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INVESTIGATION OF THE STRUCTURE OF A HETEROXYLAN FROM THE OUTER PERICARP (BEESWING BRAN) OF WHEAT KERNEL

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ABSTRACT

A heteroxylan isolated from the pericarp (beeswing bran) of wheat kernel and purified is shown to be a highly substituted glucuronoarabinoxylan in which 80% of the β-D-xylosyl residues carry one or two substituents. Single terminal α-L-arabinofuranosyl groups and short chains of 2-, 3-, 5-, and 2,3-linked arabinose residues are distributed along the β-(1→4)-linked backbone of the xylan core which also carries residues of glucuronic acid and its 4-*O*-methyl derivative. Graded acid hydrolysis and degradations with an endo-xylanase and an α-L-arabinofuranosidase indicate that both the arabinan and the xylan moieties are highly branched structures, and this is confirmed by ^{13}C-n.m.r. spectroscopy of the heteroxylan at various stages of degradation.

INTRODUCTION

Whole wheat bran (WWB), a standard dietary fibre for man, is a good source of cell-wall polysaccharides, namely, hemicelluloses (arabinoxylans)[1] and cellulose. WWB is highly heterogeneous[2] with regard to its cellular distribution since, being adjacent to the outer pericarp (beeswing bran), it contains cells from inner pericarp, seed coat, aleurone layer, and starchy endosperm. Conversely, beeswing wheat bran (BWB) comprises mainly the outer pericarp, epidermis cells being preponderant. It is highly desirable[3] in purifying a polysaccharide to start from a well defined anatomical portion of a plant. BWB is preferable to WWB as the starting material for purification of bran hemicelluloses and there have been two structural investigations[4,5] of the major BWB heteroxylan. However, BWB is not easily avail-

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able, whereas WWB can be obtained in bulk which is a prerequisite to comparative biochemical[1] and nutritional[6] studies.

In a preliminary study, we have described[7] the purification and structural analysis of the major WWB heteroxylan. We now report parallel fractionation of WWB and BWB hemicelluloses with subsequent purification of their major heteroxylans, a structural investigation of the WWB xylan, and the sensitivity of native and partially hydrolysed WWB heteroxylans to the endo-(1→4)-β-D-xylanase II from *Polyporus tulipiferae*[8] and the α-L-arabinofuranosidase from *Dichomitus squalens*[9].

RESULTS AND DISCUSSION

Fractionation of hemicelluloses from whole (WWB) and beeswing wheat bran (BWB). — Both WWB and BWB were treated[1] to eliminate non-structural material and to reduce the content of lignin by extraction with chloroform–methanol (2:1), treatment with amyloglucosidase, and delignification by sodium chlorite. The yield of BWB holocellulose (Table I) was high since this material comprises dead empty cells which are almost free of cytoplasmic material. WWB gave a low proportion of holocellulose in agreement with the high level of starch, proteins, and oligosaccharides in the raw material[1]. Hemicelluloses were extracted by M sodium hydroxide containing 1% of sodium borohydride and fractionated into hemicelluloses A and B. Marked differences appeared between the brans at their hemicellulose level. Thus, hemicelluloses (~42% and 57% of BWB and WWB holocelluloses, respectively) were almost entirely of the B type (96.5%) for BWB, whereas they showed a 30:70 (hemicellulose A/B) distribution for WWB. Therefore, hemicellulose A from WWB, which is a weakly substituted xylan[1], must originate from cells different from those of the outer pericarp layers, probably from the aleurone layer, the second cellular constituent of bran.

BWB hemicellulose B (Table I) had a high content of pentosan with xylose and arabinose in the molar ratio of 0.88, in good agreement with other data[4,5]. On the other hand, WWB hemicellulose B contained, in addition to pentoses (Xyl/Ara 1.14), some glucose originating from β-glucans[7], which are typical constituents of *gramineae* cell walls[3]. Galactose was present in each preparation.

Stepwise addition of ethanol to aqueous 1% solutions of hemicellulose B gave the precipitation profiles shown in Fig. 1; WWB hemicellulose B was efficiently fractionated into two components, the major one precipitating sharply from 65% ethanol. The homogeneity of this fraction has been demonstrated[7] and its composition is given in Table II. The precipitation profile of BWB hemicellulose B was typical of an almost homogeneous fraction[4] and was superimposable on that of the WWB heteroxylan. Of the BWB hemicellulose B, ~16% remained soluble in 97% ethanol and was not carbohydrate in nature.

The composition of WWB heteroxylan agreed well with previous findings and compared favourably with that from beeswing bran[4,5]. Similarities were also

TABLE I

DATA FROM FRACTIONATION OF WHEAT-BRAN HEMICELLULOSES

Fraction	*Beeswing wheat bran (BWB)*	*Whole wheat bran (WWB)*
Holocellulose (%)[a]	83.2	26.9
Hemicellulose A (%)	1.2	4.9
Hemicellulose B (%)	33.9	10.5
Mole ratio[b]		
Arabinose	1.00	1.00
Xylose	0.88	1.14
Galactose	0.03	0.03
Glucose	0.02	0.25

[a]Percent of starting bran (dry-weight basis). [b]Without preliminary reduction[10] of uronic acids.

found on methylation analysis (see below) and it is valid to identify the WWB heteroxylan with that from BWB.

