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Classification of Wheat Varieties Based on Structural Features of Arabinoxylans As Revealed by Endoxylanase Treatment of Flour and Grain

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Arabinoxylans (AX) are cell wall polysaccharides of complex structure involved in many aspects of wheat flour end uses. The study of the variations of AX structure can lead to the identification of genes involved in their biosynthesis, and thus in the control of the various aspects of grain quality related to their presence. A method is proposed to identify AX variations directly in whole grain by enzymatic degradation. An endoxylanase from *Trichoderma viride* was used to extract AX from a collection of 20 wheat cultivars (*Triticum aestivum* L.). Enzymatic degradation products were analyzed by HPAEC and multivariate analysis techniques (principal component analysis, canonical correlation analysis, and cluster analysis) were applied to analyze chromatographic data. The method evidenced variations in the proportion of mono- and disubstitution of the xylan backbone by arabinose side chains, allowing classification of the different varieties according to the structural features of AX. A similar classification was obtained starting from flour or whole grain, indicating that the method was specific of AX from endosperm tissues. In conclusion, the method combining endoxylanase treatment of wheat grain and the analysis of degradation products, e.g., enzymatic fingerprinting, can be applied to collections of wheat cultivars, and possibly other cereals in order to establish quantitative trait loci related to the biosynthesis of AX.

KEYWORDS: Enzymatic fingerprinting; multivariate analysis; cereal; cell wall; HPAEC

INTRODUCTION

Arabinoxylans (AX) are the main nonstarch polysaccharides (NSP) of wheat grain cell walls and are largely involved in many uses of cereal grains (*1*). They consist of a linear backbone of β-(1,4) linked xylose, which are unsubstituted (uXyl), monosubstituted (mXyl) on O-3, or disubstituted (dXyl) on O-3 and O-2 with single arabinofuranose residues (*2*). In wheat flour, total AX represents 2−3 g/100 g, and 20−30% of the AX is water-extractable (WE-AX). WE-AX presents large variation in their structure; on average, the relative proportion of uXyl, mXyl, and dXyl are 60−65%, 12−20%, and 15−30%, respectively (*2*− *6*). Structural variation can also be expressed by the arabinose to xylose molar ratio (Ara/Xyl) with a typical average value of 0.5−0.6 for the general WE-AX population (*3*), but a large gradient of structure exists (*7*). Water unextractable AX (WU-AX), which represents the major part of AX in the endosperm cell walls, have been studied after alkaline extraction (AE-AX) (*8*−*12*); their structure corresponds to that of WE-AX with only small differences concerning molecular weight, which is higher for AE-AX than for WE-AX, and somewhat higher Ara/Xyl ratio due to a stronger presence of branched regions (*12*).

Natural variation of the NSP or its constituent sugars, the main cell wall components, has been documented in various cereals and legumes (*13*−*17*). Environmental effects and interactions between genotype and environment are known to influence cell wall composition of cereal grains (*4*, *18*, *19*). Evidences of a genetic control of WE-AX were also shown and QTL (quantitative trait loci) responsible for variations in relative viscosity and Ara/Xyl ratio of WE-AX were found on chromosome 1B L of wheat (*20*, *21*). The genes located in this QTL are probably involved in the control of the molecular structure of the WE-AX (*20*); e.g., they are involved in the biosynthesis of these macromolecules. QTLs related to the content of mixed-linked β-glucan of oat and barley grains, or carbohydrate composition of the cell wall of maize, have also been reported (*22*−*24*). The measurement of the Ara/Xyl ratio alone is not sufficient to describe the complex structure of AX, and ^{1}H NMR, the method of reference to investigate the structure of AX, is rather long and tedious to run on a large number of samples. In this context, a method applicable to a large number of samples has been developed to screen the variations in the structure (e.g., proportion of mono- and disubstituted residues) of both WE-AX and WU-AX. The solubilization of WU-AX from wheat flour can be achieved by xylanase treatment (*25*). Xylanases hydrolyze (1,4) linkages between β-D-xylopyranosyl residues and the specific bonds cleaved depend on the side-chain residues

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(*26*, *27*). Therefore, endoxylanase actions are affected by the structure of AX, and in turn the degradation products of AX by endoxylanases give information on the structure of the polymers; e.g., enzyme gives a fingerprint of the polymer structure. Enzymatic solubilization and degradation of AX by an endoxylanase from *Trichoderma viride* has been carried out on isolated tissues, flour, and whole grain, and degradation products were then analyzed by HPAEC. A collection of 20 wheat cultivars were investigated, and multivariate analysis techniques (principal component analysis and canonical correlation analysis) were applied to analyze chromatographic data and classify the varieties according to AX structural features.

MATERIALS AND METHODS

Wheat Samples. French cultivars of wheat (*Triticum aestivum* L.) grown in Ménétrol (France, harvest 2001) were provided by Ulice (Riom, France). Flours were obtained using a Bühler MLU-202 laboratory mill. Viscosity of grain water extract was measured by Ulice as described (*28*). Grains were ground in a rotor speed mill to pass a 0.12 mm screen. The 20 cultivars were Albéric, Alexandre, Aligre, Apache, Baltimore, Bonpain, Crousty, Elephant, Isengrain, Magdalena, Mallaca, Meunier, Sidéral, Sisley, Soissons, Supersoft, Tamaro, Texel, Thésée, and Virtuose. In addition, a hard wheat cv. Baroudeur and a soft wheat cv. Scipion, harvested in France in 2000 were kindly provided by Dr. Xavier Rouau (INRA-IATE, Montpellier, France).

Enzyme. An endoxylanase from *Trichoderma viride* was purchased from Megazyme (Xylanase M1, Bray, Ireland). The enzyme preparation had an activity on WE-AX of 2000 U/mL (data from the supplier) corresponding to 33340 nkat/mL (1 nkat corresponds to the amount of enzyme necessary to release 1 nmol of reducing ends per second under the assay conditions).

