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Hydrolysis of wheat bran and straw by an endoxylanase: production and structural characterization of cinnamoyl-oligosaccharides

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Abstract

Hydrolysis of wheat bran and wheat straw by a 20.7 kDa thermostable endoxylanase released 35 and 18% of the cell-wall xylan content, respectively. Separation of the cinnamoyl-oligosaccharides (accounting for 6%) from the bulk of total oligosaccharides was achieved by specific anion-exchange chromatography. The cinnamoyl-oligosaccharides were further purified by preparative paper chromatography (PPC) and their molecular weight was determined by MALDI-TOF mass spectrometry. The partially purified hydrolysis end-products contained from 4 to 16 and from 4 to 12 pentose residues for wheat bran and straw, respectively, and only one cinnamic acid per molecule. The primary structure of the new feruloyl arabinoxylopentasaccharide from wheat bran hydrolysis, which has been determined using 2D NMR spectroscopy, is *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(feruloyl)-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose.

Keywords: Endoxylanase; Wheat straw; Wheat bran; Ferulic acid; *p*-Coumaric acid; Arabinoxylo-oligosaccharides

1. Introduction

Agricultural residues represent large renewable resources for lignocellulose bioconversion. Wheat straw and bran are hemicellulose-rich substrates, among which xylans represent between 25 and 40% of dry matter [1,2]. Xylans are β-(1→4) polymers in which the D-xylose residues can be substituted at O-3 or O-2 with α-L-arabinose and at O-2 with α-D-glucuronic acid or its 4-methyl ether. In addition, cinnamic acids [ferulic acid (FA) and *p*-coumaric acid (*p*CA)] are bound to arabinosyl side chains of xylans via ester bonds [3–5], and to lignin via ester and ether bonds [6,7]. Feruloyl groups can form cross-linkages between polysaccharide chains by peroxidase-catalyzed dimerization [8,9] or between lignin and polysaccharides via ether–ester bonds [10] and thus, may protect xylans against enzymatic attack [11].

Many studies have dealt with the isolation of feruloylated- and *p*-coumaroylated-oligosaccharides from Gramineae and from certain members of the genus Caryophyllales by mild acid hydrolysis or by treatment with

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polysaccharide hydrolyzing enzymes (reviewed in Ref. [12]). The isolation of such structures has allowed a better understanding of the cell-wall architecture. Furthermore, interest in these oligosaccharides is motivated by their biological activities and their technological applications. Indeed, it has been shown that these compounds can act as signaling molecules within plants [13] and between microorganisms and plants [14]. In addition, they possess the ability to inhibit induced oxidation of low-density lipoprotein (LDL) [15,16]. In the food industry, such oligosaccharides with higher DP are useful due to their gelation properties induced by oxidative cross-linking [17].

In a recent study, we analyzed the kinetics of wheat straw hydrolysis by a purified 20.7 kDa endoxylanase (EC 3.2.1.8) [18] belonging to the family 11 of glycoside hydrolases [19]. The major hydrolysis products were xylose-containing oligosaccharides of DP 2–4, although small amounts of higher DP arabinoxylo-oligosaccharides were also released.

In this work, using the same endoxylanase, we have characterized cinnamoyl-oligosaccharides released from wheat bran and wheat straw. To achieve this, we have developed a rapid and specific procedure for the purification of cinnamoyl-oligosaccharides from the bulk of the hydrolysis products. These compounds have been characterized using a variety of analytical methods. In addition, the primary structure of a new feruloylated arabinoxylo-pentasaccharide from wheat bran was determined on the basis of 2D (^{1}H and ^{13}C) NMR spectroscopy.

2. Results

Composition of wheat bran and wheat straw.—The composition of wheat bran and straw is presented in Table 1. The main differences in the biochemical composition of these substrates are found in their total lignin content (3% for wheat bran and 21% for wheat straw) and in their xylose/arabinose ratios. The degree of substitution of the main xylan chains by arabinose is higher in wheat bran (approximately 1 in 5 xylose residues are substituted in bran compared with 1 in 12 in straw). In contrast, total hemicellulose content is almost the same in bran and straw (21 and 26%, respectively). Nevertheless, if one considers that starch represents 35% of the dry weight (data not shown), then xylans represent 45% of bran.

Small amounts of cinnamic acids are also present in both substrates (less than 1% of the dry weight). Wheat straw contains approximately 30% more ester-linked cinnamic acids than wheat bran and contains approximately equal amounts of FA and *p*CA. In contrast, wheat bran contains a very small amount of *p*CA.

