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ISOLATION AND ANALYSIS OF CELL WALL MATERIAL FROM BEESWING WHEAT BRAN (*TRITICUM AESTIVUM*)

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Key Word Index—*Triticum aestivum*; wheat; beeswing bran; cell wall material; glucuronic acid and 4-*O*-Me-glucuronic acid; ferulic acid; arabinoxylans; methylation analysis.

Abstract—Cell wall material (CWM) isolated from beeswing wheat bran contains 66% carbohydrate, 12% Klason lignin, 6% protein and 4% ash. The relative proportions of sugars in the CWM are arabinose 34%, xylose 26%, galactose 2%, glucose 32% and uronic acid 6%. The uronic acid was shown to consist of glucuronic acid and its 4-*O*-Me analogue in the ratio 1.8:1. Partial acid hydrolysis of the CWM yielded neutral sugars and a uronic acid fraction. The latter was shown to contain Glc p A-(1→2)-Xyl p and Glc p A-(1→2)-*O*-Xyl p-(1→4)-Xyl p and their 4-*O*-Me substituted uronic acid analogues. Methylation analysis of the whole CWM and partially degraded methylated CWM revealed the nature of the constituent glycosidic linkages. From the combined evidence we infer that the major structural features of the non-cellulosic polysaccharides are a linear chain of xylopyranose units joined by (1→4)-linkages, and arabinofuranose, xylose, galactose (and uronic acid) end groups, which in at least some of the polysaccharides, are attached directly by (1→2)- and/or (1→3)-linkages to the xylan chain. The CWM has been fractionated by successive extractions with water at 80°, 0.2 M $(NH_4)_2C_2O_4$ at 80°, Na chlorite/HOAc at 70°, 0.2 M $(NH_4)_2C_2O_4$ at 80°, 1 M and 4 M KOH, and the neutral sugar composition of the fractions determined. It is concluded from these and other experiments that the CWM contains two main types of polysaccharides, the arabinoxylans and cellulosic polymers, and that phenolic ester linkages play a role in holding them together.

INTRODUCTION

There has been much recent interest in the physiological effects of dietary fibre in man [1, 2]. Cereal fibre (e.g. wheat bran) which is rich in arabinoxylans has been shown to increase faecal bulk and reduce transit time [3]. Two explanations have been offered to explain this effect. In one, it is suggested that the bran is microbially degraded to short chain fatty acids which promote catharsis [4]. In the other, the water-binding properties of bran (or its microbially degraded counterpart) is suggested to account for the increase in faecal bulk [3].

As a preliminary to more detailed work on the mechanism of faecal bulking, it is necessary to have a reasonably well-defined cell wall preparation from bran. The work involved isolation of cell wall material (CWM) from beeswing bran, which consists mainly of the outer coating of the wheat grain, followed by analysis of its composition and determination of the overall structural features of the cell wall polysaccharides. Fractionation of the CWM with aqueous inorganic solvents yielded information on the nature of the constituent polysaccharides. The results of these investigations are reported in this paper.

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RESULTS

Preliminary experiments with whole bran had shown that the carbohydrate composition of its CWM was similar to that of beeswing bran. Beeswing bran was chosen for further study as it could easily be prepared free from endospermic contamination. The CWM was prepared by sequentially extracting the wet ball-milled tissue with 1% aq. Na deoxycholate, PhOH–HOAc–H_2O (2:1:1, w/v/v) and 90% aq. DMSO. All the extractants solubilized a small proportion of cell wall polysaccharides, but as the combined weight of these was <3% of the total CWM, they were not looked at further. 100 g (dry) tissue gave 64 g (dry) CWM. The CWM was shown to be free of starch by its negative reaction with I_2–KI.

Analysis of unfractionated CWM

The bulk of the dry matter of the CWM consisted of polysaccharides 66%, lignin 12%, protein 6%, ash 4% and unidentified material 12%. The latter could include 'residual water', sugars lost during hydrolysis or not measured due to complete hydrolysis, and lignin not estimated by the Tappi procedure. The CWM was hydrolysed by two procedures, (1) 1 M H_2SO_4

Table 1. Chemical composition of beeswing bran CWM

Component	H_2SO_4 hydrolysis*	Saeman hydrolysis*	Component†	
			Ala	8.6
Ara	192	216	Gly	14.6
Xyl	140	168	Val	5.5
Man	—	t	Thr	5.6
Gal	7	12	Ser	8.7
Glc	10	204	Leu	7.2
			I Leu	4.2
Uronic acid‡		40.0	Pro	7.3
			Hyp	t
			Asp	11.6
			Phe	4.9
			Glu	10.5
			Lys	4.2
			Tyr	3.7
			Arg	3.6
			His	t

