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CARBOHYDRATES AND CARBOHYDRATE ESTERS OF FERULIC ACID RELEASED FROM CELL WALLS OF *LOLIUM MULTIFLORUM* BY TREATMENT WITH CELLULOLYTIC ENZYMES*

ROY D. HARTLEY and EDWIN C. JONES
Grassland Research Institute, Hurley, Berks, England

and

THOMAS M. WOOD
Rowett Research Institute, Bucksburn, Aberdeen, Scotland

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Abstract—41% of the cell walls from mature leaf blades of *Lolium multiflorum* were digested by treatment during 14 days with C_1 enzyme (cellulase) which had been purified by gel filtration and ion-exchange chromatography. Cellobiose was the main sugar released from the walls, together with some glucose and higher oligosaccharides. Considerable amounts of carbohydrate esters of ferulic and *p*-coumaric acids were also released. When the C_1 enzyme was further purified by isoelectric focusing, only 8% of the cell walls were digested. Purified C_x (CM-cellulase) containing β-glucosidase digested 51% of the cell walls in 16 hours: the major component detected in the soluble products was glucose together with some β (1→4)-xylobiose, xylose and arabinose. Higher oligosaccharides and carbohydrate esters of ferulic and *p*-coumaric acids were also present. It was shown that these acids were present in the cell walls mainly in the *trans*-configuration.

INTRODUCTION

Earlier work [1, 2] showed that H_2O-soluble carbohydrate esters of ferulic acid were released from grass cell walls by treatment with C_x (CM-cellulase). Esters were also released by treatment with C_1 (see [3] for definition) but the possibility could not be excluded that other enzymes associated with the C_1 caused this release. Study of the action of C_1 on cotton cellulose has shown that it is a cellobiohydrolase which acts synergistically with the random-acting C_x component [4]. In the present work, comparisons have been made between sugars produced by treatment of grass cell walls with different cellulolytic enzymes and the results related to release of carbohydrate esters of ferulic and *p*-coumaric acids.

RESULTS AND DISCUSSION

Sugars in filtrates obtained by treatment of grass cell walls with C_1 and/or C_x enzymes or with a crude cellulase preparation, were identified by TLC, GLC of the alditol acetate derivatives and, in the case of cellobiose and β (1→4)-xylobiose, also by GLC of their trimethylsilyl (TMSi) derivatives; further evidence for the presence of xylobiose was that, on hydrolysis, it gave only xylose. UV absorption measurements on the filtrates and estimation of the mono- and di-saccharides present are given in Table 1: in a separate experiment no reaction could be detected between glucose, arabinose, xylose, *trans*-*p*-coumaric acid and *trans*-ferulic acid in the presence of cellulolytic enzymes.

When a high concentration of C_x (containing β-glucosidase but no C_1) was used, most of the cell walls were digested. The extent of this digestion contrasts with that observed with C_x action on untreated native cotton fibre [5]. Clearly, the presence of C_1 is not required for extensive degradation of grass cell wall material which has been subjected, during preparation, to grinding and mild chemical treatment.

The soluble products resulting from C_x action on cell wall material contained glucose as the major component, but when only small amounts of C_x were used glucose accounted for a much lower proportion of the products due apparently to the presence of higher oligosaccharides. Cellobiose was absent but considerable quantities of xylobiose were present with smaller amounts of arabinose and xylose. A low MW C_x [2,5] (*ex Trichoderma koningii*, 1000 units) gave the same result as C_x while a crude commercial cellulase (*ex* Basidiomycete, 1000 units of C_x) showed higher activity than C_x. The same sugars were present in the filtrate from the digestion but a much higher proportion of xylobiose was present. This xylobiose was completely hydrolysed to xylose by a culture filtrate of *T. koningii*.