Enzymatic hydrolysis.—The optimal conditions for bran and straw hydrolyses using the thermostable endoxylanase have been determined to be 10 and 14 UI mL^{-1} in water at 60 °C, respectively (data not shown). In these

Table 1
Composition of wheat bran and wheat straw in carbohydrates, lignin and ester-bound cinnamic acids

		Wheat straw [a] (%) (dry weight)	Wheat bran [a] (%) (dry weight)
Monosaccharide composition [b]	glucose	39.6 ± 0.2	42.5 [c] ± 0.8
	xylose	24.3 ± 0.9	15.4 ± 1.3
	arabinose	2.1 ± 0.1	3.1 ± 0.2
	galactose	0.2 ± 0.1	2.7 ± 0.2
Lignin content	Klason lignin	21.0 ± 1.3	3.4 ± 0.6
Cinnamic acid content [d]	FA	0.28 ± 0.02	0.44 ± 0.02
	*p*CA	0.36 ± 0.01	0.014 ± 0.001

[a] Experiments were performed in four replicates. Mean values and standard deviation to the mean are reported.
[b] Determined by HPAEC after sulfuric acid hydrolysis.
[c] Where cellulose accounts for 20% and starch accounts for 80%.
[d] Determined by RP-HPLC.

Table 2
Composition (mol%) of wheat straw and wheat bran hydrolyzates and fractions recovered after AG 1 × 2 anion-exchange chromatography

	Wheat bran			Wheat straw		
	Sb [a]	Fb [b]	Eb [c]	Ss [a]	Fs [b]	Es [c]
Xyl	69.4	66.5	41.7	64.9	65.7	50.4
Ara	19.7	21.5	28.9	13.1	14.8	16.2
Gal	1.9	2.3	0.4	3.1	3.1	0.5
Glc	6.1	8.6	4	15.2	16.3	17.3
GlcA	0.8	1.1	/	1.9	/	/
FA	2.0	/	24.3	1.1	/	11.0
*p*CA	0.1	/	0.6	0.5	/	4.7

[a] Enzymatic hydrolyzate.
[b] Fraction recovered after elution by ammonium formiate.
[c] Fraction recovered after elution by EtOH.

conditions, maximum xylan hydrolysis was reached within 6 h for bran with 35% of hemicellulose solubilization and within 24 h for straw with 18% of hemicellulose solubilization. Cinnamic acid-bearing oligosaccharides were released in significant amounts with 29 and 20% of FA being solubilized from bran and straw, respectively. *p*CA was extracted to a lesser extent, attaining only 6% in the case of straw hydrolysis.

Purification of the cinnamoyl-oligosaccharides.—Both hydrolyzates were concentrated to a final volume of 100 mL and applied to an anion-exchange AG 1 × 2 (OH^-) column. Initially, all of the oligosaccharides were retained on the column. Application of an ammonium formiate solution (0.8 M) to the column led to the elution of approximately 82% of the total oligosaccharides. However, no ester bound cinnamic acids were detected in this fraction (Table 2). After extensive washing of the column with water to remove ammonium salts, the cinnamoyl-oligosaccharides were eluted using aqueous 50% EtOH. This fraction represented 6% of the total oligosaccharides and 75% of the total cinnamic acids. Further purification by preparative paper chromatography (PPC) led to the isolation of seven oligosaccharide-containing fractions from wheat bran hydrolyzate and six from wheat straw hydrolyzate. All of these oligosaccharides exhibited a λ_{max} at 320 nm characteristic of cinnamic acids.

Analysis of the oligosaccharides.—The composition of the different fractions obtained by PPC was determined on the basis of their carbohydrate and cinnamic acid molar composition and their MALDI-TOF mass spectra (Table 3).

The results of these analyses indicate that the endoxylanase had liberated oligosaccharides displaying a pentose content in the range 4–16 (wheat bran) and 4–12 (wheat straw). These oligosaccharides contain a single ester-linked cinnamic acid per molecule. The bran extract is almost devoid of *p*CA ester-linked oligosaccharides, except in Fraction **B4** where it was found in trace amounts. However, the purification method is not efficient for the separation of *p*CA and FA ester-linked oligosaccharides from straw extract. Chemical analysis reveals an increase of the arabinose/xylose molar ratio (Table 3) as a function of the pentose content and this ratio is higher in bran fractions than in the corresponding straw ones.

