respectively. The fructan content was 1.0% as analysed with a commercial kit (Fructan HK; Megazyme, Wicklow, Ireland).

Water was purified with a Milli-*Q*-Plus system (Millipore Corporation, Billerica, MA, USA). Sulfuric acid (H_2SO_4 95–97%), sodium citrate, $NaBH_4$, acetyl chloride, bis-trimethyltrifluoroacetamide and acetic acid anhydride were obtained from Merck (Darmstadt, Germany). Acetic acid (99–100%) was purchased from J.T. Baker (Deventer, The Netherlands), 1-methylimidazol, anhyd MeOH and chlorotrimethylsilane from Fluka (Buchs, Switzerland), pyridine from Sigma–Aldrich (Riedel-de-Haen, Seelze, Germany), heptane from Rathburn Chemicals Ltd (Walkerburn, Scotland) and dimethyl sulfoxide from Lab-Scan (Dublin, Ireland).

The commerical enzyme preparations included Celluclast 1.5 L (CCNO3078), Shearzyme 500 L (CDN00220) and Novozymes 188 (DCN00206) were obtained from Novozymes A/S (Bagsvaerd, Denmark) and Depol 740 L (Batch 2656018) from Biocatalysts (Cardiff, Wales, UK).

The low-viscosity (WAX-LV, lot 90201), medium-viscosity (WAX-MV, lot 20401) and high-viscosity (WAX-HV, lot 80601) wheat (*Triticum aestivum*) AX were obtained from Megazyme. The manufacturer certified that they contained some protein (2–3%), ash (1–3%) and moisture (3–5%). The AX content constituted 94–95% of the carbohydrates, and the Ara:Xyl ratio was 0.61. The enzyme activity assays were performed using birch xylan (Roth 7500, Carl Roth RG, Karlsruhe, Germany), *p*-nitrophenyl β-D-xylopyranoside (PNXP, N2132), *p*-nitrophenyl α-L-arabinofuranoside (PNAF, N3641) and *p*-nitrophenyl β-D-glucopyranoside (PNGP, N7006) from Sigma–Aldrich Chemie (Steinheim, Germany), and barley β-glucan (P-BGBM; Megazyme) as substrates. The monosaccharides D-arabinose, D-xylose, D-glucose, D-galactose and D-fructose from E. Merck and sorbitol from Sigma Chemicals Co. (St. Louis, MO, USA) were used as carbohydrate standards. The *myo*-inositol, levan, a (2→6)-β-D-fructose polymer from *Erwinia herbicola* (L8647), and inulin from chickory were purchased from Sigma Chemicals Co.

The values presented for all samples (flour, WAX-LV, WAX-MV, WAX-HV) were calculated on an 'as is' basis.

4.2. Methods

4.2.1. Preparation of enzyme mixture. Four commercial enzyme preparations (1 mL of each) were mixed, and 6 mL of 0.05 M sodium citrate buffer, pH 5.0, was added to obtain a final volume of 10 mL. The enzyme

mixture was purified by gel filtration using a prepacked PD-10 column according to the manufacturer's instructions (Amersham Bioscience, Uppsala, Sweden). Use of this procedure resulted in further dilution of the mixture by a factor of 1.4.

4.2.2. Enzyme activity assays. The *endo*-(1→4)-β-D-xylanase activity was assayed according to Bailey et al.[39] with 1% birch xylan in 0.05 M sodium citrate buffer, pH 5.0, using xylose as standard. The *endo*-(1→3)(1→4)-β-D-glucanase activity was assayed according to Zurbriggen,[40] using a 1% solution of P-BGPM in 0.05 M sodium citrate buffer, pH 5.0. The β-D-xylosidase, α-L-arabinofuranosidase and β-D-glucosidase activities were determined using 5 mM PNXP, 2 mM PNAF and 1 mM PNGP in 0.05 mM sodium citrate buffer, pH 5.0, according to Poutanen and Puls,[41] Poutanen et al.[42] and Bailey and Nevalainen,[43] respectively. All enzyme activity assays were performed in duplicate and carried out at 50 °C. The enzyme activities were expressed in SI units (katals).

4.2.3. Extraction of water-soluble pentosans. Water-soluble pentosans were extracted from wheat flour as described by Rouau and Surget[11] with modifications. In all, 1 g of flour was extracted with 4 mL of Milli-*Q* water at 4 °C for 15 min with magnetic stirring. The sample was centrifuged, and the pellet was discarded. The supernatant was kept in a boiling water bath for 10 min to inactivate the enzymes. The solution was centrifuged again, and the supernatant was separated and frozen. Three parallel samples of flour were extracted.

4.2.4. Degradation of arabinoxylans. Three methods were used to degrade the samples to monosaccharides: acid hydrolysis (AH), acidic methanolysis (AM) and enzymatic hydrolysis (EH). The monosaccharide results after EH of commercial WAX-HV, WAX-MV and WAX-LV were compared with those from AM. For soluble AX extracted from wheat flour, all three methods (AH, AM and EM) were compared.