*Values given as μg sugar/mg dry CWM.
†Amino acid values are given as mol/100 mol.
‡Uronic acid was estimated by the colorimetric method and calculated as μg glucuronic acid/mg dry CWM.
t = trace.

hydrolysis which will hydrolyse the bulk of the non-cellulosic polysaccharides and a small proportion (~5–10%) of the cellulose component, and (2) Saeman hydrolysis which in addition will hydrolyse the cellulose completely. An estimate of the uronic acid content of the CWM was obtained using the modified carbazole method. The monosaccharide and amino acid composition of the CWM are shown in Table 1. The amounts of major phenolic acids in the CWM were ferulic acid 4.8 and *p*-coumaric acid 0.14 mg/g dry wt.

Chemical fractionation of CWM

To obtain an indication of the types of polymers constituting the cell wall complex, the CWM was fractionated with hot water, hot aq. $(NH_4)_2C_2O_4$, acidified Na chlorite, hot aq. $(NH_4)_2C_2O_4$, 1 M and 4 M KOH containing 10 mM $NaBH_4$ to leave a residue of α-cellulose. The yield of the fractions and their carbohydrate composition are given in Table 2. The fractionation studies show that the CWM consists predominantly of pentosans (arabinoxylans) the greater part of which are not water-extractable, but alkali-soluble. The presence of smaller quantities of alkali-soluble hexosans can be inferred. To check whether the delignification step improved the yield of alkali-soluble polysaccharides, the oxalate extracted CWM was extracted directly with aq. alkali. These results which are also included in Table 2 show that there was no significant improvement in yield.

The 1 M KOH-soluble polysaccharide(s) gave single diffuse peaks on ultracentrifugation and on chromatography on Bio Gel P100 column. The material could however be resolved into three fractions by adsorption onto cellulose followed by sequential elution with water, urea and alkali. The results which are summarized in Table 3 show that the basis of this fractionation is probably the degree of substitution of the xylan backbone. The arabinoxylans with a higher degree of substitution appear not to have a high affinity for cellulose.

Since the fractionation procedure gave several fractions which have comparable composition, it was decided to investigate the structural features of the whole CWM directly.

Isolation and characterization of acidic oligosaccharides from CWM

The CWM was subjected to partial acid hydrolysis and the acidic oligosaccharides produced were separated from the neutral sugars by anion exchange chromatography. The acidic material was treated with methanolic-HCl to form methyl ester methylglycoside. The product was dissolved in tetrahydrofuran and reduced with $LiAlD_4$. The resulting neutral material was hydrolysed and the sugars released determined as their alditol acetates by GC–MS. Three peaks were present in the chromatogram corresponding to xylitol pentaacetate, 1,2,3,5,6-penta-*O*-acetyl-4-*O*-Me glucitol and glucitol hexaacetate in the ratio 5:1:1.8, respectively. The MS of the peaks showed the incorporation of two D atoms at C-6 in both glucitol derivatives showing that the glucitol was derived from glucuronic acid. In addition, the MS confirmed the position of substitution of the *O*-Me group at C-4 in one of the glucitol derivatives showing that it was derived from 4-*O*-Me glucuronic acid. The results also

Table 2. Products of fractionation of CWM of beeswing bran

Fractions	Yield (mg/g dry CWM)	Monosaccharide composition (μg/mg dry fraction)				
		Ara	Xyl	Man	Gal	Glc
Hot water—soluble	2	456	102	t	21	15
Oxalate—soluble	6	258	204	—	39	90
Chlorite-HOAc—soluble	10	309	190	—	t	t
Hot water—soluble	10	251	225	—	t	t
Oxalate—soluble	10	280	220	—	t	t
1 M KOH—soluble	440	260	230	—	12	16
4 M KOH—soluble	50	68	82	—	13	39
α-Cellulose	263	73	53	—	—	657
Oxalate-extracted CWM						
1 M KOH—soluble	370	280	270	—	15.0	8.7
1 M KOH—insoluble	610	92.5	80	—	t	320

Table 3. Binding of arabinoxylans onto cellulose

Solvent used for elution	Yield (%)	Monosaccharide composition (expressed as mol/100 mol)			
		Ara	Xyl	Gal	Glc
Water	15	60	37	3	t
7 M urea	15	47	51	2	t
1 M KOH	5	14	85	1	t

Experimental details are given in the text.

showed that xylose is present in the acidic oligosaccharides.