Two fractions arising from bran hydrolysis were purified to homogeneity as determined by their MALDI mass spectra (data not shown). The molecular weight of **B2**, the major product, is 854 (FA_1, Ara_1, Xyl_4; 33%) and that of **B1**, the minor one, is 722 (FA_1, Ara_1, Xyl_3; 5%). Compounds **B1** and **B2** were recovered in sufficient amounts to have their chemical structure fully characterized by NMR spectroscopy.

*Structural identification of compounds **B1** and **B2** by ^1H and ^{13}C NMR spectroscopy.*—^1H and ^{13}C NMR spectroscopy were used to elucidate the primary structure of **B1** and **B2**. The ^1H NMR spectrum of **B2** shows that FA is present as a cis/trans mixture in a ratio of 25:75 (Table 4). The ^1H and ^{13}C signals of the trans FA ($J_{7-8} = 16$ Hz) were readily identified by comparison with data from the literature [20–22]. The cis isomer ($J_{7-8} = 12.5$ Hz) was characterized by means of COSY, HSQC and HMBC experiments.

The feruloyl unit is connected to arabinose at its C-5 position as shown by the C-F9/H-A5(*R*) and C-F9/H-A5(*S*) HMBC correlations. The cis/trans isomerization has some impact on ^1H and ^{13}C chemical shifts in arabinose, such that two sets of data can be measured for sugar units A*t* and A*c*. The

assignment of the pro-*R* and pro-*S* A5 is based on previously obtained data [22].

The signals of H-A*t*1 (δ 5.43) and H-A*c*1 (δ 5.38) are deshielded by the carbonyl group at F9. The ^{1}H NMR signals of H-2, H-3 and H-4 in arabinose were sequentially identified from H-1 by means of the COSY spectrum.

The HSQC spectrum shows seven anomeric signals. Two of them arise from arabinose, four from β-D-xylose ($J_{1-2} \approx 8$ Hz) and the last one from the α form ($J_{1-2} \approx 3.6$ Hz) of the reducing sugar unit.

Xylose units Bβ, C, D and E present H-5e signals that can be individualized. Therefore, the signals from H-5a, H-4, H-3, H-2 et H-1 within these four sugar units were sequentially labeled by means of COSY, COSY-RCT, COSY-RCT2 and COSY-RCT3 (or TOCSY) spectra (Fig. 1). The HSQC spectrum helps to discriminate between H-5a and H-4 signals.

Table 3
Molar ratio and positive-ion *m*/*z* MALDIMS of the fractions recovered from PPC

Fraction number	$[M+Na]^+$	Pentoses	FA	pCA	Ara:Xyl:CA [a]	Total oligosaccharides [b] (%)	Cinnamoyl-oligosaccharides [b] (%)
A. Wheat bran							
B1	**745** [c]	4	1		1.4:3.4:1.0	0.4	4.9
B2	**877**	5	1		1.1:3.7:1.0	2.6	33.4
B3	*877*; **1009**	6	1		2.3:4.9:1.0	1.1	14.3
B4	*979*; **1009**	6	1	*1*			
	1111; **1141**	7	1	*1*	2.3:5.6:1.0	0.5	6.5
B5	**1009**	6	1				
	1141	7	1		2.2:5.6:1.0	1.2	15.0
B6	*1141*; **1273**	8	1				
	1405; *1537*	9	1		2.5:4.9:1.0	0.3	3.6
B7	**1537**	10	1				
	1669	11	1				
	1801	12	1				
	1933	13	1		4.8:7.6:1.0	1.8	22.3
	2065	14	1				
	2197	15	1				
	2329	16	1				
B. Wheat straw							
S1	**715**; **847**	4/5		1			
	745; **877**	4/5	1		1.1:2.9:1.0	1.7	30.7
S2	**847**; *979*	5		1			
	877; *1009*	5	1		1.0:4.4:1.0	0.5	9.0
S3	*847*; *979*	5/6		*1*			
	877; *1009*	5	1		0.9:4.0:1.0	0.9	16.7
S4	**979**; *1111*	6		1			
	1009; *1141*	6	1		1.8:4.3:1.0	1.1	19.5
S5	**1111**; *1243*	7		1			
	1141; **1273**	7/8	1		1.8:5.3:1.0	0.5	8.8
S6	**1243**	8		1			
	1273	8	1				
	1375	9		1	3.0:9.2:1.0	0.8	15.3
	1405	9	1				
	1507	10		1			
	1537	10	1				
	1669	11	1				
	1801	12	1				

[a] Molar ratio relative to (CA: cinnamic acid) determined by chemical analysis.
[b] Determined by the phenol–sulfuric method for carbohydrates.
[c] Bold and italic figures correspond to major and trace compounds, respectively.

