Phytochemistry, 1973, Vol. 12, pp. 661–665. Pergamon Press. Printed in England.

CARBOHYDRATE ESTERS OF FERULIC ACID AS COMPONENTS OF CELL-WALLS OF *LOLIUM MULTIFLORUM**

R. D. HARTLEY

Grassland Research Institute, Hurley, Berks.

(*Received* 29 *September* 1972. *Accepted* 12 *October* 1972)

Key Word Index—*Lolium multiflorum*; Gramineae; cell-walls; cellulolysis; lignin-carbohydrate linkages; ferulic acid.

Abstract—Carbohydrate esters of ferulic acid were released from grass cell-walls by cellulase action as a mixture of at least four H_2O-soluble compounds in which the carboxylic group of the acid was bound to carbohydrate by ester bonds. The MW of these esters varied from a few hundred to over 50 000. Hydrolysis of one of the carbohydrate moieties showed that it contained xylose, arabinose and glucose units. The linkage of the carbohydrate esters of ferulic acid to other cell-wall constituents is discussed.

INTRODUCTION

SEVERAL workers[1–3] have reported the release of *p*-coumaric acid (PCA), ferulic acid (FA) and other phenolic acids from plant cell-walls by treatment with NaOH solution. In earlier work[4] it was shown that PCA, FA and small amounts of vanillin (V) were released from the cell-walls of leaf blades and sheaths of ryegrass by this treatment. Some or all of the PCA and FA released might be linkage units between the lignin 'core' and the structural carbohydrates. Such lignin-carbohydrate linkages could be responsible for the well-established decrease in the digestibility of the structural carbohydrates of cell-walls which occurs with increasing lignin content.[5] In the present work we have investigated the action of a commercial cellulase preparation on grass cell-walls and examined the H_2O-soluble phenolic compounds obtained.

RESULTS

The relationships between weight of cellulase taken, 'cell-wall' digested and absorbance (A) of the filtrate at λ_{max} 290 and 324 nm are shown in Table 1. Maximum A at λ_{max} 324 nm was obtained using approx. 2000 units of C_x (CM–cellulase) activity.

There were correlations between weight of cellulase employed and % digested ($r = 0{\cdot}68$, $p < 0{\cdot}01$) and between % digested and A of the filtrate at λ_{max} 290 nm ($r = 0{\cdot}99$, $p < 0{\cdot}001$) or at λ_{max} 324 nm ($r = 0{\cdot}99$, $p < 0{\cdot}001$). Using similar conditions of cellulolytic degradation, UV spectra of filtrates from 'purified' cell-walls and from an 'acetone powder' were compared with the cell-wall material. Cell-walls and purified cell-walls gave similar UV-spectra; 'acetone powder' gave a shoulder at 321 nm. The corresponding values of A at 324 nm of the filtrates were 1·41 for cell-walls, 1·59 for purified cell-walls and 1·28 for

* Part I in the projected series "Lignin-carbohydrate linkages in plant cell-walls".

[1] S. A. BROWN, *Ann. Rev. Plant Physiol.* **17**, 223 (1966).
[2] T. HIGUCHI, Y. ITO and I. KAWAMURA, *Phytochem.* **6**, 875 (1967).
[3] T. HIGUCHI, Y. ITO, M. SHIMADA and I. KAWAMURA, *Phytochem.* **6**, 1551 (1967).
[4] R. D. HARTLEY, *J. Sci. Food Agric* **23**, 1347 (1972).
[5] P. J. VAN SOEST, *J. Anim. Sci.* **23**, 838 (1964).

acetone powder (based on the same weight of cell-walls). Treatment of cell-walls with pronase, Cleland's reagent or 2 M NaCl failed to liberate compounds showing absorption between 260 and 400 nm.