C_1 which had been purified by gel filtration and ion exchange chromatography on DEAE-Sephadex, but not by isoelectric focusing, showed considerable capacity for digesting grass cell walls after incubation for 14 days (see Table 1) compared with its capacity for digesting cellulose substrates (maximum of 7% of cotton cellulose

* Part 3 in the series *Lignin-carbohydrate linkages in plant cell walls*. For Part 2 Ref. [2].

Table 1. UV Absorption and GLC estimation of carbohydrates in filtrates from treatment of grass cell walls with partially purified enzymes and with a crude enzyme mixture

Enzyme and amount	Cell wall digested (%)	A of filtrate at λ_{max} 324 nm (%)*	Carbohydrates (% of cell wall) in filtrate†					
			Glc	Cbi	Ara	Xyl	Xbi	Total
C_x (5 units)	9	21	0·8	0	<0·1	0·1	0·3	1·2
C_x (1000 units)	51	80	24·4	0	3·1	3·2	8·0	38·7
Cellulase‡ (1000 units C_x)	68	95	34·8	0	2·8	2·2	15·7	55·5
C_1 (0·18 mg protein)§	41	24	5·3	20·8	Tr	Tr	0	26·1
C_1 (0·18 mg protein)	5	3	ND	ND	ND	ND	ND	ND
C_1 (0·18 mg protein) + C_x (5 units)	23	29	3·7	12·5	0·1	0·2	0·6	17·1
C_1 purified by isoelectric focusing (0·18 mg protein)§	8	1	ND	ND	ND	ND	ND	ND

Tr = trace; ND = not determined. For conditions of incubation with enzymes see text: incubation time 16 hr (except § 14 days). Unless otherwise stated, C_1 was not purified by isoelectric focusing. * Calc. as % of maximum value of absorbance (A) obtained with crude cellulase (*ex* Basidiomycete, 2000 units) [1]. † GLC conditions in text: Glc—glucose; Cbi—cellobiose; Ara—arabinose; Xyl—xylose; Xbi—β (1 → 4)-xylobiose. ‡ Crude cellulase (*ex* Basidiomycete).

digested in 28 days) [4]. The main reaction product, cellobiose (*ca* 50% of the soluble material), was the same as from cotton but the amount of glucose was much higher (*ca* 13% of the soluble material). TLC examination showed that the other components of the soluble products consisted of several higher oligosaccharides, some of which were esters of ferulic acid: two of the components of the soluble products had the same R_f values as cellotriose and cellotetraose. In contrast to C_x treatment of cell walls, C_1 did not produce xylobiose from xylan chains. Hydrolysis of the soluble products with C_x (containing β-glucosidase) gave glucose, and with acid, glucose with small amounts of xylose and arabinose suggesting that the higher oligosaccharides were mainly cello-oligosaccharides.

It was shown earlier [2] that synergism occurs between C_1 and C_x when grass cell walls are digested. The present work using C_1 and C_x showed that the products of digestion were qualitatively similar to those obtained using C_1 alone except that xylobiose was also obtained.

When C_1 was further purified by isoelectric focusing, only a small amount of the cell walls were digested even after 14 days incubation (Table 1). TLC examination of the filtrate indicated that cellobiose was the major component; small amounts of glucose and cellotetraose were also present.

The carbohydrate esters of phenolic acids in filtrates from C_x treatment of cell walls were saponified and the phenols shown to be mainly *trans*-ferulic acid with small amounts of *trans*-*p*-coumaric acid and traces of other unidentified phenols. Traces of the corresponding *cis* compounds of ferulic and *p*-coumaric acids were also present. Precautions were taken to prevent isomerisation from *trans* to *cis* forms as this can be rapid under certain conditions [6]. For example we have found [7] that methanolic solutions of *trans*-ferulic acid or *trans*-*p*-coumaric acid are rapidly converted to mixtures of their *cis* and *trans* isomers in daylight in the laboratory.