Composition and structure of the heteroxylan. — The heteroxylan was 94.5% polysaccharidic in nature, arabinose and xylose constituting 92% of the molecule. A preliminary reduction of uronic acids using carbodi-imide[10] and then sodium borohydride improved the yield of xylose (40→42.5%) after acid hydrolysis. Galactose, a common minor constituent of galactoarabinoxylans from *gramineae*[11,12], was present in small amount (1.6%) as well as glucose (0.7%) which arises from a β-glucan contaminant[7]. Analysis of uronic acids by the *m*-phenyl-

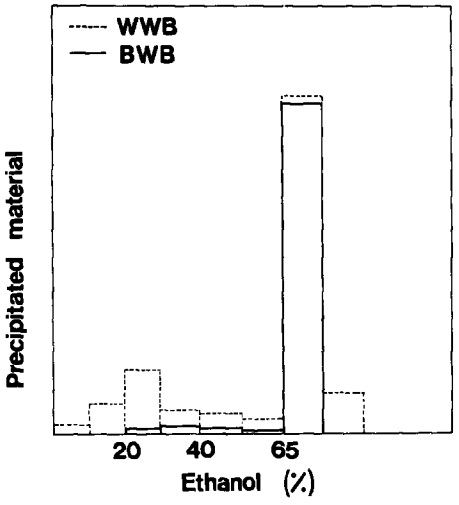

Fig. 1. Stepwise ethanol fractionation of aqueous 1% solutions of hemicellulose B from whole wheat bran (WWB) and beeswing wheat bran (BWB).

TABLE II

COMPOSITION OF HETEROXYLAN

Constituent	
Ashes (%)[a]	2.0
Nitrogen	<0.1
Neutral sugars[b] (%)	90.2
Uronic acids[c,d] (%)	4.3
Sugar distribution (mole ratio)	
Rhamnose	Traces
Arabinose	1
Xylose	0.98 (0.91)[e]
Galactose	0.04
Glucose	0.02
Glucuronic acid	0.08
4-*O*-Methylglucuronic acid	0.02

[a]Dry-weight basis. [b]Determined by the orcinol method. [c,d]Determined by the *m*-phenylphenol colorimetric technique and by titration (acid consumption) after reaction with carbodi-imide. [e]Value in brackets is the "anhydroxylose" content measured after hydrolysis without uronic acid reduction.

phenol[13] colorimetric procedure, titration of the consumption of acid after reaction with carbodi-imide[10] (CMC), and g.l.c. of the alditol acetates afforded similar values averaging 4.3%. Glucuronic acid represented ~80% of total uronides, the remainder being its 4-*O*-methyl derivative.

The heteroxylan and the carboxyl-reduced heteroxylan were each treated with 1.9M sodium methylsulfinylmethanide[14] and then methylated by methyl iodide. After extensive dialysis, each polymer was subjected to two Purdie methylations[15] and then hydrolysed, and the products were converted into the partially methylated alditol acetates which were analysed by g.l.c. and g.l.c.–m.s. (see Experimental). The native heteroxylan was subjected to two Hakomori methylations in order to promote β-elimination of uronic acid residues[16] and to locate their sites of attachment. The results are shown in Table III.

The native heteroxylan gave derivatives of twelve partially methylated sugars, but 2- and 3-*O*-methylxylose could not be separated under our conditions. The polysaccharide had a highly branched structure as indicated by the high proportions of non-reducing end-groups: 2,3,5-tri-*O*-methylarabinose, 2,3,4-tri-*O*-methylxylose, 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4-tri-*O*-methylrhamnose, and, for the carboxyl-reduced xylan, 2,3,4,6-tetra-*O*-methylglucose. This was confirmed by the high proportion of branching points, reflected by the derivatives of 2(3)-*O*-methylxylose, xylose, and 5-*O*-methylarabinose. The ratio (0.93) between terminal unsubstituted residues and branched units suggested that the occurrence of xylose did not reflect undermethylation. This structural feature was also observed in nearly the same proportion when the native heteroxylan was subjected to Haworth[17] and then two Purdie methylations. The efficiency of the methylation was checked by the absence of i.r. absorption for hydroxyl. The presence of doubly substituted

TABLE III

METHYLATION ANALYSIS OF HETEROXYLAN

Methyl ether	T[a]			*Native heteroxylan*	*Carboxyl-reduced heteroxylan*	*Heteroxylan submitted to β-elimination*
	A	*B*	*C*			
2,3,4-Tri-*O*-methylrhamnose	0.35	0.52	0.46	0.9[b]	0.5[b]	0.6[b]
2,3,5-Tri-*O*-methylarabinose	0.44	0.49	0.47	30.6	29.5	30.0
2,3,4-Tri-*O*-methylxylose	0.57	0.66	0.67	8.2	8.8	9.1
3,5-Di-*O*-methylarabinose	0.79	0.89	0.89	3.0	2.5	4.0
2,5-Di-*O*-methylarabinose	0.89	1.07	1.09	8.7	7.8	9.5
2,3,4,6-Tetra-*O*-methylglucose	1.00	1.00	1.00	—	4.8	—
2,3-Di-*O*-methylarabinose	1.06	1.31	1.26	5.3	4.5	4.0
2,3,4,6-Tetra-*O*-methylgalactose	1.19	1.36	1.26	1.1	1.2	1.6
2,3-Di-*O*-methylxylose	1.19	1.60	1.45	7.8	6.5	11.7
5-*O*-Methylarabinose	1.31	1.60	1.56	6.3	5.6	7.0
2(3)-*O*-Methylxylose	2.17	3.16	2.92	13.3	14.8	10.0
Xylose	3.63	5.44	5.23	14.8	13.5	12.5

[a]Retention times relative to that of 2,3,4,6-tetra-*O*-methylglucose on *A*, 3% OV-225; *B*, 3% SP 2340; *C*, 3% ECNSS-M. [b]Relative mole ratio.

xylose has also been reported in arabinoxylans from wheat[18] and rice[19] endosperm, barley[20] aleurone cells, sorghum husk[21], and corn[22] and rice[23] bran. Thus, this xylan is built of a central core of (1→4)-linked β-D-xylopyranosyl residues, ~22% being unsubstituted, ~37% carrying a single substitution at O-2 or O-3, and ~41% being doubly branched on O-2 and O-3. Therefore, this xylan has no open region in contrast to the more linear xylans from *gramineae*, for example, the heteroxylan from bamboo leaves[24] (degree of substitution ~24%) or xylan from cereal stem[12] (d.s. ~36%). This pattern of substitution resembles that observed[4] for a similar BWB hemicellulose. It seems that heteroxylans from the brans of cereal grains[4,22,23] have a very high degree of substitution. Indeed, a purified xylan from rice bran[22] was 79% branched, mainly at position 3 of xylose. The formation of a high proportion of 2,3,4-tri-*O*-methylxylose suggests that part of the xylose occupied terminal position in short side-chains, as previously shown in bamboo-leaf heteroxylan[24].