Table 4
Assignments of signals in the ^{1}H NMR spectra of **B1** and **B2**

Chemical shifts (ppm), coupling pattern description, and coupling constants (Hz)						
Compound **B2**						
Unit	H-1	H-2	H-3	H-4	H-5e (*R*)	H-5a (*S*)
A*t*	5.43 bs	4.22 bs	4.00 bd 5.1	4.55 m	4.50 bd 12.4	4.35 dd 7.4, 12.4
A*c*	5.38 bs	4.18 bs	3.90 bd 5.3	4.48 m	4.43 bd 12	4.30 dd 7, 12
Bα	5.19 d 3.6	3.55 m	3.77 m	3.76 m	3.83 m	3.76 m
Bβ	4.59 d 8.4	3.27 t 8.5	3.56 t 8.7	3.79 m	4.07 dd 11.7, 5.5	3.39 m
C	4.47 d 7.7	3.32 m	3.55 t 7.5	3.79 m	4.11 dd 11.7, 5.1	3.37 m
D	4.51 d 8	3.46 t 8.5	3.73 t 9.5	3.83 m	4.13 dd 11.7, 5.1	3.41 m
E	4.44 d 7.7	3.29 t 8.5	3.42 t 9.5	3.60 m	3.92 dd 11.5, 5.5	3.28 t 12
	H-2	H-5	H-6	H-7	H-8	OCH_3
F*t*	7.24 s	6.93 d 8.4	7.18 d 8.4	7.70 d 16	6.46 d 16	3.90 s
F*c*	7.43 s	6.91 d 8.4	7.12 d 8.4	7.05 d 12.5	5.97 d 12.5	3.87 s
Compound **B1**						
A*t*	5.42 bs	4.22 bs	3.99 bd 5.1	4.54 m	4.50 bd 12	4.34 dd 7.7,12
Bα	5.19 d 3.7	3.54 m	3.76 m	3.75 m	3.81 m	3.75 m
Bβ	4.58 d 8	3.26 t 8.2	3.55 t 9.5	3.76 m	4.05 dd 11.7, 5.5	3.37 t 11
C	4.50 d 8	3.45 t 9.5	3.73 t 10	3.80 m	4.13 dd 11.7, 5.3	3.41 t 12.2
D	4.44 d 7.7	3.28 t 8.7	3.42 t 9.5	3.59 m	3.91 dd 11.7, 5.5	3.28 t 11.2
	H-2	H-5	H-6	H-7	H-8	OCH_3
F*t*	7.26 s	6.91 d 8	7.17 d 8.1	7.69 d 16.1	6.44 d 16.1	3.89 s

In the Bα sugar, H-2 and H-3 signals were located using H-1 by means of the COSY spectrum, while H-5 signals were determined from the HSQC spectrum. The latter allowed the assignment of all ^{13}C sugar resonance. The C-Bα4/H-Bα4 correlation was found by elimination.

Signals from H-4 in Bβ, C and D rings appear at 3.8 ppm while H-E4 appears at 3.6 ppm, showing that sugar E is not substituted at C-4. The substitution of sugar D at position 3 is inferred by the chemical shift of H-D3 (δ 3.73), which is greater than that of H-Bβ3 (δ 3.56), H-C3 (δ 3.55) and H-E3 (δ 3.42). The low value of δ (C-1) in ring Bβ (δ 98.92), when compared with those in rings C, D and E (δ 104.03 and 104.07), indicates that B is the reducing sugar unit. This is confirmed by the lack of inter-residue HMBC correlation of H-B1 signals.

The HMBC correlations C-A*t*1/H-D3, C-Bβ4/H-C1, C-C4/H-D1 and C-D4/H-E1 confirm the position of the branching points between sugars. The structure determination of compound **B1** was achieved using the same strategy. The bond location between sugar units was revealed by the C-A*t*1/H-C3; C-Bβ4/H-C1 and C-C4/H-D1 HMBC correlations (Tables 4 and 5). The chemical shifts values are in good agreement with those reported for similar compounds [22].