The following experiments confirmed the above data and threw further light on the acidic oligosaccharides. The oligosaccharides were reduced with $NaBD_4$, methylated with CD_3I and the resulting methylated oligosaccharide alditol methyl esters were examined by GC on OV-1. Two main peaks were present and these were identified by MS using the principles outlined by Kärkkäinen [5, 6] and Kováčik *et al.* [7]. The following data were used, (a) *RR*, with reference to methylated cellobiitol, (b) the presence of diagnostic fragment ions in the MS, and (c) the sugar composition of the oligosaccharide mixture. In the following, only the pertinent ions in the MS of the peaks are given followed by their (%) relative intensities within brackets.

Peak 1 (max *RR*, 1.20). This component eluted in the methylated disaccharide alditol methyl ester region. The origins of some pertinent fragments are shown in Fig. 1. The component gave ions at *m/e* 48 (49), 88 (10.5), 95 (31.9), 107, i.e. 101+6 (100), 142 (10.8), 172 (2.5), 175 (13.8), 204 (64.6), 207 (25.4), 210 (26.7), 242 (2.4), 245 (6.3), 370 (0.2) and 417 (0.2). The M^+ ion was not detected in the MS. An indirect way of calculating MW is on the basis of the ions *m/e* 417(M^+-48) and 370(M^+-95) formed through primary cleavage of the C–C bonds in the alditol moiety. Alternatively the MW could be calculated from the *m/e* values of the fragments aA_1 and the alditol moiety: $M = aA_1 + 204 + 16 = 465$. The alditol moiety afforded ions at *m/e* 48, 95, 142 and 204. From these results and the virtual absence of the ion at *m/e* 189, the occurrence of a 2-linked pentitol moiety in the molecule could be inferred.

Ions of the A series are formed through cleavage of the glycosidic bond between the uronic acid and the alditol moiety to give rise to the ion aA_1 at *m/e* 245 (or 242), which, after elimination of CD_3OH, afford ions aA_2 and aA_3 at *m/e* 210 (or 207) and 175 (or 172). The ions at 242, 207 and 172 show the presence of -OMe group in the uronic acid moiety. The abundance ratio $aA_2/aA_1 = 26.7/6.3 \gg 1$, indicates that a glucuronic and not a galacturonic acid is involved. From these results and those of the preceding section, the structure of the compound in this peak could be inferred to be 1,3,4,5-tetra-*O*-Me-*O*-(methyl 2,3,4-tri-*O*-Me glucopyranosyluronate)-xylitol. The parent compounds are therefore Glc p A-(1→2)-Xyl p and its 4-*O*-Me substituted uronic acid analogue. These compounds have been isolated from acid hydrolysates of wheat bran hemicellulose by Adams and Bishop [8].

Peak 2 (max *RR*, 2.85). This component eluted in the methylated trisaccharide alditol methyl ester region. The origins of some pertinent fragments are shown in Fig. 2. The component gave ions at *m/e* 48 (34.6), 49 (16.3), 88 (13.5), 95 (14.8), 96 (16.4), 107, i.e. 101+6 (66.2), 104 (8.1), 101 (3.8), 142 (3.4), 143 (9.9), 172 (2.1), 175 (18.8), 204 (100.0), 207 (30.0), 210 (95.2), 242 (2.6), 245 (7.7), 300 (0.2), 335 (0.5), 341 (2.1), 370 (2.6), 376 (11.5), 411 (0.2), 433 (1.6); and 582(0.2). This MS also did not show a M^+ peak. Disintegration of the M^+ proceeded in rings a and b and in the alditol c. The ions shown in the structure afforded useful information on the structural features of the molecule. The presence of the ion $cbaA_1$ at *m/e* 582 (M^+-49) enabled the MW to be determined. As the abundance of the ion was small, the MW was calculated from the *m/e* values of the A_1 fragments: $M = aA_1 + bcA_1 + 16 = 631$. From the MW, as well as from the *m/e* values of the ions $cbaA_1$ and $abcJ_1$, the