The formation of 2,3,4,6-tetra-*O*-methylglucose from the carboxyl-reduced xylan means that uronic acid residues were located essentially in terminal non-reducing positions. After alkaline β-elimination, the yield of 2,3-di-*O*-methylxylose was increased by ~4%, corresponding to the content of uronic acid. Therefore, glucuronic acid and its 4-methyl ether, which were not distinguished, were directly attached to the xylan backbone as single units, confirming previous data[5,25] which showed both uronic acids to be directly linked to O-2 of xylose in the main core of BWB xylan.

Arabinose occurred mainly as terminal residues, but was also linked through O-2, O-3, and O-5. These minor structural features are commonly encountered aside terminal arabinosyl groups in some *gramineae* xylans[19–24]. Arabinofuranose residues are present in short side-chains of multiple types in the xylans from oat stem[12] and bamboo leaves[24]. 5-*O*-Methylarabinose is of unique occurrence in cereal xylans. It was detected in a similar BWB xylan[4] but was not detected in previous work[7] probably because of overlapping with 2,3-di-*O*-methylxylose on the 3% SP 2340 column. Therefore, some short side-chains must have branching points through O-2 or O-3 of arabinofuranosyl residues.

Galactose was observed in non-reducing end positions, possibly terminating short side-chains. Terminal rhamnose has no evident structural significance, but is consistently observed as a trace component in highly purified acidic galactoarabinoxylans from oat leaf[11] and stem[12], and in total soluble hemicelluloses from bamboo leaves[24].

The heteroxylan consumed 0.75 mol of periodate during 48 h (calc. 0.70 mol). Reduction of the polyaldehyde, hydrolysis, and then conversion of the products into alditol acetates gave xylose and arabinose in the ratio 1.5:1, consistent with a highly branched structure.

Partial acid hydrolysis of native heteroxylan. — Mild acid hydrolysis of the heteroxylan with boiling 2.5mM sulfuric acid (pH 2.44) gave the results shown in Fig. 2. T.l.c. of diffusates from partial hydrolysates revealed that arabinose was liberated progressively in major amount with a very slight release of xylose which

corroborated the usual furanose structure of arabinose. A homologous series of xylo-oligosaccharides (xylobiose–xylopentaose) was detected together with other minor unidentified products only after hydrolysis for ≥8 h but was detected qualitatively in minute amounts after ≥1 h by h.p.l.c. on an Aminex HPX-87P column. The presence of xylo-oligomers suggests that, even if a preferential splitting of α-L-arabinofuranosyl linkages occurred under the above conditions, the central xylan backbone underwent some cleavage. This view was corroborated by the continuous release of diffusable uronic acids (Fig. 2). Rhamnose was also liberated from the outset of partial hydrolysis (Fig. 2).

Of the arabinose, 70% was lost within the first 6 h but ~92% only after 24 h. This double-phase nature of the release of arabinose has also been observed in the graded hydrolyses of a similar BWB hemicellulose[4], and of oat-stem[12] and bamboo-leaf[24] xylans. This behaviour could reflect the different locations of the arabinose residues, the readily split portion corresponding to terminal non-reducing end-groups (Table III) with the more resistant fraction corresponding to arabinosyl residues in the short side-chains. Methylation analysis of the degraded non-diffusable xylans partially supports this viewpoint (Fig. 3). By considering the yields of methylated sugars formed from each degraded xylan (up to 6-h hydrolysis) relative to those from the native heteroxylan and taking into account losses as diffusable material (Fig. 2), the disappearance of each structural feature can be tentatively assessed. Thus, all the arabinose (Fig. 3A) was removed, except for the 2-linked residues only one-third of which had been lost after 6 h. The resistance of 2-linked arabinose residues has been observed during controlled acid hydrolysis of heteroxylans from sorghum husk[21] and bamboo leaves[24]. In the latter heteroxylan, the

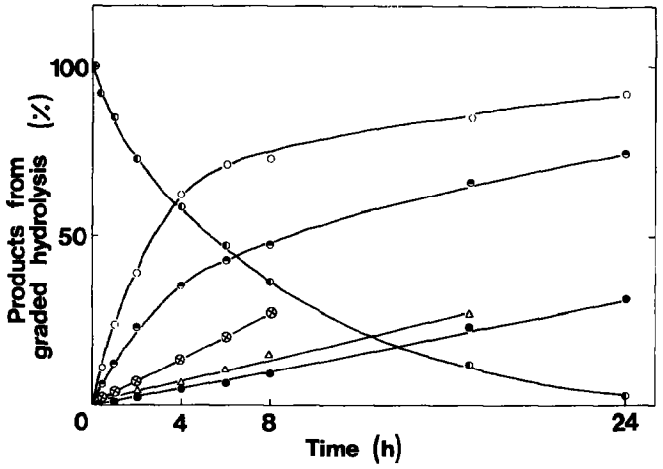

Fig. 2. Graded acid hydrolysis of native wheat-bran heteroxylan by boiling 2.5mM sulfuric acid: —●—, liberated (total) reducing sugars; —○—, arabinose; —●—, xylose; and —⊗—, diffusable uronic acids as percentages of maximum amount potentially releasable from native xylan; —◐—, non-diffusable xylans and —△—, diffusable oligosaccharides as percentages of the starting WWB heteroxylan.

resistant feature was part of the *O*-D-xylosyl-(1→2)-L-arabinosyl-(1→2)-L-arabinose side-chains. The rates of disappearance of the 2,5-di-, 2,3-di-, and 5-methyl ethers decreased on prolonged incubations, whereas the terminal arabinose residues were eliminated at the same rate.