Spectral data of the minor isomer containing a cis feruloyl unit are not reported, due to the limited sample quantity available.

3. Discussion

In this study, we have shown that a 20.7 kDa endoxylanase released 20–30% of ester-linked FA from wheat bran and straw. In the case of wheat straw, a smaller proportion of *p*CA analogs are also liberated. With respect to xylan conversion, this enzyme hydrolyzed 35% of xylans in wheat bran and 18% in straw. Since wheat bran xylan is more substituted by arabinose than wheat straw, the presence of this sugar does not directly limit xylan hydrolysis. Likewise, since cinnamic acids are liberated to the same extent as the xylan products, it appears that the cinnamoyl residues do not block access of the enzyme to the xylan.

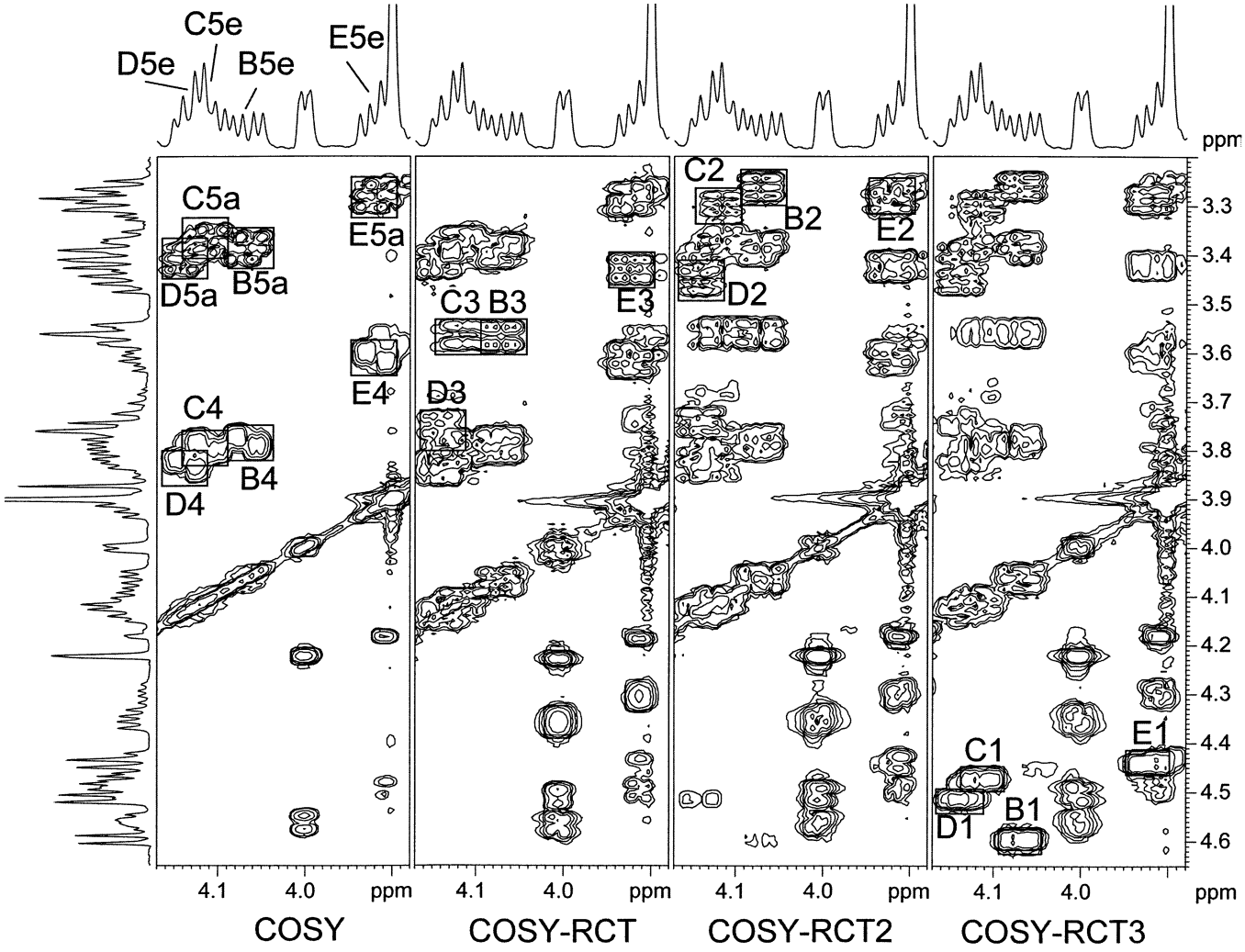


Fig. 1. Sequential identification of xylose protons in **B2**.