Oligosaccharides. Oligoxylosides (DP 2−6) were purchased from Megazyme (Bray, Ireland). Oligoxylosides substituted by arabinose were prepared by enzymatic digestion of WE-AX and characterized as previously described (*29*).

Enzymatic Extraction of AX from Grain Tissues. Wheat bran tissues (pericarp, intermediate layer, and aleurone layer) from cultivars Baroudeur and Scipion were kindly provided by Dr. X. Rouau, (INRA-IATE, Montpellier, France) and were obtained as described (*30*). Endosperm tissue from the same grain cultivars were prepared as follows: grains were soaked in 50% aqueous ethanol overnight at room temperature, after soaking germ and brush were removed, then crease incision was made and endosperm was recovered using a scalpel by scraping the outer layers. The absence of outer layers in the endosperm was checked by examination with a microscope. Finally, endosperm was homogenized in a mortar. Before enzymatic degradation each tissue was treated with boiling 80% aqueous EtOH as described below. Once dried, samples of the different tissues (amounts were calculated to contain 2 mg of AX) were suspended in 1.5 mL of 25 mM sodium acetate buffer pH 5.0 containing 80 nkat of xylanase (40 nkat/mg of AX); the mixture was incubated for 16 h at 40 °C under continuous stirring. The mixture was then centrifuged (10 min at 6300*g*). The supernatant was recovered and boiled for 10 min; after cooling, samples were frozen. Supernatants were then analyzed for their carbohydrate content and injected on HPAEC.

Enzymatic Extraction of AX from Flour and Grain. Flour samples (1 g) were treated with 5 mL of boiling 80% aqueous EtOH for 10 min; the insoluble residue was isolated by centrifugation (6300*g*; 15 min). The procedure was repeated once and the residue was washed with 5 mL of 95% EtOH and again isolated by centrifugation (6300*g*; 15 min). The supernatant was discarded and the residue was dried in two steps: first in an oven at 40 °C for 48 h and second with P_2O_5 in a vacuum oven at 40 °C for 48 h.

Five milliliters of 25 mM pH 5.0 sodium acetate buffer containing 800 nkat of xylanase (40 nkat/mg of AX considering an average AX content of 2% w/w for the flours) were added to dried samples. The mixture was incubated at 40 °C for 16 h under continuous stirring. The reaction mixture was centrifuged (6300*g*, 20 min) and 3.5 mL of supernatant was recovered and boiled for 10 min to inactivate enzyme. After cooling and centrifugation (6300g, 15 min), 3 mL of supernatant was recovered and frozen. Degradation was performed in triplicates for each cultivar.

Milled whole grain (0.5 g) was essentially treated as flour sample, except for the following modifications: for enzymatic extraction of AX, 1200 nkat of xylanase was added (40 nkat/mg of AX; considering an average AX content of 6% in wheat grain). Degradation was performed in triplicate for each cultivar.

Gas−Liquid Chromatography (GLC). AX content of flour, milled grain, and supernatant was determined by gas chromatography of alditol acetates, after sulfuric acid hydrolysis (1 M H_2SO_4, 100 °C, 2 h). AX content and Ara/Xyl molar ratio were corrected for the presence of arabinose (Ara_{AG}) from arabinogalactans (AG; % Ara_{AG} = 0.7 * % Gal). The extent of AX extraction (% AX) from flour or milled grain was calculated as follows: % AX = (XE-AX/total AX) × 100, where XE-AX takes into account WE-AX initially present in the flour or grain.

High-Performance Anion-Exchange Chromatography (HPAEC). Supernatant obtained after xylanase treatment was filtered through 0.45 μm filters (Millipore) and a 20 μL aliquot was analyzed by HPAEC on a Waters quaternary gradient system (626 pump, 600S controller, 717 autosampler, Waters, Milford-United States), using a Carbopac PA-1 analytical column (2 × 250 mm, Dionex, Sunnyvale, United States) at 25 °C with a flow rate of 0.25 mL/min and gradient elution (0−5 min: 53% A, 2% B, 45% C; 5−10 min: 83% A, 7% B, 10% C; 10−20 min: 75% A, 15% B, 10% C; 20−30 min: 80% A, 15% B, 5% C; 30−50 min: 53% A, 2% B, 45% C where A is water, B is 1 M NaOAc, and C is 0.5 M NaOH). Detection was made with a TSP EC2000 pulse amperometric detector (PAD, Thermo Separation Products) using the following pulse potentials: $E_1 = +0.05$ V, $E_2 = +0.6$ V, and $E_3 = -0.6$ V. Assignments of peaks were made by comparison of their retention time with oligomers of known structure. Eight peaks corresponding to xylose, xylobiose, and arabino-xylo-oligosaccharides previously identified as $A1_2X3$, $A1_3X4$, $A2_{3d}X4$, $A2_{4,3}$-X5, $A3_{4,3d}X5$, and $A4_{4d,3d}X5$ (*29*). Afterward, these peaks were called X1, X2, DP4, DP5, DP6, DP7, DP8, and DP9, respectively.

Software package Borwin (JMBS, France) was used to collect and process chromatographic data. Peak surface was calculated by the software and was normalized for each chromatogram as follows: $S_NPeak_i = (SPeak_i/\sum_{i=1}^{i=8} SPeak_i) \times 100$, with S_NPeak_i being the normalized surface of peak i and $SPeak_i$ the surface of peak i.

Data Analysis. Chromatographic data tables were considered for which the 8 normalized peak surfaces were the variables and the three replicates × 20 wheat cultivars were the individuals. One set of data was obtained for flour samples and one for whole grain samples.