Xylose was released linearly in very small amounts from the start of hydrolysis, as previously observed[7]. Here too, the extent of xylose appearance was far less pronounced than from the hemicellulose purified by Adams[4].

Variations of the xylose methyl ethers formed on methylation analysis of the products of graded hydrolysis (Fig. 3B) throw light on the structure of the central core. The unmethylated sugar disappeared at the highest rate, similar to that of 2,3,5-tri-*O*-methylarabinose. Previously[7], it was assumed that most of the doubly branched xylose residues were substituted with single terminal arabinosyl groups since, in conformity with the present data, a partially degraded WWB xylan was freed in parallel from terminal arabinose and fully substituted xylose with a concomitant increase of 2,3-di-*O*-methylxylose. However, no direct proof of this statement was obtained. The (1→4)-linked xylose was extensively produced during

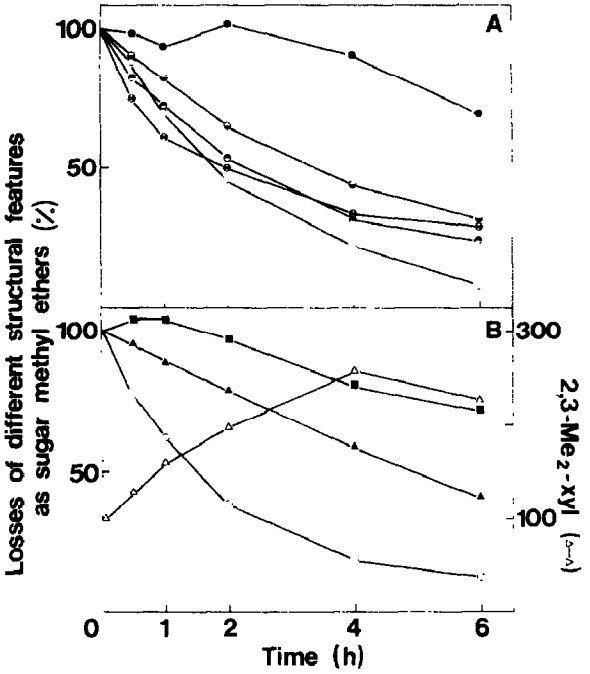

Fig. 3. Changes in the products of methylation analysis of native heteroxylan during graded hydrolysis by boiling 2.5mM sulfuric acid, taking into account losses of diffusable material (see Fig. 2). A. Arabinose series: —○—, 2,3,5-Me_3; —●—, 3,5-Me_2; —◐—, 2,5-Me_2; —⊗—, 2,3-Me_2; —◒—, 5-Me; B, xylose series: —▲—, 2,3,4-Me_3; —△—, 2,3-Me_2; —■—, 2(3)-Me; —○—, unmethylated; 2,3-Me_2-Xyl must be related to the 0–300% scale.

the first 4 h by progressive cleavage of the side-chains and then decreased when the main xylan chain was degraded into dialysable oligosaccharides (Fig. 2).

Non-diffusable degraded xylans had the following xylose/arabinose ratios: 1.07 (0.5-h hydrolysis), 1.17 (1 h), 1.50 (2 h), 2.47 (4 h), 3.73 (6 h), 4.06 (8 h), 8.63 (17 h), and 1.23 (24 h).

Since prolonged, graded hydrolysis resulted in extensive cleavage of the central backbone, only xylans being degraded for <6 h have been used for n.m.r. and enzymic studies.

^{13}C-N.m.r. spectroscopy. — The spectra of the purified native heteroxylan and its partially hydrolysed forms were interpreted on the basis of the results of methylation analysis. The progressive disappearance of signals in the spectra recorded at each step of the graded hydrolysis allowed a detailed assignment (Table IV) of the complex spectrum furnished by the native heteroxylan. In these spectra (Fig. 4), there are two main groups of signals corresponding to L-arabinose and D-xylose. The group of signals centered at 108–109 p.p.m. belongs to C-1 of α-L-arabinofuranose residues and the multiplicity of the resonances reflects the highly branched structure of the arabinan moiety[26] of the heteroxylan. The same is true for the complex of signals for C-1 of the β-D-xylopyranose residues centered at 102–103 p.p.m. More precise assignments could be effected on the basis of literature data[26–30] and by comparing the spectra of the products obtained as the hydrolysis progressed. After 6 h, nearly all of the arabinose had been removed, but the spectrum of the residual xylan still showed some complexity. Two sets of the most prominent signals were assigned to internal (1→4)-linked β-D-xylosyl residues and terminal β-D-xylosyl groups, respectively. The signals at 102.5, 77.2, 74.5, 73.7, and 63.8 p.p.m. correspond to C-1, C-4, C-3, C-2, and C-5, respectively, of the internal units of the xylan main-chain. The signals at 103.5, 76.4, 73.7, 70.0, and 66.1 p.p.m. are characteristic of C-1, C-3, C-2, C-4, and C-5, respectively, of the terminal xylosyl groups. The proportions of the xylose end-groups relative to the internal residues (including the branch points) suggest either a low d.p. of the residual material after hydrolysis for 6 h or a highly branched structure. The former hypothesis is ruled out since no signal for C-1α or C-1β, at 92.8 or 97.3 p.p.m., of the reducing end-groups can be seen.

The branching of the xylose side-chains at C-2 of the main-chain units is indicated by the shift of the signal for substituted C-2 from 73.7 to 77.2 p.p.m. which shows a high integrated intensity. Thus, it is concluded that not only is the main xylan core substituted with arabinose side-chains but also with xylosyl groups. This structural feature is confirmed by the high yield of 2,3,4-tri-*O*-methylxylose obtained in the methylation analysis of the native heteroxylan. The substitution by the arabinose residues at O-3 of the xylosyl residues in the backbone is evidenced by the concomitant disappearance of the signal at 82.95 p.p.m. (substituted C-3 of xylosyl residues) and of the arabinan moiety. This modification of the spectra was clearly observed after hydrolysis for only 1 h. Another feature which agrees with the methylation analysis is the absence of signal at 67–68 p.p.m.