However, the presence of larger DP, highly arabinose-substituted oligosaccharides in the final reaction products does indicate that a high level of arabinose substitution does hinder the action of the endoxylanase. Therefore, it is possible that the remaining, non-hydrolyzed xylan (65% of total xylan in bran and 82% in straw) is highly substituted. However, a major hindrance to enzyme action is probably due to the presence of lignin. This is indicated by the total lignin content of both substrates, which correlates well with the percentage of non-hydrolyzed material. It is also known that diferulic acid (diFA) bridges and ester–ether bound FA provide intimate contacts between polysaccharides and lignin. Therefore, polysaccharide associated to lignin may well be inaccessible to the endoxylanase, due to the physical barrier that the lignin represents [23–26]. In either case, our results are in good agreement with recent results obtained by Grabber and co-workers, which indicate that while diferulate cross-links constitute a limiting factor for enzyme degradation, ferulate esters of non-lignified [27] and synthetically lignified maize cell wall [28] do not. Other factors linked to the cell-wall architecture or the tissular organization may also be partly responsible for the limitation of the hydrolysis [29,30]. However, our previous data, obtained using immunogold labelled-endoxylanase, showed that the enzyme was evenly distributed through the straw cell wall [31].

In order to separate the cinnamoyl-oligosaccharides from the bulk of oligosaccharides, we have employed anion-exchange chromatography, which allowed the specific separation of a

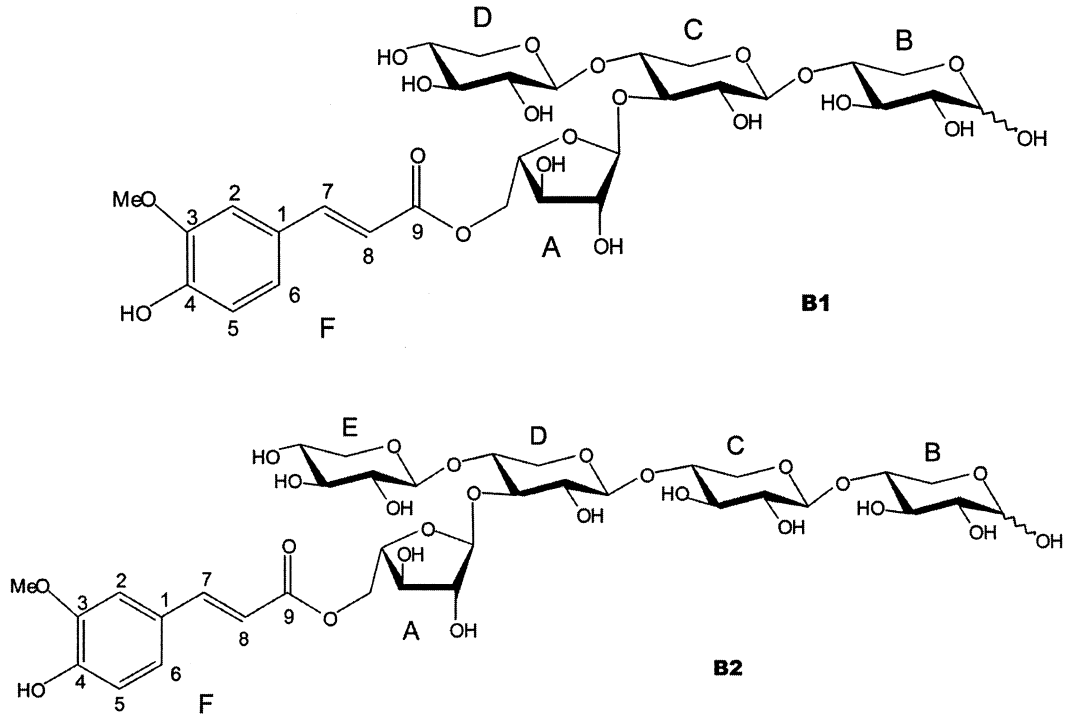
Table 5
Assignments of signals in the ^{13}C NMR spectra of **B1** and **B2**