Principal component analysis was applied on each data table. Principal component analysis is a multidimensional data treatment that reveals similarities within individuals by taking all variables into account (*31*, *32*). Similarity maps, drawn from principal component scores, are used to visualize the similarities between samples. Principal component loadings highlight similarities between variables.

Canonical correlation analysis was applied to compare whole grain and flour data sets. This multivariate data treatment describes the correlations between two sets of variables. Canonical variates are assessed for each data table as linear combinations of the original variables so that they are as correlated as possible (*31*, *32*). Canonical correlation analysis was applied on principal components in order to eliminate correlation within original variables. By this way correlation between data tables are highlighted. Canonical similarity maps can be drawn to show the canonical scores of individuals. Canonical patterns were assessed as previously described (*33*) in order to relate canonical variates to the original variables.

Cluster analysis using Ward's method was applied to help finding groups in similarity maps. The squared Euclidean distance was used between individuals.

One-way ANOVA and mean comparison test using Fischer's least significant difference (95% LSD) were used to compare the structural characteristics of AX between clusters.

RESULTS AND DISCUSSION

Xylanase Treatment of Flour, Grain, and Isolated Tissues. The conditions for enzymatic extraction of AX from wheat

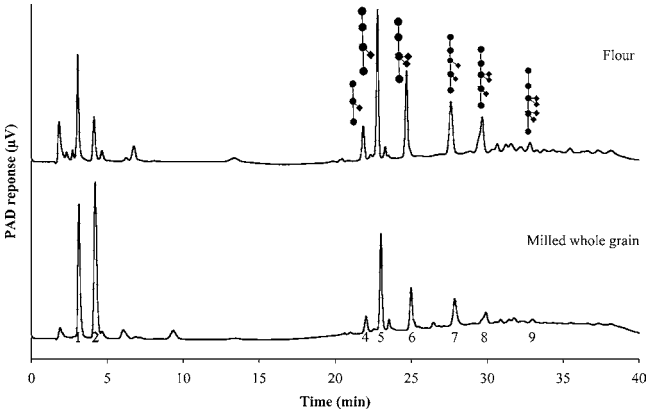

Figure 1. HPAEC-PAD profile of endoxylanase degradation of cultivar Supersoft performed on flour sample and milled whole grain in the same cultivar. Numbers 1–9 indicate oligosaccharides DP and represent respectively xylose, xylobiose, and oligomers $A1_2X3$, $A1_3X4$, $A2_{3d}X4$, $A2_{4,3}X5$, $A3_{4,3d}X5$, and $A4_{4d,3d}X5$ as outlined in ref *29*.

flours have been previously determined (*25*). These conditions allowed first reaching optimal solubilization of AX, and second obtaining end products (oligosaccharides) of xylanase action. xylose (X1), xylobiose (X2), and various arabino-xylo-oligosaccharides of complex structure are obtained (**Figure 1**). The main peaks have been isolated and identified as $A1_2X3$ (DP4), $A1_3X4$ (DP5), $A2_{3d}X4$ (DP6), $A2_{4,3}X5$ (DP7), $A3_{4,3d}X5$ (DP8), and $A4_{4d,3d}X5$ (DP9) (*29*). Other peaks were detected but their surfaces represented less than 2% of the total surface of peaks and they were not used for subsequent analysis.

The extent of AX extraction from flours was on average 75%. Baltimore and Supersoft flours presented the smallest and Soissons flour the highest extent of AX extraction (**Table 1**). Similar coefficient of variation (15%) was observed for total AX content and extent of extraction. Furthermore, total AX content and extent of extraction were negatively correlated ($r = -0.75$), suggesting that flours with the highest AX content could contain higher amount of outer layers, or higher proportion of highly substituted AX fractions in endosperm, both resistant to enzyme degradation. Similar peaks were observed in chromatograms obtained after xylanase treatment of grain or flour (**Figure 1**), but the proportion of X1 and X2 peaks was higher in grain than in flour. The extent of AX extraction from grain was on average 65%, only slightly lower than that from flour. Coefficient of variation was smaller than for flours (10%) and no correlation was observed between extent of extraction and total AX content of grain (**Table 1**).

To better understand the origin and the relative proportions of the peaks observed after xylanase treatment of flour and grain, the treatment was performed on tissues isolated by dissection of wheat grain, e.g., endosperm, aleurone layer, intermediate layer, and pericarp. Analyses were performed on the tissues from cultivars Baroudeur and Scipion and their flours (**Table 2**; **Figure 2**).

The extent of AX extraction and HPAEC chromatograms were very similar for both cultivars but large variations were observed between the different tissues. The extent of AX extraction was slightly higher from flour (97%) than from isolated endosperm tissue (91%) (**Table 2**). In both cases extracted AX exhibited a slightly lower degree of substitution than AX of the starting tissue. Xylanase treatment of the endosperm tissue and flour gave by order of importance similar proportion of DP5, DP6, DP7, X1, and X2. An inversion in the relative abundance of X1 and DP7 was observed for the flour (**Table 2**). DP8 and DP9 were found in a lower abundance.

The extent of AX extraction was lower in aleurone layer than in flour and endosperm. The degree of substitution of extracted AX (Ara/Xyl: 0.33) was lower than in flour or endosperm and very similar to the value obtained with a xylanase from *Thermobacillus xylanilyticus* (*34*, *35*). X2, DP5, X1, and DP7 were the main peaks observed (>10% of total surface), whereas DP6 was observed in low proportion and DP8 and DP9 were not detected. DP5 and DP7 only contain monosubstituted xylose residues. This result is in agreement with the structure of AX from the aleurone layer where most of the arabinose residues are found as monosubstitution on position (O)-3 of xylose residues, whereas few disubstituted xylose residues are observed (*36*, *37*).