TABLE IV

^{13}C-N.M.R. DATA OF NATIVE AND PARTIALLY DEGRADED WWB HETEROXYLANS

Chemical shift[a] δ *(p.p.m.)*	*Assignment*
109.2	C-1 α-L-Ara*f*
108.6	
103.5	C-1 Terminal non-reducing β-D-Xyl*p*
102.5	C-1 (1→4)-β-D-Xyl*p*, main chain
	C-1 (1→4)-β-D-Xyl*p* substituted at O-2 by another β-D-Xyl*p*
100.6	n.a.[b]
98.4	C-1 α-D-glucuronic (and 4-*O*-Me) acid
89.7	n.a.[b]
84.8	C-4 α-L-Ara*f*
82.95	C-3 (1→4)-β-D-Xyl*p* substituted at O-3 by α-L-Ara*f*
81.9	C-2 α-L-Ara*f* and C-4 4-*O*-Me-α-D-Glc*p*A
77.4	C-3 α-L-Ara*f*
77.2	C-4 (1→4)-β-D-Xyl*p*, main chain
	C-2 (1→4)-β-D-Xyl*p*, substituted at O-2 by β-D-Xyl*p*
76.4	C-3 Terminal non-reducing β-D-Xyl*p*
74.5	C-3 (1→4)-β-D-Xyl*p*, main chain
73.7	C-2 (1→4)-β-D-Xyl*p*, main chain
	C-2 Terminal non-reducing β-D-Xyl*p*
72.1	C-3 4-*O*-Me-α-D-Glc*p*A
70.0	C-4 Terminal non-reducing β-D-Xyl*p*
66.1	C-5 Terminal non-reducing β-D-Xyl*p*
63.8	C-5 (1→4)-β-D-Xyl*p*, main chain
62.0	C-5 α-L-Ara*f*
61.8	
59.5	OCH_3

[a]Relative to acetone as the internal reference (31.07 p.p.m.). [b]Not assigned.

corresponding to C-5-linked arabinofuranose residues. The low proportion of substitution at O-5 of the arabinan residues suggested by the methylation analysis is thus confirmed by the ^{13}C-n.m.r. spectrum of the native heteroxylan.

Hydrolysis of native and partially degraded xylans by α-L-arabinofuranosidase. — The native and degraded xylans were treated with α-L-arabinofuranosidase from *Dichomitus squalens*[9], an enzyme which cleaves terminal α-L-arabinofuranosidic linkages. Small quantities of arabinose (Fig. 5), determined by the NAD$^+$/galactose dehydrogenase system[31], were liberated from the native xylan, reflecting the presence of terminal α-L-arabinofuranose residues; ~5% of the total arabinose (or ~9% of the terminal arabinose) had been removed after 120 h. When a five-fold amount of enzyme was used, there was only a moderate increase in the production of arabinose which suggests that the arabinosyl end-groups were not easily accessible to the enzyme, a surprising observation since one-third of the molecule comprised terminal arabinosyl groups (Table III). These observations must be related to the rather limited action of α-L-arabinofuranosidase from *Aspergillus niger* (Pectinol 59-L) on a wheat endosperm arabinoxylan[32] (maximum extent of hydrol-

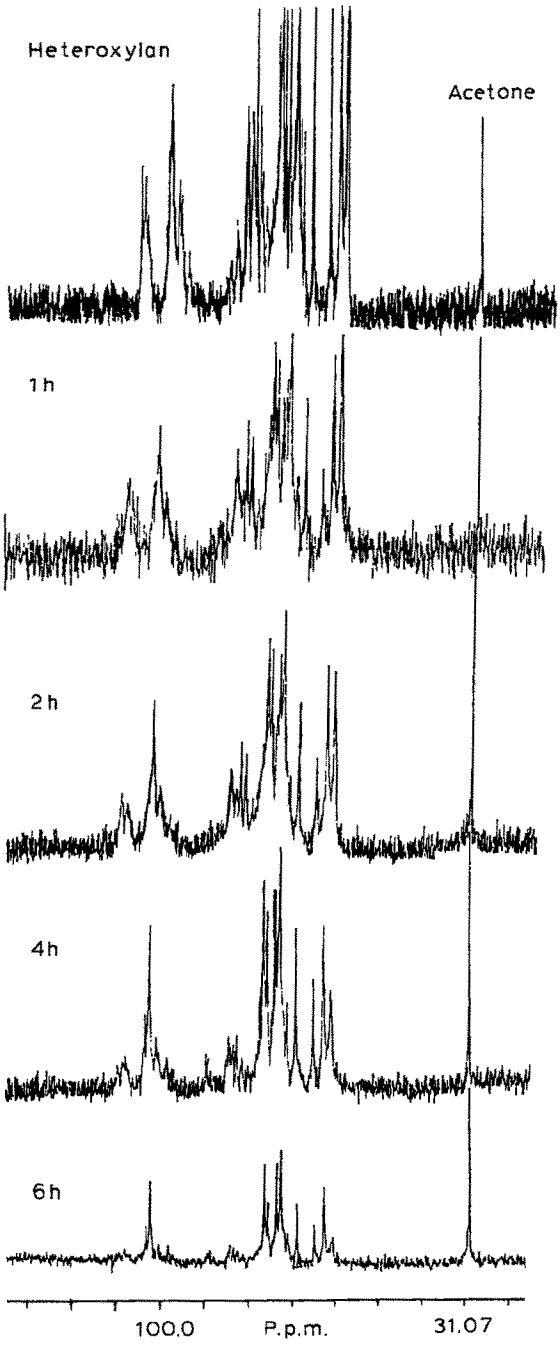

Fig. 4. ^{13}C-N.m.r. spectra of the non-dialysable heteroxylan at different stages of graded acid hydrolysis.