Chemical shifts (ppm)					
Compound **B2** Unit	C-1	C-2	C-3	C-4	C-5
A*t*	110.28	83.13	80.15	84.32	66.61
A*c*	110.28	83.13	80.06	84.22	66.61
Bα	94.40	73.77	73.33	78.96	61.20
Bβ	98.92	76.38	76.31	78.80	65.35
C	104.07	75.07	76.05	78.80	65.38
D	104.03	75.69	80.11	76.11	65.26
E	104.07	75.44	78.00	71.67	67.50
F*t*	129.02	113.65	150.14	150.74	118.04
F*c*	129.44	116.10	149.14	150.74	117.46
	C-6	C-7	C-8	C-9	OCH_3
F*t*	126.01	149.13	116.33	171.47	58.18
F*c*	127.06	147.28	118.24	170.66	58.18
Compound **B1**					
A*t*	110.29	83.10	80.13	84.24	66.58
Bα	94.38	73.76	73.32	79.02	61.19
Bβ	98.90	76.36	76.30	78.83	65.34
C	104.00	75.67	80.05	76.12	65.23
D	104.06	75.42	77.98	71.60	65.49
F*t*	128.63	113.59	160.30	151.41	118.17
	C-6	C-7	C-8	C-9	OCH_3
F*t*	126.12	149.19	116.05	171.49	58.16

large spectrum of cinnamoyl-oligosaccharides displaying high DP values. This report constitutes the first preparation of such oligosaccharides using a single, purified polysaccharide hydrolase. It is noteworthy that none of the feruloyl-oligosaccharides purified from bran contained glucuronic acid.

Primary structures of one major (**B2**) and one minor (**B1**) oligosaccharide were determined by ^{1}H and ^{13}C NMR. **B2** has been shown to be a feruloyl ester-linked pentasaccharide which bears one arabinose residue at the C-3 position of the third xylose residue from the reducing end. FA is linked to the arabinose at the C-5 position by an ester bond (Scheme 1). Trans and cis isomers of this FA in a ratio of 3:1 have also been identified by NMR spectroscopy. The relatively large proportion of the cis isomer is rather perplexing, but may suggest that this isomer is already present in significant proportion in the original plant material. The oligosaccharide **B1** is similar to **B2**, but is a tetrasaccharide with the xylose in the second position bearing the feruloyl-arabinose substituent (Scheme 1). To the best of our knowledge, this is the first report of a feruloylated pentasaccharide such as **B2** isolated from a plant cell-wall hydrolyzate. In contrast, compound **B1** has previously been isolated from bamboo [32] and sugar cane bagasse [33] and *p*-coumaroylated analogs were found in oat straw [5] and bamboo shoot hydrolyzates [34]. Interestingly, oligosaccharides having structures similar to those identified in many grass species [22,35], in which the substituting feruloyl-arabinose residue is itself substituted by a xylose residue, were not found in the wheat bran hydrolyzate. Therefore, it may be possible that these structures are resistant to enzymatic activity [36].

In the two structures that we have identified, no substitution is found at the reducing or non-reducing termini, as is the case for the hydrolysis products of other endoxylanases that have been previously studied [37]. This suggests that our enzyme shares a common cleavage mode with other members of the family 11 of polysaccharide hydrolases, which is different as compared with the enzymes of family 10 [38]. Indeed, these latter enzymes are



Scheme 1. Structure of compounds **B1** and **B2**. **B1**: *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(feruloyl)-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose. **B2**: *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-(feruloyl)-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose.

able to cleave the linkage between substituted and unsubstituted xylosyl residues [39].

4. Experimental

Materials.—Wheat straw (*Triticum aestivum*) was harvested at full maturity and air-dried. Internodes were separated, collected, and reduced to small particles (size: < 2 mm). Wheat bran was obtained from flour milling. Wheat bran and wheat straw were autoclaved for 45 min at 121 °C in order to destroy any endogenous enzymatic activities [18] and were subsequently swollen at 60 °C for 16 h in water with continuous stirring.

Endo-β-(1→4)-xylanase (EC 3.2.1.8) was produced from a thermophilic *Bacillus sp.* strain I-1018 (CNCM, Institut Pasteur, Paris, reference I-1018) [40]. Purification to homogeneity was performed as previously described [41]. The purified protein had an activity of 400 UI mL^{-1}, a specific activity of 2000 UI mg^{-1} of protein at 60 °C, a molecular mass of 20.7 kDa, and a pI of 7.7. Endo β-(1→4)-xylanase activity was determined as previously described [42].