The intermediate layers are formed of inner pericarp (cross and tubular cells), testa, and nucellar tissue (*38*). X2 and X1 were the main peaks observed after xylanase treatment whereas DP5 was only detectable as traces (**Figure 2**). The extent of AX extraction was 54% and 58% for Baroudeur and Scipion, respectively, similar to the value reported on the same tissues on cultivar Isengrain (*34*). The very low degree of substitution (Ara/Xyl: 0.07) observed for solubilized AX confirmed that poorly substituted AX are found in the nucellar layer (*34*). Furthermore, previous studies (*34*, *35*) demonstrated that xylanase degrades only this latter layer and keeps the inner pericarp intact.

Finally, the outer pericarp that presents the highest Ara/Xyl ratio (1.15) was not degraded at all by the enzyme. This layer is constituted of AX highly substituted by arabinose, glucuronic, and acetic acids, is lignified, and also exhibits the highest amount of dehydrodimers of ferulic acid which are known to be a major limiting factor to the enzymatic degradation of AX (*39*).

In conclusion, oligosaccharides released from flour are essentially similar for their amount and proportion to oligosaccharides released from both the endosperm tissue and the aleurone layer. AX content in flour of both cultivars (1.6 and 1.2 g/100 g, for Baroudeur and Scipion, respectively) was higher than that in endosperm tissues (1.4 and 0.94 g/100 g for Baroudeur and Scipion, respectively), indicating contamination of flours by outer tissues of the grain, probably mainly by the aleurone layer. In other words, xylanase treatment of flours reflects essentially the structural features of AX from the endosperm tissue. However, beyond the peaks observed, X1 and X2 originate mainly from maternal tissues (testa and nucellar tissue) and their variation in flours could be related to the different extraction rate of the flour (contamination with outer layers), whereas in grains variation of X1 and X2 could be mainly related to different proportions of tissues (endosperm vs testa) rather than to differences in the AX structure of a specific tissue.

Data Analysis. *Principal Component Analysis of Flour and Grain (All Peaks).* For each of the 20 cultivars, three chromatograms were obtained from the flour and the grain (representing three independent assays). In a first approach, variations of peaks X1, X2, DP4, DP5, DP6, DP7, DP8, and DP9 were considered for the analysis.

The first two principal components accounted for 90.7% of the total variance for flour and 92.8% for grain. For flour, the three replicates of the same cultivars were grouped on the similarity map (**Figure 3**) and a similar result was observed for grain (score plot not shown). This clustering of replicates attested that the enzymatic digestion and subsequent HPAEC analysis were highly reproducible for both flour and grain.

Table 3 show principal component analysis loadings from flour and grain. For flour, the first principal component was

Table 1. Total AX, WE-AX, and Extent of AX Extraction by Xylanase in Wheat Flour and Milled Whole Grain[a]

	flour					grain				
cultivar	total AX[a] g/100 g	WE-AX[b] g/100 g	XE-AX[c] g/100 g	Ara/Xyl	extraction[d] %	total AX[e] g/100 g	XE-AX[f] g/100 g	Ara/Xyl	extraction[g] %	viscosity[h]
Alberic	2.12	0.45	1.71	0.62	80.66	6.13	4.45	0.32	72.68	1.70
Alexandre	2.03	0.66	1.77	0.60	87.13	6.92	3.99	0.36	57.69	2.99
Aligre	2.09	0.35	1.66	0.65	79.72	5.71	3.55	0.32	62.11	1.57
Apache	2.36	0.52	1.74	0.60	73.56	6.49	4.04	0.33	62.33	1.86
Baltimore	2.67	0.36	1.49	0.51	55.99	5.67	3.73	0.33	65.82	1.58
Bonpain	1.89	0.54	1.66	0.55	88.00	6.48	3.49	0.34	53.79	2.03
Crousty	1.85	0.27	1.15	0.57	61.98	5.04	2.82	0.37	55.83	1.42
Elephant	2.37	0.41	1.78	0.60	75.06	5.11	4.21	0.32	82.45	1.68
Isengrain	1.94	0.26	1.30	0.58	67.21	4.79	2.93	0.32	61.20	1.34
Magdalena	2.46	0.75	1.59	0.51	64.41	6.57	3.71	0.32	56.44	3.30
Mallaca	1.93	0.59	1.69	0.62	87.62	6.01	3.99	0.36	66.29	2.34
Meunier	2.18	0.70	1.63	0.59	74.99	5.52	3.89	0.35	70.47	3.27
Sidéral	1.95	0.63	1.78	0.59	91.12	5.93	4.13	0.36	69.58	2.33
Sisley	1.69	0.49	1.45	0.60	85.53	5.94	3.80	0.30	63.94	1.96
Soissons	1.66	0.28	1.62	0.66	97.38	4.88	3.41	0.35	69.91	1.44
Supersoft	2.87	0.73	1.65	0.50	57.51	6.44	4.20	0.34	65.21	2.91
Tamaro	2.22	0.39	1.46	0.56	65.60	4.99	3.11	0.34	62.32	1.71
Texel	2.10	0.56	1.75	0.54	83.17	5.60	3.64	0.33	64.89	2.13
Thesee	2.68	0.60	1.75	0.59	65.22	5.12	3.05	0.39	59.66	1.90
Virtuose	2.52	0.65	1.55	0.47	61.64	5.87	4.03	0.35	68.68	2.46
<chgrow;lp;4q>SD	0.34	0.16	0.17	0.05	12.23	0.63	0.46	0.02	6.70	0.61
mean	2.18	0.51	1.61	0.58	75.18	5.76	3.71	0.34	64.57	2.10
cv (%)	15.43	30.86	10.45	8.73	16.27	10.96	12.39	6.11	10.38	29.24
min	1.66	0.26	1.15	0.47	55.99	4.79	2.82	0.30	53.79	1.34
max	2.87	0.75	1.78	0.66	97.38	6.92	4.45	0.39	82.45	3.30