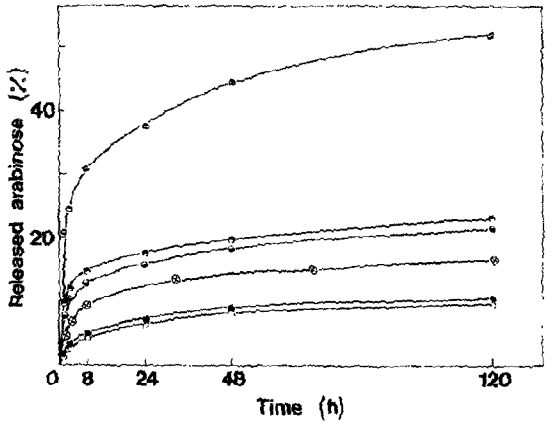

Fig. 5. Hydrolysis of native (—○—) and partially degraded xylans [1 h- (—⊗—), 2 h- (—◐—), 4 h- (—●—), and 6 h-treated (—◓—)] by α-L-arabinofuranosidase from *Dichomitus squalens*. Arabinose, measured by the galactose dehydrogenase/NAD^+ system, is expressed as a percentage of the releasable 2,3,5-Me_3-Ara. Native xylan (—●—) treated with a 5-fold amount of enzyme.

ysis, 18%). In contrast, an arabinosidase from another strain of *A. niger* (Pectinol R-10) effected 100% hydrolysis[33] of a similar wheat endosperm arabinoxylan and 35% hydrolysis[34] of a highly branched heteroxylan (Ara/Xyl ratio of 1) from rice bran. No satisfactory explanation can be given at present for these variable modes of action.

The arabinosidase progressively removed terminal arabinosyl groups (based on the yield of 2,3,5-Me_3-Ara) from the non-diffusable xylans as graded acid hydrolysis proceeded. Some terminal arabinosyl groups might have been produced by progressive peeling of side-chains, but it seems clear that gradual stripping of the xylan backbone improved the accessibility of the arabinofuranosidase to its substrate.

Hydrolysis of native and partially degraded xylans by xylanase II. — Native and partially debranched xylans were treated with an endo-(1→4)-β-D-xylanase from *Polyporus tulipiferae*[8], an enzyme which cleaved (1→4)-β-D-xylopyranosidic linkages of the xylan core. The course of the hydrolysis was followed by measuring reducing sugars released[35] (*Fig. 6*). Xylanase had a very restricted action on native xylan, only ~1% of the linkages being split after 24 h [~2.7% of the total (1→4)-β-D-xylosidic linkages, ~17% of bonds between unsubstituted xylose residues (on the basis of the yield of 2,3-Me_2-Xyl; see Table III)]. This result is in good agreement with methylation analysis data showing that a maximum of ~8% of the xylan would be theoretically susceptible to hydrolysis. The far lower figure actually measured reflects the steric hindrance existing in the neighbourhood of unbranched areas as opposed to weakly substituted xylans[36,37] having exposed regions which are

hydrolysed up to ~40% (on the basis of the yield of 2,3-Me_2-Xyl). Adams[4] also observed a lack of activity of a xylanase from *Myrothecium verrucaria* on a similar bran hemicellulose. Sapote gum[38], which is also a highly branched xylan (~84% substitution) having similarities with our xylan, is also resistant to various hemicellulases. More recently, Shibuya *et al.*[19] have shown that highly substituted arabinoxylans from the cell walls of rice endosperm were not readily hydrolysed by an endo-xylanase from *Streptomyces sp.* Degraded xylans became progressively more sensitive to enzyme as the time of treatment with dilute sulfuric acid increased. Schmorak *et al.*[39] noted the same behaviour of the *Myrothecium verrucaria* enzyme on increasingly debranched BWB heteroxylans. These findings corroborate the data discussed above on the progressive debranching of bran xylan by controlled acid hydrolysis. Not only the percentage hydrolysis increased but also the proportion of bonds between unsubstituted xylosyl residues (based on the yield of 2,3-Me_2-Xyl); after hydrolysis for 6 h, the xylan had undergone ~10% hydrolysis (~18% on a xylose basis; 32% on a 2,3-Me_2-Xyl basis). This progressively increasing sensitivity of (1→4)-linkages reflects the exposure of unsubstituted regions along the xylan backbone, thus improving the access of xylanase. The average d.p. of the native xylan was 83, and those for the hydrolysis products were 30 (1 h), 23 (2 h), 16 (4 h), and 10 (6 h). The rather high d.p. of the products formed by the xylanase acting on 6-h-treated xylan must be compared with the final d.p. (~3) obtained with weakly substituted xylan[37], and t.l.c. of hydrolysates from the 4-h- and 6-h-degraded xylans gave identical results and confirmed the presence of slowly migrating material as well as a significant proportion of thymol-positive material at the origin even after treatment with the xylanase for 24 h. Digestion media from other briefly treated xylans were not analysed because the amounts of oligosaccharides present were too small. Six components were consistently observed at any time of hydrolysis, three of them belonging to a homologous series of xylo-oligosaccharides, namely: xylose, xylobiose (R_{Xyl} 0.77), and xylotriose (R_{Xyl} 0.55). Xylose and xylobiose, the major product, were produced in increasing amounts and xylotriose decreased after 1 h. Xylotetraose (R_{Xyl} 0.36) was not observed by t.l.c. but was seen transiently in trace amounts by h.p.l.c. of a 0.3-h-digestion medium from the 6-h-treated xylan (Fig. 7). The virtual absence of xylotetraose, a usual early product of the action of xylanase on xylans[37,40] with limited branching, indicates that the open regions exposed by cleavage of the side-chains must have been of limited average length. The profiles of the release of xylose, xylobiose, and xylotriose are typical of an endo-mechanism of action of xylanase II. However, a xylotriose/xylobiose ratio of ≥1 is usually observed early (<4 h) in the degradation of more linear xylans[37,41] which again sustains the above assumption. The xylanase tends to produce xylobiose because of the restricted exposed domains available for its action.