The location and function of the carbohydrate esters of phenolic acids in cell walls are under investigation. Earlier work [1] had shown that the carbohydrate portion of one of the esters contained glucose, xylose and arabinose units. The esters could be attached to various cell wall constituents. Firstly, they might arise from xyloglucans [8] but the soluble products released from grass cell walls using C_x gave no colour reaction with iodine [9] for xyloglucans either before or after separation by TLC (cellulose plates, Solvent 4). Secondly, the esters could form part of xylan chains and arise by C_x acting as a β (1 → 4) hydrolase or by hemicellulase contaminants being present in C_x. A commercial hemicellulase released only traces of the esters; these traces could have been produced by the presence of cellulase impurity known to be present in the enzyme preparation. Thirdly, the esters could be attached to cellulose chains and are released by cellulolytic action. The function of the phenolic acid components of the esters could be to act as intermediates in the process of lignification.

EXPERIMENTAL

Plant material. Mature leaf blades of Italian ryegrass (*Lolium multiflorum* L.), cv. RVP, were employed and cell walls separated as previously described [1].

Methods. Sources of culture filtrate (*ex Trichoderma koningii*) and enzymes, determination of enzyme concn and methods of incubation were the same as those described earlier [2]. Hemicellulase (*ex* fungal sources) was obtained from Koch-Light (activity *ca* 5000 units/g). The purified cellulolytic enzymes were from *T. koningii*: C_x contained β-glucosidase as impurity (10·2 units to 100 units C_x) [2]. Further purification of C_1 by isoelectric focusing was carried out using ampholyte covering the range pH 3–6 [4]. Incubation of cell walls with cellulolytic enzymes was carried out at 37° using 30 mg batches of walls with 0·2 M NaOH–HOAc buffer, pH 4·8, containing 0·02% NaN_3. Incubations (72 hr) with hemicellulase (100 mg) were by the same method.

Incubations (16 hr) of mixtures of glucose (Glc), xylose (Xyl), arabinose (Ara), *trans*-*p*-coumaric acid (*trans*-PCA) and *trans*-ferulic acid (*trans*-FA), using 10 mg of each component, were carried out with cellulolytic enzymes and the mixtures subjected to TLC.

Identifications of sugars in plant extracts by TLC and GLC were confirmed by co-chromatography with references.

TLC of plant extracts. Schleicher and Schüll plates were employed with the following: (1) for neutral sugars, Solvent 1, *n*-BuOH–HOAc–H_2O (62:15:23) and Solvent 2, aq. phenol (*ca* 90%)–H_2O (89:11) + 0·002% oxine [10]. Aniline phthalate and *p*-anisidine phthalate were used as spray reagents [11]. R_f in Solvent 1 (cellulose plates, F1440) of Glc was 0·22, cellobiose (Cbi) 0·10, cellotriose (Ctri) 0·02, cellotetraose (Ctet) 0·01, galactose (Gal) 0·20, Ara 0·24, Xyl 0·27, and xylobiose (Xbi) 0·15; in solvent 1 (Sil gel plates, F1500) Glc 0·35, Cbi 0·22, Ctri 0·12, Ctet 0·07, Gal 0·32, Ara 0·40, Xyl 0·46 and Xbi 0·25; in Solvent 2 (F1440) Glc 0·28, Cbi 0·21, Ctri 0·09, Ctet

0·01, Gal 0·36, Ara 0·45, Xyl 0·38 and Xbi 0·35. (2) for uronic acids Solvent 3, Me_2CO–$CHCl_3$–MeOH–H_2O (75:10:10:5) [12] and Solvent 4, EtOAc–pyridine–H_2O (60:30:20) [13]. R_f in Solvent 3 (F1500) of glucuronic was 0·02, galacturonic 0·02 and in Solvent 4 (F1440) glucuronic 0·09, galacturonic 0·07. (3) for phenolic acids, Solvent 5, PhMe–HCOOH–H_2O (40:45:15, upper) and Solvent 6, HCOOH–H_2O (4:96). R_f in Solvent 5 (F1440) of *cis* and *trans*-PCA was 0·07 and *cis* and *trans*-FA 0·26 and in Solvent 6 (F1440) *cis*-PCA 0·74, *trans*-PCA 0·26, *cis*-FA 0·60 and *trans*-FA 0·20. Diazotised sulphanilamide was used as spray reagent [14].