[a] Means from duplicates for *a*, *b*, and *f*, coefficient of variation was <10%; triplicates for *c*, *d*, *g*, and *h*, coefficient of variation <5%. [b] Values from ref *25*. Calculated as the sum of Ara and Xyl in flours. [c] Values from ref *25*. Calculated as the sum of Ara and Xyl extracted by water. Ara is corrected for the presence of arabinogalactans. [d] Calculated as the sum of Ara and Xyl extracted by xylanase from flour. [e] Calculated as (XE-AX[c]/total AX[a]) × 100. [f] Calculated as the sum of Ara and Xyl in grain. [g] Calculated as the sum of Ara and Xyl extracted by xylanase from grain. [h] Calculated as (XE-AX[f]/total AX[e]) × 100. [i] Relative viscosity of grain water extract.

Table 2. Extent of AX Extraction and Relative Abundance of Oligosaccharides Obtained after Enzymatic Degradation by Endoxylanase from Tissues of Wheat Grain[a]

					relative peaks area (%)							
tissue	cultivar	extraction %	XE-AX[b] Ara/Xyl	total AX[c] Ara/Xyl	X1[d]	X2	DP4	DP5	DP6	DP7	DP8	DP9
flour	Scipion	96.20	0.55	0.61	15	10	6	21	21	13	11	3
	Baroudeur	98.80	0.56	0.60	18	10	7	22	17	14	10	2
endosperm	Scipion	91.00	0.51	0.58	13	11	5	23	20	15	11	2
	Baroudeur	90.10	0.49	0.56	13	9	6	24	19	16	12	2
aleurone layer	Scipion	89.40	0.33	0.39[e]	19	38	6	24	3	11		
	Baroudeur	79.00	0.34	0.38[e]	19	36	6	25	3	11		
intermediate layer	Scipion	58.10	0.01	0.34[e]	30	70						
	Baroudeur	54.00	0.08	0.37[e]	31	69						
pericarp	Scipion	0.00	0.00	1.15[e]								
	Baroudeur	0.00	0.00	1.13[e]								

[a] Results obtained from duplicates, coefficient of variation <10%. [b] Arabinose to xylose molar ratio of AX extracted by the xylanase. [c] Arabinose to xylose molar ratio of AX in the starting tissue. [d] HPAEC-PAD signals are expressed as % area of each peak relative to the total area. X1 and X2 denotes xylose and xylobiose, respectively, DP4 = $A1_2X3$, DP5 = $A1_3X4$, DP6 = $A2_{3d}X4$, DP7 = $A2_{4,3}X5$, DP8 = $A3_{4,3d}X5$, DP9 = $A4_{4d,3d}X5$, as described in ref *27*. [e] Values from ref *38*.

assessed from DP6, DP7, and DP5. DP5 and DP7 were negatively correlated to DP6. Principal component 2 mainly revealed variations of DP5. For grain, the first component was mainly assessed from X2 and DP5, while the second component was assessed from DP6. Principal component analysis of flour separated cultivars according to their relative proportion of mono- and disubstitution on the first principal component. Interestingly, X1 and X2 were little involved in the separation of flour samples. To the contrary, variations in peak X2 mainly influenced the principal component analysis of grain. X2 is mostly released from testa and nucellar layers and did not separate samples according to the structure of endosperm AX. Consequently, X1 and X2 data were not taken into account in subsequent analysis in order to focus only on the structural variations occurring in AX from endosperm tissue. For clarity and owing to the high reproducibility of the xylanase treatment, data of the three replicates for each cultivar have been averaged.

Principal Component Analysis of Flour and Grain without X1 and X2. Similarity maps and loadings of flour and grain without X1 and X2 are presented in **Figure 4** and **Table 3**. The first two principal components accounted for 95.9% and 95.3% of total variance for flour and grain, respectively. For flour, the first two principal components were assessed from DP6, DP7, and DP5 as previously observed (**Table 3**). Similar loading was obtained for the first component of the whole grain data set. The second component slightly differed by the contribution of DP7. The similarity maps of flour and grain (**Figure 4**) showed similar distribution of cultivars along component 1 and some differences along component 2. Correlations between principal components of the two data sets were

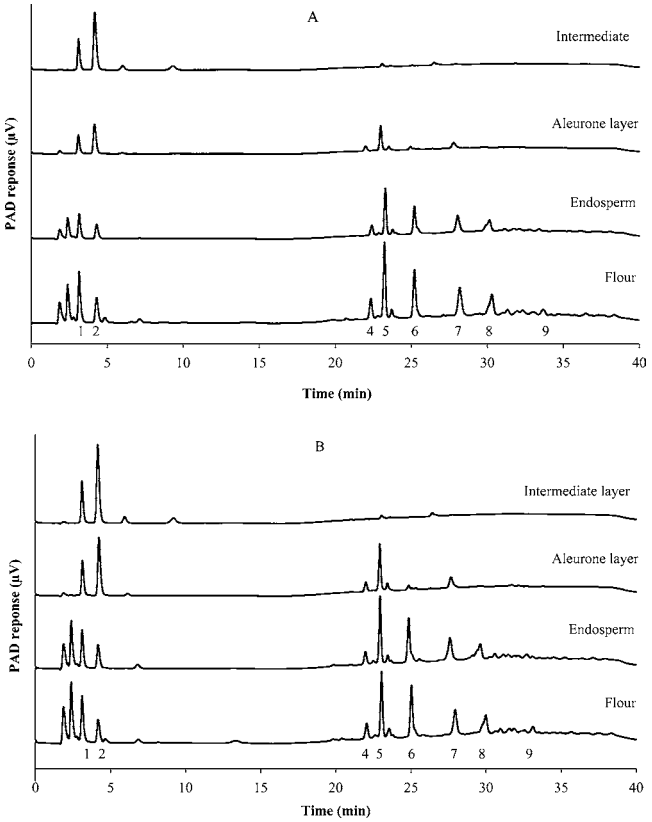

Figure 2. HPAEC-PAD profile of endoxylanase degradation of A = cultivar Baroudeur tissues and B = cultivar Scipion. Numbers 1–9 indicate oligosaccharides DP and represent respectively xylose, xylobiose, and oligomers $A1_2X3$, $A1_3X4$, $A2_{3d}X4$, $A2_{4,3}X5$, $A3_{4,3d}X5$, and $A4_{4d,3d}X5$, as outlined in ref *29*.