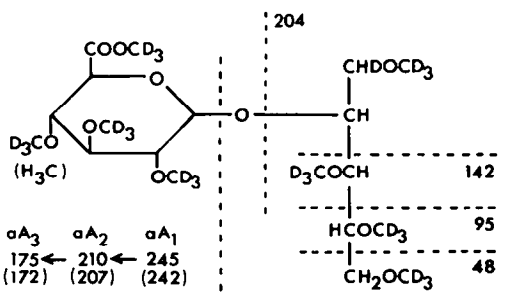


Fig. 1

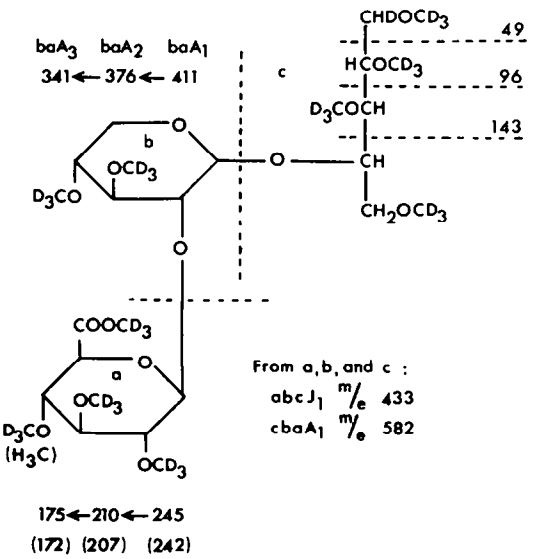


Fig. 2

number of hexose and pentose units in the trimer was determined. The ions baA_{1-3}, bcA_{1-3} and $abcJ_1$, are the most prominent in the mass range *m/e* 300 to 450. The pentitol moiety afforded a M^+ at *m/e* 204 and ions at *m/e* 48, 95 and 143; the ion at *m/e* 189 is virtually absent. From these results, the occurrence of a 4-linked pentitol was inferred.

The characteristic for (1→3)- and (1→2)-linkages in an ab position is the predominance of baA ions over bcA ions. A distinguishing feature for (1→2)-linked compounds is the greatly increased intensity of the baA_2 ion. The baA_2 ion at *m/e* 376 suggests that a→b linkage is 1→2. The a→b linkage can also be inferred from the structure of the compound in peak 1. From the above set of data, the structure of the compound in peak 2 could be inferred to be: *O*-(methyl 2,3,4-tri-*O*-methyl-glucopyranosyluronate)-(1→2)-*O*-(3,4-di-*O*-methyl xylopyranosyl)-(1→4)-1,2,3,5-tetra-*O*-methylxylitol. The parent compounds are therefore, Glc p A-(1→2)-*O*-Xyl p-(1→4)-Xyl p and its 4-*O*-methyl substituted uronic acid analogue.

Methylation analysis of unfractionated CWM

The CWM was methylated using modifications of the Hakomori procedure [9] and the products separated into $CHCl_3$–MeOH-soluble and -insoluble fractions. The insoluble residue contained lignin-like material and had some protein associated with it. Since the insoluble residue contained some methylated arabinoxylans, it was subjected to a second methylation and the product re-extracted with $CHCl_3$–MeOH. The methylated polysaccharides present in the combined $CHCl_3$–MeOH-soluble fractions (80%) and insoluble residue (20%) were hydrolysed and the partially methylated sugars in the hydrolysates determined as their alditol acetates by GC–MS. The results are summarized in Table 4. The methylation conditions appear to methylate 'completely' all the non-cellulosic polysaccharides in the $CHCl_3$–MeOH-soluble and -insoluble fractions and a large proportion of the cellulose. This can be inferred from the fact that there is good agreement between the non-reducing end groups (represented by tri- and tetra-*O*-methyl ethers of pentoses and hexoses, respectively) and branch points (as determined by the amount of mono-*O*-methyl ethers of pentoses and xylitol ×2). It is unlikely that the xylitol (or xylose) arises merely from incomplete methylation, since it was observed in *ca* the same concentration in triplicate experiments and is necessary to account for the end groups. It would appear that the methylated highly branched arabinoxylans in the $CHCl_3$–MeOH-insoluble residue are closely associated with or linked to lignin and/or wall proteins. The methylated cellulose appears to be completely soluble in $CHCl_3$–MeOH and the presence of small amounts of di-*O*-methyl glucose derivatives indicate some undermethylation of the cellulose. Similar observations have been made with cellulose of CWM of runner beans [10].