AH was performed with 1.0 M H_2SO_4 for 2 h in a boiling water bath.[11] After hydrolysis the solutions were neutralized with 25% ammonia, and the monosaccharides were analysed as alditol acetates with GC.[15] The stock solutions of standards (10 mg/mL) were treated in the same way as the samples and standard curves were made from the dilutions of these monosaccharide solutions.[44]

AM was carried out according to the method of Sundberg et al.[16] using 10–15 mg of the commercial wheat AX or flour. The water extracts of wheat flour were first dried in a Speed-Vac Plus equipped with a Universal Vacuum System Plus with Vapornet UVS400 (Savant Instruments, Inc., Holbrook, NY, USA) at 65 °C for 72 h. The samples and standards were treated with anhyd HCl/methanol at 100 °C for 3 h. The monosaccharides released were analysed after trimethylsilylation using GC.

The amount of the enzyme mixture used in the hydrolysis of isolated AX was dosed according to the α-L-arabinofuranosidase activity (1000 nkat/g), which was regarded as the limiting enzyme activity in the mixture. The commercial AX (5 mg/mL) was dissolved in 0.05 mM sodium citrate buffer, pH 5.0. The enzyme mixture (60 μL, 2.5 nkat) was added to 0.5 mL of the AX solution, and the volume was adjusted to 1 mL with 0.05 mM sodium citrate buffer, pH 5.0. The final concentration of AX in the reaction was 2.5 mg/mL. For the flour extracts, the same amount of enzyme (60 μL) as used for commercial AX was added to 0.5 mL of the extract, and the volume was adjusted to 1 mL with 0.05 mM sodium citrate buffer, pH 5.0. The hydrolysis was carried out at 40 °C for 48 h. The reaction was stopped by maintaining the samples in a boiling water bath for 10 min to inactivate the enzymes. The monosaccharides released were analysed as alditol acetates using GC. The monosaccharides liberated from the water-soluble wheat flour extracts were also analysed with HPAEC–PAD.

4.2.5. Degradation of fructan. The ability of the enzyme mixture to hydrolyse fructans was tested using inulin and levan as substrates. The substrate (2 mg/mL) was dissolved in sodium citrate buffer, pH 5.0, and hydrolysis was carried out with the enzyme mixture as described above for AX. The amount of fructose released was determined using HPAEC–PAD.

4.2.6. Gas chromatography. GC was used to analyse the monosaccharides obtained from AH, AM and EH. The GC instrument used was a Hewlett–Packard 5890 series II gas chromatograph equipped with a flame ionization detector (FID) using splitless injection, injector and autosampler (Hewlett–Packard, Palo Alto, CA, USA). The column used for all separations was an HP 5 capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness; Agilent Technologies, CA, USA). The monosaccharide standards used in all methods were arabinose, xylose, glucose and galactose. The amounts of the monosaccharides were calculated as anhydro sugars (0.88 for pentoses, 0.90 for hexoses).

The samples from AH and EH were first reduced with $NaBH_4$ and then acetylated with acetic acid anhydride according to Blakeney et al.[15] and Johansson et al.[44] For quantitation, a calibration curve was produced for each sugar at five concentration levels; *myo*-inositol was used as the internal standard. All analyses were performed in triplicate. The analyses of the acetylated alditols were performed isothermally at 200 °C for

20 min with a detector temperature of 280 °C and injector temperature of 250 °C.

The AM products were trimethylsilylated as described by Sundberg et al.[16] The GC analyses with splitless injection were performed with the same device and column as for the acetylated alditols. Gradient programming was used, which included an isotherm at 150 °C for 5 min followed by a gradient to 200 °C. The total running time was 40 min. For quantitation a calibration curve was produced for each sugar at five concentration levels, with sorbitol as the internal standard. All analyses were performed in triplicate.

4.2.7. HPAEC–PAD. HPAEC–PAD was used to determine the amount of monosaccharides after EH of wheat-flour extract.[30] The analytical column was a CarboPac PA1 (250 × 4 mm, i.d.), and the guard column a PA1 (25 × 3 mm, i.d.) (Dionex, Sunnyvale, CA, USA) maintained at 30 °C. The analyses were performed using isocratic elution with 1.5 mM NaOH. Quantification was performed for each sugar with standard solutions at five concentration levels. Three parallel samples were analysed. The amounts of monosaccharides were calculated to anhydro sugars (0.88 for pentoses, 0.90 for hexoses). The presence of oligosaccharides after EH was analysed with HPAEC–PAD.[30]

4.2.8. Total organic carbon analysis. The TOC analyses were performed from the water solutions of the commercial AX preparation using a Shimadzu TOC-5000 (Kioto, Japan). Three parallel samples were analysed. The calculated content of carbon in AX was 45.45%. This value was used to estimate the maximum carbohydrate content in the AX samples.

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