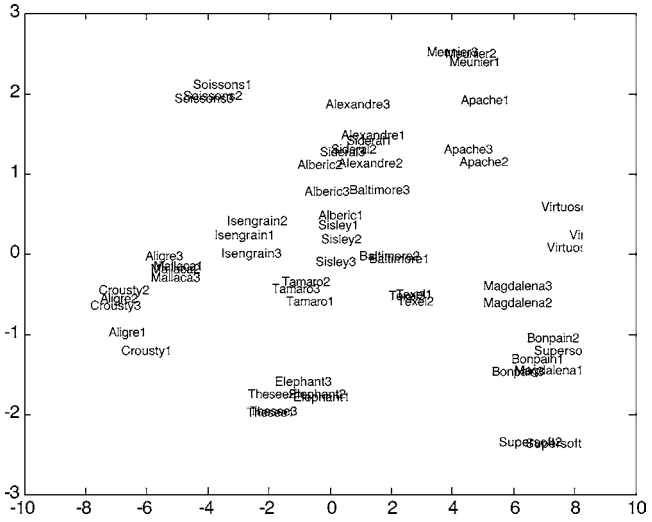

Figure 3. Score plot from principal component analysis of flour samples analyzed by HPAEC-PAD; each cultivar is performed in triplicates.

examined. Component 1 of flour data was highly correlated with component 1 of whole grain data: $r = 0.90$. Component 3 of flour data was correlated with components 2 and 3 of the whole grain data set: $r = 0.60$ and $r = 0.56$, respectively. To better describe the correlations between flour and grain data sets, canonical correlation analysis was performed between the principal components of both data sets.

Canonical Correlation Analysis of Flour and Grain. The six principal components of flour and grain were used. The first four canonical correlation coefficients were $r^2 = 0.97$, 0.83, 0.82, and 0.62 and the first two canonical patterns are given in **Table 4**. Canonical variate 1 was mainly driven by DP6 and DP9 (negative scores) and DP7 (positive score) for both data sets. Cultivars were mainly sorted along variate 1 according to

Table 3. Loadings of the Variables for the First Two Principal Components from Flours and Whole Grain Data Set

	flour		grain		flour		grain	
variable	PC1	PC2	PC1	PC2	PC1	PC2	PC1	PC2
X1	–0.177	0.283	–0.279	0.067				
X2	–0.083	–0.344	–0.758	–0.350				
DP4	0.099	0.182	0.090	0.033	–0.077	0.189	–0.020	0.086
DP5	0.478	–0.676	0.439	–0.435	–0.413	–0.767	–0.388	–0.802
DP6	–0.659	–0.269	0.062	0.741	0.747	–0.323	0.749	–0.033
DP7	0.512	0.368	0.364	–0.321	–0.481	0.261	–0.517	0.565
DP8	–0.005	0.215	0.102	0.140	0.043	0.361	0.039	0.170
DP9	–0.161	0.239	–0.019	0.102	0.180	0.271	0.137	0.004

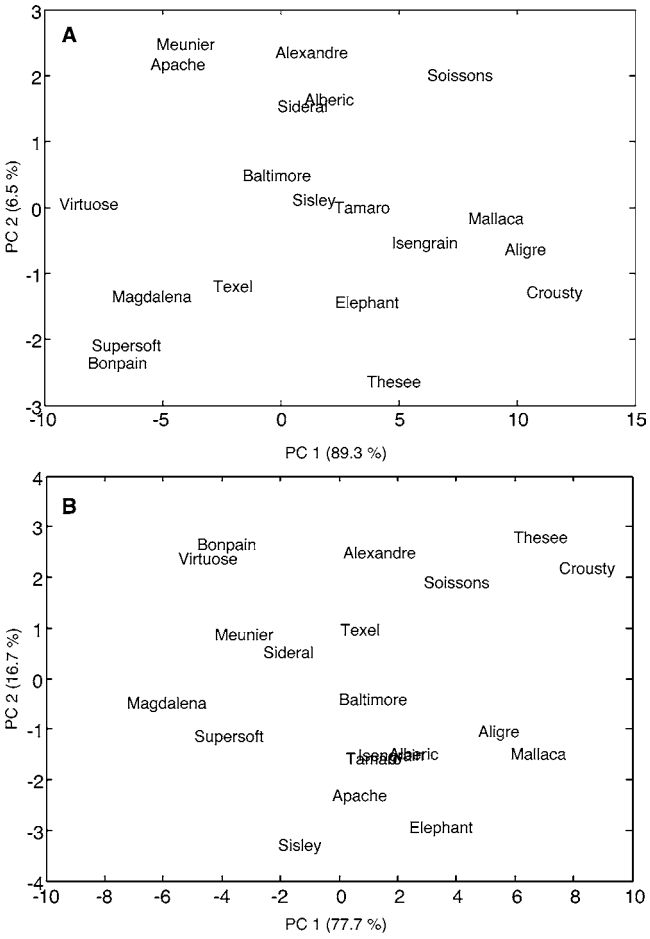

Figure 4. Score plot from principal component analysis of flour (**A**) and milled whole grain samples (**B**) analyzed by HPAEC-PAD (without xylose and xylobiose peaks).