Identification of glucose and cellobiose from treatment of cell walls with C_1. A sample of the filtrate from reaction with C_1 was treated with Zerolit 325 (H form) to remove Na^+ and soln dried in vac. over NaOH. TLC indicated that the residue contained mainly Cbi with some Glc. A sample of the residue (1 mg) was treated with Tri-Sil (0·25 ml) and the mixture heated at 37° for 16 hr with occasional shaking. TMSi derivatives were examined by GLC using an FID and 1·50 m × 4 mm glass column containing 3% SE-30 on Diatomite 'CQ' with Ar at 80 ml/min. The column was programmed at 4°/min from 160–195° then at 48°/min to 230° (detector temp. 240°), R_t (min) Glc 8·5 and 10·5 and Cbi 31 and 40·5. A sample of the filtrate (2 ml) before removal of Na^+ was used to prepare the alditol acetate derivatives [1]. The derivatives were separated by a similar method to that used for the TMSi derivatives except that the column packing contained 1% SE-30 and the gas flow rate was 70 ml/min; the column was maintained at 180° for 6 min then programmed at 48°/min from 180–240° (detector temp. 250°), R_t (min) Glc 3·0, Cbi 20·0.

Identification of β (1 → 4)-xylobiose from treatment of cell walls with C_x or crude cellulase (ex *Basidiomycete*). Suspected Xbi was isolated by column chromatography using Ag1-X8 (formate form) [15]. A sample (1 mg) was incubated at 37° for 16 hr using culture filtrate (*ex T. koningii*, 200 units of C_x); reference Xbi was treated similarly. Na^+ was removed by Zerolit and the solns examined for sugars by TLC; in each case only Xyl was present. In a second expt, a further sample of suspected Xbi (1 mg) was treated with N HCl (1 ml) at 80° for 8 hr and the acid removed by evaporation in vac. over NaOH; reference Xbi was treated similarly. Again TLC showed that the only sugar in the residues was Xyl. TMSi derivatives of suspected and reference Xbi were prepared and examined as above; the GLC column was maintained at 210° (detector temp. 220°), R_t (min) Xbi 26 and 28. RR_t (sucrose = unity) were 0·87 and 0·94 identical with the reference β (1 → 4) linked compound but different from the β (1 → 3) linked xylobiose [16]. The alditol acetate derivative of both reference Xbi and suspected Xbi were prepared and chromatographed by the method used for Cbi. The R_t of reference and suspected Xbi was 12·0 min.

Estimation of sugars by GLC. The method of monosaccharide analysis using their alditol acetate derivatives has been described previously [1]. Cbi and Xbi were estimated by conversion to their alditol acetate derivatives and chromatographed as described above.

Acid hydrolysis of H_2O-soluble compounds from treatment of cell walls with C_1. H_2SO_4 (2N, 1 ml) was added to the soln of H_2O-soluble compounds (1 ml) then hydrolysed for 4 hr at 100° and examined by TLC.

Saponification of H_2O-soluble compounds from treatment of cell walls with C_x. All manipulations of solns were carried out in "white" fluorescent light to prevent *cis-trans* isomerisation of FA and PCA and their derivatives [7]. NaOH soln (10 N, 0·2 ml) was added with shaking to the soln of H_2O-soluble compounds (2 ml) and left for 45 min at 20° until saponification of esters containing PCA and FA was complete [1]. The soln was deionised with Zerolit, concentrated by freeze-drying and examined for *cis* and *trans*-FA and -PCA by GLC [7] and TLC.

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