TABLE 1. RELATIONSHIPS BETWEEN AMOUNT OF CELLULASE, CELL-WALL DIGESTIBILITY AND UV ABSORPTION OF THE FILTRATE

Cellulase (units)*	Cell-wall digested (%)	A† at λ_{max} 290 nm	324 nm	Cellulase (units)*	Cell-wall digested (%)	A† at λ_{max} 290 nm	324 nm
2·2	9·6	0·13	0·18	518	63·1	0·86	1·15
7·4	11·7	0·21	0·29	740	66·6	1·00	1·28
22·2	17·5	0·33	0·44	1110	68·5	1·06	1·34
74·0	27·5	0·48	0·63	1480	69·4	1·06	1·34
148	41·5	0·61	0·80	1850	70·3	1·07	1·36
259	54·4	0·73	0·95	2590	70·8	1·08	1·41
370	60·6	0·83	1·08	3700	71·6	1·11	1·41

Time of incubation 16 hr. Temp. of incubation 37°.
* 370 units C_x/mg cellulase.
† Absorbance of filtrate diluted to 20 ml (1 cm cell).
The relationship $Y = 23{\cdot}04 - 47{\cdot}78X + 40{\cdot}93X^2 - 6{\cdot}67X^3$ was fitted for Y = cell-wall digested (%) and $X = \log_{10}$ cellulase, also $Y = 12{\cdot}58 - 52{\cdot}09X + 247{\cdot}50X^2 - 138{\cdot}91X^3$ for Y as above and X = A at 290 nm and $Y = 12{\cdot}62 - 41{\cdot}41X + 146{\cdot}84X^2 - 62{\cdot}78X^3$ for Y as above and X = A at 324 nm.

The UV spectrum of Solution 1, which was obtained by reaction of grass cell-walls with cellulase, was compared with that of FA, both before and after treatment with NaOH (see Table 2). The compounds in Solution 1 of λ_{max} 324 nm could not be removed by Et_2O extraction but after reaction with N NaOH and acidification, the compounds of λ_{max} 316 nm were completely soluble in Et_2O. The Et_2O-soluble material was identified as FA by TLC (Solvents 1 and 2) and GLC; only traces of PCA could be detected. The weight of FA/g of cell-wall, determined by UV absorption as sodium ferulate at λ_{max} 324 nm, was 8·3 mg. By comparison, direct N NaOH treatment of cell-walls gave 8·1 mg FA + 1·0 mg PCA/g of cell-wall.

TABLE 2. SPECTRAL PROPERTIES OF SOLUTION 1 OBTAINED BY REACTION OF CELLULASE WITH GRASS CELL-WALLS

	λ_{max} (nm) in H_2O	N NaOH (10 min)	N NaOH (6 or 24 hr)	N NaOH (24 hr) acidified with HCl
Solution 1	290	298	300	288
	324	359	342	316
Ferulic acid	—	306*	308*	290
	—	348	340	316

Reaction times with NaOH shown in parenthesis. Scanned between 260 and 400 nm. * Shoulder.

TLC of Solution 1 using Solvent 1, gave 4 spots (R_f zero, 0·14, 0·30 and 0·43) which fluoresced in UV light. The fluorescence was enhanced by exposure to NH_3. Only the 4 spots gave positive phenolic tests (violet colorations). The spots of R_f zero and 0·43 gave

positive tests for pentose (maroon coloration with *p*-anisidine phthalate reagent); the reaction of the spots of R_f 0·14 and 0·30 could not be determined by this method owing to the presence of other interfering sugars. The above reactions indicated that the compounds in Solution 1 having λ_{max} 324 nm were carbohydrate esters of FA.[6,7]

Samples of Solution 1 were separated by Sephadex gel chromatography: with G10, 33·6% of the absorption at 324 nm was eluted at the void volume (i.e. $K_{av} = 0$) compared with 21·3% for G15, 19·2% for G25, 5·5% for G50 and 5·6% for G75. These separations indicated that the carbohydrate esters of FA had MWs varying from a few hundred to over 50 000.

Substance A from separation of Solution 1 on Sephadex G25 had λ_{max} 290 and 315 nm, K_{av} 1·76. This high K_{av} value was probably due to gel absorption effects brought about by the presence of a phenolic OH group in Substance A. A sample of Substance A was subjected to TLC (Solvent 1). A single spot (R_f 0·43) was obtained which fluoresced in UV light and which gave a violet coloration with the phenolic reagent and a maroon coloration with the reducing sugar reagent. After N NaOH treatment, the reaction product, after removal of Na^+, gave two spots on TLC (Solvent 1), one of which was identified as FA (R_f 0·90). The second spot (R_f 0·20) gave a positive pentose test (maroon coloration with reducing sugar reagent). When Substance A was hydrolysed with NaOH followed by HCl, TLC (Solvents 1 and 3) and GLC indicated that the pentosan of R_f 0·20 (Solvent 1) was composed of xylose, arabinose and glucose units. The molar ratio of these 3 compounds in the acid hydrolysate was 2·1 : 1·0 : 1·4.