Table 4. Canonical Patterns for the First Two Canonical Variates from Flours and Whole Grain Data Set

	flour		grain	
variable	CC1	CC2	CC1	CC2
DP4	–0.18	0.39	0.02	0.2
DP5	0.34	–0.01	–0.03	0.92
DP6	–0.52	–0.77	–0.66	–0.26
DP7	0.65	–0.49	0.58	–0.12
DP8	–0.02	–0.13	0.33	–0.01
DP9	–0.40	0.02	–0.35	0.2

Table 5. Physicochemical and Structural Features for 20 Wheat Cultivars; Mean and Standard Deviation for Each Cluster[a]

feature	cluster 1	cluster 2	cluster 3	cluster 4	cluster 5	CC1[b]
mXyl (%)	12.5 ± 0.4a	14.1 ± 1.1 ab	14.9 ± 1.6bc	17.2 ± 0.8cd	18.7 ± 0.5d	0.91
dXyl (%)	22.5 ± 0.3a	20.2 ± 2.0ab	20.2 ± 1.0a	18.2 ± 2.1b	14.7 ± 0.7c	–0.86
Ara/Xyl [XE-AX (flour)]	0.64 ± 0.02a	0.61 ± 0.05ab	0.59 ± 0.02ab	0.57 ± 0.04b	0.51 ± 0.03c	–0.72
Ara/Xyl (WE-AX)	0.62 ± 0.007a	0.56 ± 0.04a	0.57 ± 0.03a	0.55 ± 0.05a	0.47 ± 0.008b	–0.69
WE-AX (g/100 g)	0.47 ± 0.17ab	0.38 ± 0.19a	0.42 ± 0.09a	0.58 ± 0.13ab	0.67 ± 0.09b	0.72
viscosity	1.96 ± 0.5abc	1.59 ± 0.3a	1.71 ± 0.2a	2.46 ± 0.7abc	2.68 ± 0.5c	0.66

[a] Means with same letters are not significantly different based on Fisher's least significant difference (LSD) mean comparison test. Cluster 1: Aligre, Mallaca; Cluster 2: Crousty, Soissons, Thésée; Cluster 3: Alberic, Apache, Elephant, Isengrain, Sisley, Tamaro; Cluster 4: Alexandre, Baltimore, Meunier, Sidéral, Texel; Cluster 5: Bonpain, Magdalena, Supersoft, Virtuose. [b] Correlation coefficient between CC1 and physicochemical features assessed for the 20 cultivars.

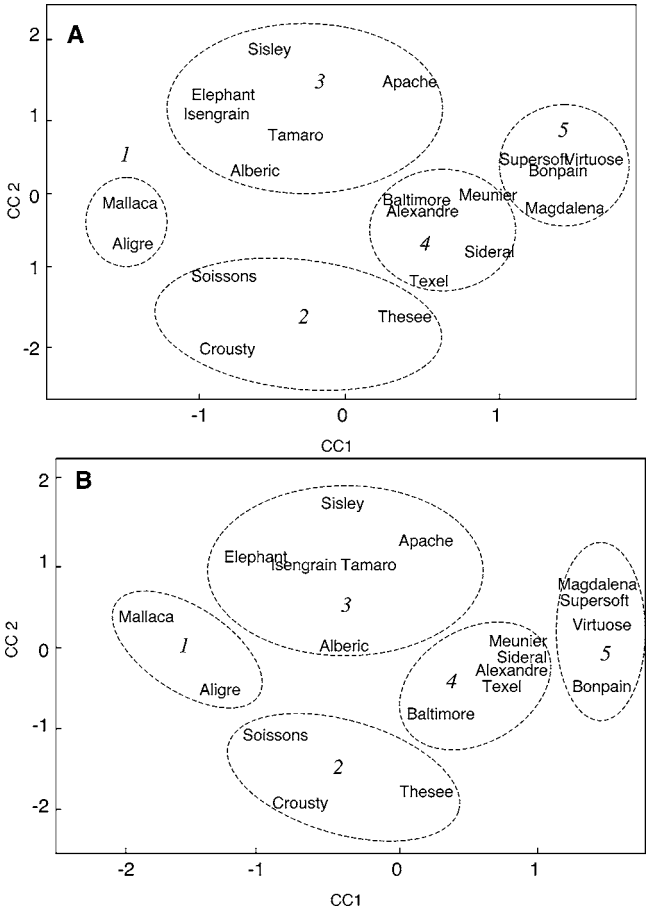

Figure 5. Score plot from canonical correlation analysis of flours (**A**) and milled whole grain samples (**B**) analyzed by HPAEC-PAD (without xylose and xylobiose peaks). Dotted lines represent the different clusters outlined by the ordination.

the relative proportion of their monosubstituted and disubstituted xylose residues for flour as well as for whole grain. Canonical variate 2 was mainly driven by DP6 for flour data and strongly by DP5 for whole grain data. In whole grains, variations in DP5 could be related to variations in the proportion of aleurone layer. In flour, aleurone layer is only found as a contamination of endosperm by outer layers that depend on milling properties of cultivars. DP6 indicates a higher content of disubstituted residues. AX in aleurone cell walls are mostly monosubstituted (*37*). This suggested that cultivars with positive scores of canonical variate 2 contained a higher content of aleurone in whole grain and subsequently in flour.

The similarity maps generated from each data set were plotted, and a similar distribution of cultivars was observed in both cases (**Figure 5**). Cluster analysis has been performed to identify clusters of cultivars exhibiting similar structural characteristics of their AX. Scores of whole grain observed on the first two canonical variates were used and the result is shown in **Figure 6**. Five groups were selected from this analysis and identified on canonical similarity maps as dashed circles (**Figure 5**).