The non-cellulosic polysaccharides are highly branched arabinoxylans. The occurrence of 2- (and 3-) mono-*O*-methyl xylose derivatives indicate branching through O-3 (and O-2), whereas the presence of xylose shows that some residues are branched through both O-2 and O-3. The branch points are terminated by arabinose, xylose and galactose non-reducing end groups; arabinose in the furanose form, xylose and galactose in the pyranose form. The following experiments threw further light on the nature of the end groups. Advantage was taken of the fact that only the arabinose residues are involved in furanosidic linkages and are therefore far more susceptible to acid hydrolysis. The methylated polysaccharides in the $CHCl_3$–MeOH-soluble fraction were subjected to mild acid hydrolysis, and the product reduced with $LiAlD_4$ and remethylated using CD_3I. Any free -OH groups generated by mild hydrolysis would now be labelled with a deuterated methyl group. The methylated polysaccharides were hydrolysed and the products examined as their alditol acetates by GC–MS. The results are shown in Table 5. The results indicate that controlled

Table 4. Alditol acetates obtained from methylated CWM of beeswing bran

Alditolacetate	*RR*,*	Relative amounts† $CHCl_3$–MeOH-soluble	$CHCl_3$–MeOH-insoluble
2,3,5-Tri-*O*-methylarabinitol	0.46	14.6	32
3,5-Di-*O*-methylarabinitol	0.83	1.9	4.3
2,5-Di-*O*-methylarabinitol	0.91	3.6	7.3
2,3-Di-*O*-methylarabinitol	1.07	0.5	1.1
5-Mono-*O*-methylarabinitol	1.34	4.3	9.1
Arabinitol	2.32	t	2.3
2,3,4-Tri-*O*-methylxylitol	0.6	4.8	8.7
2,3-Di-*O*-methylxylitol	1.2	11.3	12.0
2- and 3-Mono-*O*-methylxylitol	2.14	7.6	11.0
Xylitol	3.47	5.7	12.0
2,3,4,6-Tetra-*O*-methylgalactitol	1.20	1.0	
2,3,6-Tri-*O*-methylmannitol	2.03	1.0	
2,3,6-Tri-*O*-methylglucitol	2.32	37.7	
2,3-Di-*O*-methylglucitol	3.65	4.6	
3,6-Di-*O*-methylglucitol	4.35	1.0	

*Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on OV-225 at 180°.
†Data expressed as relative mol %.

Table 5. Composition of the partially degraded permethylated cell wall polysaccharides*

Alditol acetate	RR_t	Relative amounts	Pertinent fragment ions†
2,3,4-Tri-*O*-methylxylitol	0.6	4.0	171 (13), 118 (17), 120 (13), 121 (7), 161 (3), 162 (3.2), 164 (1), 165 (1.3), 167 (0.8)
2,3-Di-*O*-Methylxylitol	1.2	14.0	118 (15), 121 (20), 129 (11), 132 (22), 189 (2), 192 (5)
3-Mono-*O*-methylxylitol	2.15	2.0	189 (2), 190 (2), 192 (2), 193 (2)
2,3,4,6-Tetra-*O*-methylglucitol	1.0	13.0	118 (20), 132 (28), 162 (9), 164 (8), 208 (5)
2,3,6-Tri-*O*-methylglucitol	2.32	67.0	118 (30), 162 (3), 173 (3), 233 (10)

*The methylated polysaccharides in the $CHCl_3$–MeOH-soluble fraction were subjected to mild acid hydrolysis, reduced with $LiAlD_4$ and remethylated with CD_3I.

†Pertinent fragment ions in the MS are followed by their (%) relative intensities within brackets.