Enzymatic hydrolysis.—Hydrolysis of wheat straw 2% (w/v) and wheat bran 3% (w/v) in deionized water (1 L) was performed using endoxylanase (14 UI mL^{-1} and 10 UI mL^{-1}, respectively, where 1 UI is the amount of xylanase required to release 1 μmol min^{-1} xylose reducing equivalent from birchwood xylan) at 60 °C for 24 h with constant stirring. To remove starch, wheat bran hydrolyzate was precipitated overnight in 1 vol of abs EtOH at 4 °C. Both hydrolyzates were concentrated at 40 °C under reduced pressure.

Sugar analysis.—Total sugars were determined using the phenol/sulfuric acid method [43]. Monosaccharide composition was determined by HPAEC. Soluble fractions were hydrolyzed in CF_3COOH (2 N, 1 h, 121 °C), whereas solid samples were prehydrolyzed using H_2SO_4 (72% (w/v), 1 h, 25 °C), diluted to 2 N and heated (2 h, 100 °C) [44]. Fucose was added as an internal standard. The samples were diluted 50-fold, filtered, and injected onto a CarboPac PA1 anion-exchange column (4 × 250 mm, Dionex). Neutral monosaccharides were separated in 5 mM NaOH for 12 min and for a further 7 min with a linear

gradient up to 100 mM. Elution of acidic monosaccharides was achieved using a linear gradient up to 300 mM NaOAc in 100 mM NaOH for 30 min. The flow rate was 1 mL min^{-1} and detection was performed by pulsed amperometry (PAD 2, Dionex) after mixing of eluent with 300 mM NaOH (0.7 mL min^{-1}).

Lignin content.—The Klason lignin content was estimated according to a previously described procedure using 100 mg of sample [45].

Cinnamic acid.—The content in ester-linked FA and *p*CA was determined as described in [7]. 3,4,5-Trimethoxycinnamic acid was used as an internal standard. Separation was obtained with a linear gradient from 10 to 40% CH_3CN in 0.1% H_3PO_4 at a flow rate of 1 mL min^{-1} and detection was monitored at 280 and 313 nm.

Chromatographic methods.—Chromatography on an AG 1×2 anion-exchange resin using OH^- as counter-ion (Bio-Rad Laboratories) was performed on a column (40×1.6 cm) equilibrated with distilled water at a flow rate of 72 mL h^{-1}. Samples (100 mL) were loaded onto the column and the gel was washed with distilled water. A two-step elution was performed to remove bound material. First, an ammonium formiate (0.8 M) solution was applied to the column. Then, after extensive washing of the gel with distilled water to remove ammonium salts, the remaining oligosaccharides were eluted with aq EtOH (50%, v/v). This fraction was further purified by PPC.

PPC was performed on Whatman 3 MM paper by the descending method with 12:3:5 *n*-ButOH–HOAc–H_2O [36] as the mobile phase. Approximately 50 mg (estimated as total sugar) of sample were applied to the paper. After migration, cinnamoyl-bound oligosaccharides were detected under UV and stained with an oxalate/aniline reagent [2 vol of aniline 2% (v/v) in EtOH and 3 vol of oxalic acid 2.5% (w/v)]. Cinnamoyl-oligosaccharide-bearing regions were excised as paper strips and the samples were eluted in H_2O.

Mass spectrometry (MALDIMS).—Each of the samples (1 μL, 3g L^{-1}) was mixed with 50 μL of the matrix solution [2-5 dihydroxybenzoic acid 15 g L^{-1} in ACN:H_2O (3:1; v/v)]. A total of 1 μL of this solution was applied to a polished gold sample slide and allowed to dry at room temperature.

Positive ion MALDI mass spectra were recorded using a PerSeptive Biosystems Voyager Elite (Framingham, USA) time of flight mass spectrometer equipped with a 337 nm nitrogen laser (VSL 337ND).

NMR spectrometry.—Samples (between 8 and 10 mg) were dissolved in D_2O (99.996 at%) and NMR spectra were recorded on a Bruker DRX spectrometer operating at 500.13 and 125.75 MHz for ^1H and ^{13}C, respectively. Chemical shifts, in parts per million (ppm) were measured relative to internal acetone-d_6 (δ 2.20 for ^1H, 31.88 for ^{13}C) (50 μL in 500 μL D_2O). The number of t_1 increments in all 2D spectra is 512, except in the HSQC spectrum where it is 1024.

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