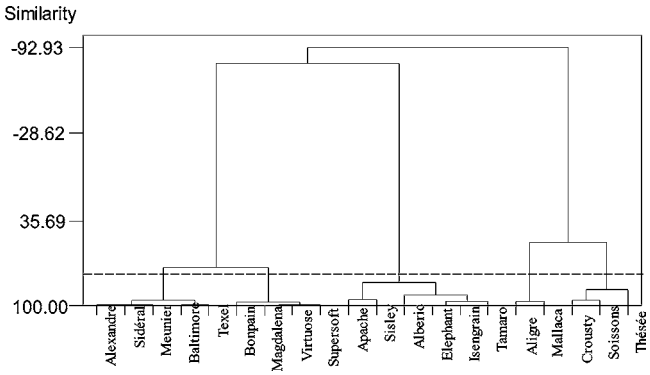

Figure 6. Ward's cluster analysis from flour and whole grain AX solubilized by endoxylanase.

Wheat Clusters. Structural features of WE-AX and XE-AX have been previously determined for the different cultivars (*25*). Percentages of mono- and disubstituted xylose residues determined for XE-AX and Ara/Xyl ratio of WE-AX and XE-AX have been measured on flours. In addition, relative viscosity has been assessed in grain water extract.

As expected from patterns of canonical variate 1, scores of the cultivars were positively correlated with % of monosubstituted and negatively correlated with disubstituted xylose residues, showing that enzymatic fingerprinting allowed a discrimination of cultivars according to the structural features of their AX. Correlations were observed to a lesser extent with features related to WE-AX such as their content, Ara/Xyl ratio, or the viscosity of grain water extract (**Table 5**). No significant correlations were observed with canonical variate 2.

The mean values of the features were calculated for the five clusters (**Table 5**). Clusters 1, 4, and 5 separated along canonical variate 1 were characterized by different proportions of mono- and disubstitution (**Table 5**). Ara/Xyl ratios were not sufficient to discriminate the three clusters. Cluster 5 formed by cultivars Bonpain, Magdalena, Supersoft, and Virtuose gathered cultivars presenting a significantly higher content of WE-AX.

Cultivars Alberic, Apache, Elephant, Isengrain, Sisley, and Tamaro have been grouped in cluster 3 and cultivars Crousty, Soissons, and Thésée in cluster 2. These clusters were not statistically different for the level of mono- and disubstitution, Ara/Xyl ratios, and WE-AX content. However, enzymatic fingerprinting revealed additional differences that allowed the separation of the two clusters along CC2. The hypothesis that these differences were caused by variations in aleurone content should be further investigated.

Conclusion. Several methods have been developed for the screening and identification of plants with altered cell wall polysaccharide structure. As such, Fourier transform infrared microspectroscopy (*40*, *41*) has been developed for the selection of cell wall mutants. Methods based on the quantification of neutral monosaccharide have also been used for the screening of cell wall mutants (*42*). The characterization of wall polysaccharide alteration usually involves its purification using differential extraction procedures and additional structural characterization using glycosidic linkage compositions by methylation analysis or NMR spectrometry. These procedures are unfortunately labor intensive and generally require large amounts of material. The enzyme treatment and subsequent chromatographic analysis, e.g., enzymatic fingerprinting, is an alternative method to study structural variation of plant polysaccharide on a large number of samples, which has already been successfully applied to reveal structural modifications of xyloglucan in mutants of *Arabidopsis thaliana* (*43*).

Enzymatic fingerprinting of wheat grain AX was assessed using an endoxylanase. The resulting fragments, recovered in the enzyme buffer, were analyzed by liquid chromatography (HPAEC). To validate the technique, xylanase treatment was used on various tissues: endosperm, aleurone layer, intermediate layer, and pericarp, isolated by dissection of grains as well as on flours. The great structural heterogeneity of AX according to tissues was highlighted. Flour enzymatic fingerprinting treatment was found very close to that of pure endosperm, and it was shown that substituted arabino-xylo-oligosaccharides (DP4−DP9) were the best markers of endosperm tissues, whereas X1 and X2 essentially came from maternal tissues (testa and nucellar layer). Some peaks were excellent markers of mono- (DP4 and DP5 and DP7) and disubstitution (DP6, DP8, and DP9).

In the present work, structural variations were not related to the lack of a particular structure but to variation in the proportion of mono- and disubstitution. Different peaks in the chromatogram varied at the same time, which increase dramatically the number of variables to analyze. Multivariate analysis techniques are useful for analyzing large and complex data sets and allowed the revealing of similar classifications of wheat cultivars from flour and whole grain enzymatic fingerprinting. This showed that the enzymatic fingerprinting procedure proposed in this paper enables study of the structure of AX from endosperm tissues without the necessity of making flour.

Cultivars with AX exhibiting the same Ara/Xyl ratio have been differentiated. This information could be reached because both the mode of substitution of xylose residues and the polymerization degree of oligosaccharides released by the enzyme were taken into account. Indeed, differences in the distribution of arabinose residues along the xylan backbone lead to various proportions of oligosaccharides released.

Although the present procedure has been developed on wheat grain, it is likely to work with other cereal grains such as rye, maize, and possibly barley or oat. First attempts on rice grains were unsuccessful, the enzyme from *Trichoderma viride* being unable to extract a significant amount of AX from purified endosperm or milled grain. This might be due to a different structure of AX in rice grain.

In conclusion, enzymatic fingerprinting is proposed as a sensitive technique for revealing structural variations of AX in cereal grains. The method has been developed for working with flour or grain from wheat and will be applied to establish QTL on a large wheat population in order to identify chromosomic regions controlling the structural features of AX.

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