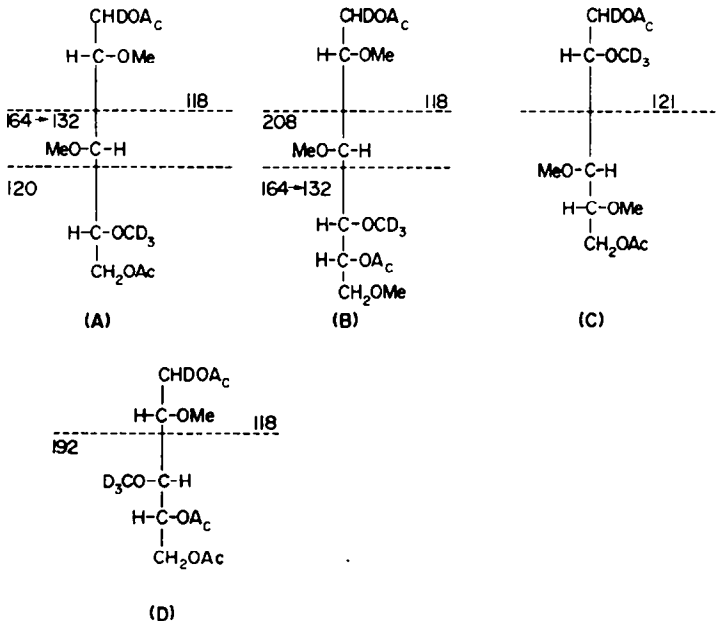
hydrolysis resulted in selective cleavage of the arabinofuranoside linkages and some destruction of the xylan and cellulose backbones as well. The latter can be inferred from the incorporation of $-CD_3$ group into O-4 of 2,3,4-tri-*O*-methyl xylose (**A**) and 2,3,4,6-tetra-*O*-methyl glucose (**B**) derivatives. The incorporation of $-CD_3$ into O-2 and O-3 of 2,3,4-tri-*O*-methyl (**C**) and 2,3-di-*O*-methyl xylose (**D**) indicates the point of attachment of the arabinofuranoside (and other?) side chains. The main distinguishing ions are shown in the structures **A**, **B**, **C** and **D**.

From the combined evidence we infer that the major structural features of the non-cellulosic polysaccharides are a linear chain of xylopyranose units joined by (1→4)-linkages, and arabinofuranose, xylose, galactose (and uronic acid) end groups, which, in at least some of the polysaccharides, are attached directly by (1→2)- and/or (1→3)-linkages to the xylan chain.

DISCUSSION

The composition of the CWM of beeswing bran is similar to that of wheat [11, 12] and barley [13] endosperm cell walls. The small amount of protein present can be regarded as a wall component since the intracellular proteins would have been solubilized by SDC and PAW. The wall protein is rich in glycine, aspartic and glutamic acids, but contains little or no hydroxyproline. In this respect it is similar to the cell wall protein from the endosperm of wheat [14], barley [13, 15] and oats [16] and is in contrast to results obtained with cell wall proteins of most higher plant tissues which are usually rich in hydroxyproline [17].

There are two main types of polysaccharides present in the bran CWM, the arabinoxylans and the cellulosic polymers. The arabinoxylans are based on a linear (1→4)-linked xylan backbone which is highly substituted at positions C-2 and/or C-3. Some of the xylose



residues are quadruply substituted; other workers have reported the occurrence of similar xylose residues in arabinoxylans from cereal cell walls [18–20]. The most abundant side chain is a single unit arabinofuranosyl residue which can be linked to either position. Glucuronic acid and its 4-*O*-methyl substituted analogue are present as single unit side chains linked to C-2. The presence of terminal xylose and galactose and 3- and 2-linked arabinose indicates the presence of longer side chains similar to those described by Wilkie and Woo [21]. Work is being done to clarify the structure of the arabinoxylans.

The structural features of the arabinoxylans are in general agreement with those previously reported for bran [22, 23] and they are similar to the arabinoxylans found in barley [24, 25], rye [26] and suspension-cultured monocotyledonous cell walls [27]. The arabinoxylans once isolated from the CWM are water-soluble. On ultracentrifugation they give a single diffuse peak—hypersharp peaks indicative of aggregation are absent [28]. Removal of the arabinose side chains by mild acid hydrolysis leads to the formation of linear degraded xylans which are insoluble in water. These results can be explained on the basis that the removal of the side chains permits closer association of the residual xylan chains resulting in less soluble aggregates.

The degree of substitution of the main xylan chain also affects the strength of the association between arabinoxylans and cellulose. Arabinoxylans with a high degree of association were eluted from a cellulose column with water, but with decreasing substitution the strength of the association increases; urea and finally alkali were needed to elute the arabinoxylans with low degrees of substitution.