Component I (R_{Xyl} 0.43, trace) was unknown, whereas components II (R_{Xyl} 0.32) and III (R_{Xyl} 0.19), which accumulated in the medium, migrated at the same rate as authentic 2-(4-*O*-methyl-α-D-glucuronosyl)-β-D-xylotriose and -β-D-

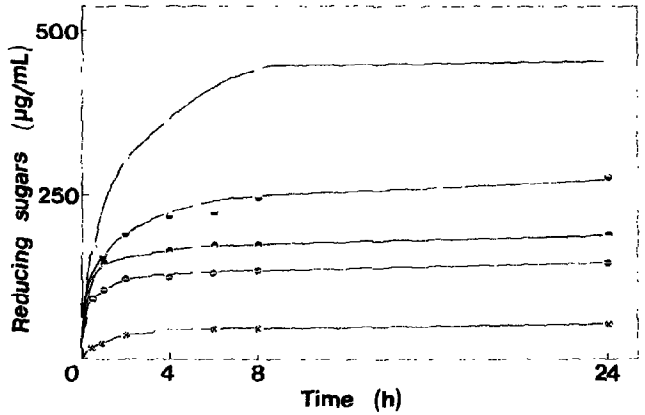

Fig. 6. Hydrolysis of native (—⊗—) and partially degraded xylans [1 h- (—◒—), 2 h- (—◒—), 4 h- (—◒—), and 6 h-treated (—○—)] by endo-xylanase II from *Polyporus tulipiferae*. Reducing sugars were measured by the Nelson procedure.

xylotetraose, respectively. The arabinoxylo-oligosaccharides AX2 (R_{Xyl} 0.90) and AX3 (R_{Xyl} 0.69) were observed only as traces during the hydrolysis which supports the previous assertion that single arabinosyl side-chains are attached to positions 2 and 3 of xylosyl residues. Another indication supporting the above hypothesis was obtained by submitting xylanase-hydrolysates of 4- and 6-h-treated xylans to the action of arabinofuranosidase. Although no comparative kinetic studies were car-

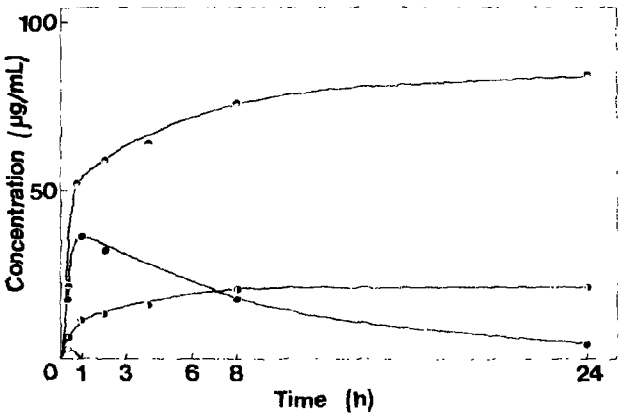

Fig. 7. Profiles of xylo-oligosaccharides produced by action of endo-xylanase II from *Polyporus tulipiferae* on (6 h-treated) heteroxylan: xylose, —◑—; xylobiose, —◒—; xylotriose, —●—; xylotetraose, —○—. Xylo-oligosaccharides were analysed, after borohydride reduction, by h.p.l.c. on RSil C18 (see Experimental).

ried out with this enzyme[9], like most arabinosidases[42], it is much more active on oligosaccharides or synthetic glycosides containing terminal α-L-arabinofuranoside linkages than on polysaccharides which could also explain the low yield in arabinose previously observed. In both experiments, only traces of arabinose (R_{Xyl} 0.89) appeared even after hydrolysis for 60 h, in contrast to hydrolysates from xylans bearing single arabinosyl branches[37] which yield massive amounts of arabinose on treatment with arabinosidase.

A more unexpected result was the liberation of rhamnose (R_{Xyl} 1.20) by the arabinosidase from xylanase-produced dextrins. Arabinofuranosidase has been checked for absence of α-L-rhamnosidase (using naringin as the substrate) but not for possible contamination with β-rhamnosidase. Although this last eventuality cannot be excluded, another possibility would be that the arabinosidase liberated arabinose from a minor undetected *O*-rhamnosyl-α-L-arabinofuranoside, the presence of which in the xylanase hydrolysate of partially degraded xylans remains unclear.

EXPERIMENTAL

General. — Polysaccharides (~5 mg) were hydrolysed with 2M trifluoroacetic acid[43] (2 mL) for 1.25 h at 120°. Each hydrolysate was concentrated to dryness under reduced pressure (40°), and the sugars liberated were converted into their alditol acetates[44,45] and then analysed by g.l.c. (using inositol as the standard) on a glass column (180 × 0.2 cm) packed with 3% SP 2340 coated on 100–120 mesh Supelcoport at 225°. Neutral sugars were also determined by the orcinol method, using xylose for calibration[46], and reducing sugars were measured by the Nelson[35] procedure. Arabinose was measured by the NAD^+/galactose dehydrogenase system[31].

T.l.c. was performed on silica gel (Schleicher and Schüll, F 1500), using 1-propanol–ethyl acetate–ethanol–pyridine–acetic acid–water (7:3:3:2:2:1) and detection with 0.5% thymol in sulfuric acid–ethanol (5:95) at 90° for 10 min.

Endo-xylanase II (specific activity, 1206 nkat.mg^{-1}) was obtained from *Polyporus tulipiferae*[8] and α-L-arabinofuranosidase (specific activity, 426 nkat.mg^{-1}) was purified from a culture supernatant of *Dichomitus squalens*[9]. β-D-Galactose dehydrogenase (5 U.mg^{-1}) from *Pseudomonas fluorescens* was obtained from Boehringer.

Plant material. — Wheat (Capitole variety) was milled and screened[1] to give whole wheat bran (<0.5 mm). Wheat grains were also soaked in distilled water, split in a Waring Blendor, and separated by hand to provide beeswing bran[5] (outer pericarp).

Isolation of wheat-bran heteroxylan. — Heteroxylan was isolated from both outer pericarp (beeswing bran) and total wheat bran by an enzymic and chemical fractionation procedure[1] including de-starching by amyloglucosidase, delignification by sodium chlorite, and an extraction of hemicelluloses by M sodium hydroxide

containing 1% of sodium borohydride. The wheat-bran heteroxylan was obtained as material still soluble after addition, to an aqueous 1% solution of hemicellulose B, of ethanol to 65% final concentration[7].

Partial acid hydrolysis of the native heteroxylan. — Eight solutions, each containing the native xylan (4 mg) in 2.5mM sulfuric acid (4 mL; pH 2.44), were kept at 100° for various times up to 24 h. Liberated arabinose and reducing power were then determined. Each hydrolysate was dialysed against distilled water, then concentrated, and analysed for total neutral sugars (orcinol), acidic sugars (*m*-phenylphenol), and mono- and oligo-saccharides (t.l.c.). Substantial amounts of partially degraded xylans (up to 6-h hydrolysis) were prepared by the same procedure starting from samples (50 mg) of native heteroxylan, and they were further used for methylation and n.m.r. investigations.

Determination of uronic acids. — Uronic acids were determined colorimetrically by the *m*-phenylphenol technique[13], using D-glucuronic acid as the standard. The native heteroxylan was also reduced as follows[10]. A solution of xylan (10 mg) in distilled water (10 mL) was adjusted to pH 4.75 using 0.01M sodium hydroxide, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulfonate (CMC, 42 mg) was added. The pH was adjusted continuously to 4.75 by automatic delivery of 2mM HCl and the reaction was allowed to proceed for 2.5 h. Reduction was then effected by the progressive addition during 45 min of sodium borodeuteride (1 g) whilst maintaining the pH at 7.0 by dropwise addition of 2M HCl (1-octanol as anti-foaming agent). After 1 h of reduction, the solution was dialysed extensively against distilled water, the carboxyl-reduced xylan was reduced again under the same conditions and then hydrolysed, and the products were converted into the alditol acetates and analysed by g.l.c. D-Glucose arising from glucuronic acid was also determined enzymically by the D-glucose oxidase–peroxidase–ABTS system[47]. The reduced heteroxylan was also methylated as described above. The native xylan was methylated twice by the Hakomori procedure for β-elimination of uronic acid groups[16].

Methylation analysis. — Native and partially degraded heteroxylans were methylated once by the Hakomori[14] method and twice with methyl iodide and silver oxide[15]. The product was hydrolysed with aqueous 90% formic acid (1 h, 100°) and then 0.25M sulfuric acid (12 h, 100°)[48], and the partially methylated sugars were converted into the alditol acetates and analysed by g.l.c. on glass columns[49] (280 × 0.2 cm) packed with *A*, 3% OV-225 on 100–120 mesh Gas Chrom Q; *B*, 3% SP 2340 on 100–120 mesh Supelcoport; and *C*, 3% ECNSS-M on 100–120 mesh Gas Chrom Q. For g.l.c.–m.s., the 3% OV-225 glass column was coupled to a Varian mass spectrometer[5]. Authentic partially methylated alditol acetates (PMAA) from methylated crude sugar-beet arabinan (Koch–Light, 52867), gum arabic (Sigma G-9752), and oat-spelt arabinoglucuronoxylan (Sigma, X-0376) were also used for identification.

Periodate oxidation. — To an aqueous solution (5 mL) of the native heteroxylan (10 mg) was added 15mM sodium periodate (5 mL), and the mixture was

stirred in the dark at ambient temperature. Consumption of periodate was monitored[50] at 225 nm. After completion of the reaction (48 h), ethylene glycol (0.2 mL) was added and the mixture was dialysed. The composition of the oxidised xylan was determined after acid hydrolysis as described above.

Action of arabinofuranosidase and xylanase II on native and degraded heteroxylans. — Solutions (400 μL) of native and partially degraded xylans (1% in 0.1M acetate buffer, pH 5.0, containing 0.02% of sodium azide) were each treated with 2 μL of a solution of α-L-arabinofuranosidase (0.4 nkat) and incubated at 40°. Samples (50 μL) were periodically withdrawn and analysed for liberated arabinose as described above.

Samples (2 mL) of native and degraded xylans (0.4% in 0.1M acetate buffer, pH 5.0, containing 0.02% of sodium azide) were incubated (40°) with 5 μL of endoxylanase (6 nkat). Aliquots (200 μL) were heated for 10 min at ~100° and analysed for reducing and total sugars and for oligosaccharides by t.l.c. and h.p.l.c.

H.p.l.c. — This was performed on a column (300 × 7.8 mm) packed with Aminex HPX 87P (Pb^{2+}) (Bio-Rad) at 85°. Elution was with degassed distilled water (0.6 mL/min) and detection was achieved by an ERMA ERC-7510 differential refractometer (flow path cuvette at 50°). Mono- and oligo-saccharides (d.p. ≤5) were analysed in the same way. Oligosaccharides produced by action of xylanase II on the degraded heteroxylan (treated for 6 h) were analysed, after reduction with sodium borohydride[51], on a column (250 × 4 cm) packed with RSil C18 HL (Alltech Associates, Inc.).

^{13}C-N.m.r. spectroscopy. — The spectra were recorded from solutions in D_2O/H_2O at 80° with complete proton decoupling using a Bruker WP 100 spectrometer (25.2 MHz). Chemical shifts were measured relative to that of internal acetone and converted into values relative to Me_4Si: $\delta(Me_4Si) = \delta(\text{acetone}) + 31.07$.

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