Since the arabinoxylans do not interact strongly either with themselves or with cellulose, it is likely that the bonds holding the arabinoxylans in the cell wall complex are mainly covalent. Delignification of the CWM does not significantly increase the yield of the alkali-extractable material. The covalent bonds holding the arabinoxylans in the cell wall must therefore be alkali-labile [29], suggesting the presence of ester linkages. The presence of phenolic acid ester linkages in cell walls of monocotyledonous plants has been suggested [30]. Ferulic acid has been found in wheat flour pentosans [31] and we have found appreciable amounts of ferulic and coumaric acids in the whole and hot water-extracted CWM of bran. The phenolic acid ester hypothesis is also supported by the fact that the alkali extract is dark brown and is decolourized to a pale yellow solution on acidification. Further, Morrison has shown that a mild treatment of a grass cell wall preparation with Na methoxide allows a portion of the arabinoxylan to become extractable with water [29]. Thus the present results would suggest that a more detailed examination of the wall will be necessary before possible carbohydrate–lignin (or carbohydrate–protein) linkages can be identified. It should be noted that certain glucans [32] and arabinogalactans [33] are linked to proteins and it is possible that some of the wall polysaccharides are associated with proteins.

In the solid state, a polysaccharide matrix held together by covalent linkages with minimum H-bonding will have a large capacity to H-bond with water through free -OH groups [34]. The CWM of bran appears to be such a complex. The structure and solubility characteristics of the bran polysaccharides may explain the water-binding capacity of the CWM and that of the whole tissue. Thus our studies are relevant in understanding the physiological role of dietary fibre.

EXPERIMENTAL

Chemicals. $LiAlD_4$, $NaBD_4$ and CD_3I were purchased from Fluka, DMSO, tetrahydrofuran, NaH and $NaBH_4$ were obtained from BDH. DMSO was vacuum-distilled over CaH_2 and stored over molecular sieve 3A. THF was distilled over $LiAlH_4$ and stored over argon.

Plant material. Wheat grain (cv Bouquet) was bought locally. The grain was thoroughly washed, suspended in H_2O and gently blended in a Waring blender for 3 min. The mixture was allowed to settle and the beeswing bran was collected by filtering the supernatant through a 3 mm sieve. This method of preparation did not cause any appreciable disruption of cellular structure.

General methods. Evapns were carried out at 40° or less. Dialysis was performed with continual stirring against dist. H_2O; toluene was added to inhibit microbial growth. GC was carried out as described in ref. [9] using columns (2.8 m × 2.2 mm) containing JJ's diatomite CQ coated with (a) 3% OV-225, (b) 3% ECNSS-M and (c) 3% OV-1. Columns a and b were used for the separation of alditol acetates and column c for the separation of methylated oligosaccharide alditol Me esters. RR_t of partially methylated alditol acetates are given relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-Me glucitol and those of the methylated oligosaccharide alditol Me esters relative to fully methylated cellobiitol. For GC–MS the columns were attached to a medium resolution spectrometer operated at an inlet temp. of 250°, ionization potential 70 eV and ion source temp. 200°. Data processing was used with continuous scanning at 10 sec/decade.

Preparation of CWM. CWM of beeswing bran was prepared by sequential extraction of the fresh wet ball-milled tissue with 1% aq. Na deoxycholate (SDC), PhOH–HOAc–H_2O (2:1:1, w/v/v) and 90% aq. DMSO [9, 35]. The material solubilized by the solvents used include low MW intracellular compounds, cytoplasmic proteins, a small proportion of starch and some non-starch polysaccharides which are rich in arabinose and xylose. Macromolecules present in the extracts were isolated after dialysis by precipitation with EtOH. From 1 g (dry) beeswing bran, SDC, PAW and aq. DMSO solubilized 10, 1 and 1.2 mg non-starch polysaccharides, respectively.

Fractionation of CWM using aq. inorganic solvents. CWM (2 g) was extracted with 100 ml H_2O at 80° for 2 hr and the mixture was filtered through a sintered glass funnel and the residue washed with 20 ml warm H_2O. The filtrate was freeze-dried and the residue extracted with 200 ml of 1% (w/v) aq. $(NH_4)C_2O_4$ at 80° for 2 hr and filtered as before. The polymers in the filtrate were isolated after dialysis by freeze-drying. The residue remaining after oxalate extraction was delignified with acidified Na chlorite soln at 70° for 1 hr. The resulting holocellulose was re-extracted with hot aq. $(NH_4)_2C_2O_4$ and then with 1 M and 4 M KOH containing 10 mM $NaBH_4$ in an argon atmosphere at 20° for 2 hr each, to leave a residue of α-cellulose. The alkaline extracts were acidified to pH 5 and the polymers isolated as before. To see the effect of the delignification procedure on the subsequent extraction of the CWM with alkali, the oxalate extracted

CWM was extracted directly with 1 M and 4 M KOH and the polymers isolated as before.

Examination of 1 M KOH-*soluble material.* A 2% (w/v) soln of 1 M KOH-soluble material was examined in a Beckman Model E analytical ultracentrifuge at 25° and 48 000 rpm. The Schlieren photographs showed a single diffuse peak.

Biogel chromatography. The 1 M KOH-soluble material (20 mg) was chromatographed on a Bio Gel P100 column (2.5 × 100 cm; flow rate 17 ml/hr) using H_2O as eluant. The fractions (5 ml) were collected and the carbohydrate analysed by the PhOH–H_2SO_4 method [36]. The polysaccharide(s) eluted as a single asymmetrical peak.

Binding to cellulose. The 1 M KOH-soluble arabinoxylan (10 mg) was dissolved in H_2O (1 ml) and then freeze-dried on 1 g of cellulose powder CFl (Whatman). The powder was then dispersed in H_2O, made into a column (1 × 6 cm) and successively eluted with 6 bed vol. of H_2O, 7 M urea and 1 M KOH. The polymers in the eluates were purified by dialysis and isolated as freeze-dried solids.

Partial acid hydrolysis of CWM and isolation of acidic fragments. CWM (500 mg) was hydrolysed with 0.2 N TFA (50 ml) for 2 hr at 100°. The hydrolysate was filtered and TFA removed by co-distillation with H_2O. The dry residue was dissolved in H_2O (10 ml), adjusted to pH 8 and applied to a column (1 × 7 cm) of Bio-Rad AG1 × 2 resin (acetate form; 200–400 mesh) and any neutral material not adsorbed eliminated by washing with 10 bed vol. of H_2O. The column was then eluted with 10 bed vol. of 1 M HOAc and the eluate evapd to dryness under red. pres. to yield 30 mg solid. A portion (5 mg) of this material was subjected to sugar analysis. Another portion was methanolysed, reduced and then subjected to sugar analysis as below. Sample (5 mg) was dried overnight *in vacuo* and then treated with 2.5% methanolic HCl (2 ml) and the tube flushed with argon, sealed and left overnight at 80°. The reaction mixture was cooled and Ag acetate (0.25 g) was added to the product, left for 3 min, and filtered. The residue was washed with MeOH (1 ml) and the combined filtrates evapd to dryness and dissolved in THF (5 ml). $LiAlD_4$ (30 mg) was added to the product and the mixture refluxed for 4 hr. Excess reducing agent was destroyed by adding in turn a few drops of EtOAc, EtOH and H_2O and the soln neutralized with 2 M H_3PO_4. The mixture was filtered, evapd to dryness and then subjected to sugar analysis. The resulting alditol acetates were examined by GC–MS. A third portion (10 mg) was reduced with $NaBD_4$(10 mg) and then subjected to Hakomori methylation using CD_3I. The resulting oligosaccharide alditol Me esters were separated by GC on a 3% OV-1 column, which was temp. programmed 220 to 320° at 2°/min, and their identity was established by MS.

Methylation analysis. Methylation of the CWM was carried out as previously described [9, 37]. Methylation of oligomeric samples was performed as described in ref. [38]. The sugar components of the methylated polysaccharides were analysed as their partially methylated alditol acetates by GC–MS [38].

Partial acid hydrolysis of methylated polysaccharides. The methylated polysaccharides from the whole CWM (10 mg) were hydrolysed with 90% HCO_2H (5 ml) for 45 min at 70°. The acid was removed by co-distillation with H_2O and the dry residue treated with THF (5 ml) and the mixture refluxed for 4 hr after the addition of $LiAlD_4$ (20 mg). Excess $LiAlD_4$ was removed as described above. The product was remethylated with CD_3I and the sugar components analysed as before.

Sugar analysis. Neutral sugars were released from the CWM using 1 M H_2SO_4 and Saeman hydrolysis [39] for 2.5 hr, and the liberated sugars analysed as their alditol acetates by GC [39]. Uronic acid content was estimated by the modified carbazole method and the values were corrected for interference from neutral sugars [39].

Amino acid analysis. Amino acids were released from the CWM using 6 M HCl at 110° for 24 hr in a sealed tube. Corrections for losses over this period were not made. Liberated amino acids were analysed as their heptafluorobutyric *n*-propyl derivatives by GC [40].

Phenolic acids were estimated by GC as described by Hartley [41].

Lignin was determined by the Tappi modification of the Klason procedure [42].

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