DISCUSSION

As purification of the cell-wall fraction did not cause a loss in yield of carbohydrate esters of FA obtained by cellulase treatment, it appears that the esters are chemically bound to the cell-walls and are not contaminants derived from cell contents. This conclusion is consistent with the finding that the digestibility of the cell-walls was correlated with release of the esters. The possibility that the esters are artifacts formed during separation of the cell-wall fraction appears unlikely, as an acetone powder which was prepared by a mild technique gave a similar yield of the esters to that obtained with cell-walls.

The maximum yield of FA liberated from the esters by NaOH treatment was similar to that obtained by treating cell-walls directly with alkali. Only traces of the PCA which was bound in the cell-walls, and which could be liberated by direct NaOH treatment, could be detected after NaOH treatment of filtrates obtained by cellulase action on the walls. By similar techniques, neither sinapic nor caffeic acid, which are closely related to FA, could be detected in the NaOH-treated filtrates.

Release of the esters of FA and of traces of PCA units from cell-walls by cellulase treatment was consistent with earlier work[4] which showed that FA units in similar cell-wall material reacted more rapidly with Na_2SO_3 solutions than the PCA units and that the FA units were lost more rapidly during passage through the alimentary tract of sheep. Hence the FA units in the cell-walls which can be liberated by NaOH are apparently more 'accessible' than the corresponding PCA units.

Although hydrolysis of Substance A, which was a carbohydrate ester of FA, indicated that its carbohydrate moiety consisted of xylose, arabinose and glucose units, the possibility

[6] J. B. Harborne and J. J. Corner, *Biochem. J.* **81**, 242 (1961).
[7] V. C. Runeckles and K. Woolrich, *Phytochem.* **2**, 1 (1963).

can not be excluded that carbohydrate contaminants were present with Substance A leading to the production, on hydrolysis, of monosaccharide units.

The possibility was investigated of the carbohydrate esters of FA being bound to protein in cell-walls. Pronase, Cleland's reagent and 2 M NaCl have been employed[8,9] for the rupture of protein linkages or removal of proteins from cell-walls, but with our cell-wall material none of these reagents caused release of bound or free FA. Hence it seems unlikely that the esters are bound to proteins unless they are linked also with carbohydrates.

There is evidence to suggest that these esters could be lignin-carbohydrate linkage units. In earlier work[4] it was shown that oxidation of the lignin 'core' material left after N NaOH treatment of a similar cell-wall fraction gave rise to PCA, FA and V, the latter being an oxidation product of FA. Hence the FA esters obtained by cellulase action have the same 1-phenylpropene unit found in the corresponding lignin core material. Other workers[10] have recently shown that lignin-carbohydrate complexes of MW of about 150 000 can be obtained by solvent extraction of grass cell-walls. These complexes contained lignin, phenolic monomers, and carbohydrates containing glucose, xylose and arabinose units; nitrogen was absent. It was thought that the phenolic monomers in the complexes were linked to carbohydrates by ester linkages. It seems likely that the carbohydrate esters of FA obtained in our work by cellulase treatment form part of this phenolic monomer-carbohydrate fraction present in the lignin-carbohydrate complexes.

EXPERIMENTAL

Source of enzymes. Cellulase (*ex* Basidiomycete) was obtained from E. Merck, Darmstadt, and found to contain 370 units C_x/mg by the method of Wood.[11] Pronase (*ex Streptomyces griseus, ca.* 45 000 PUK units/g) was supplied by Koch-Light.

Plant material. Mature leaf blades of Italian ryegrass (*Lolium multiflorum* L.), cv. RVP, were employed. Growth conditions, harvesting, drying, cell-wall, lignin and Si contents have been given elsewhere.[4]

Separation of cell-walls. This fraction was prepared from the dry grass material by the method of Van Soest and Wine,[12] except that Na_2SO_3 was omitted. The cell-walls were washed successively with hot H_2O, Me_2CO and Et_2O and air-dried. The whole procedure was repeated and the cell-walls dried over silica gel: yield 35·6% (based on the original dried grass). Electron microscopy indicated the presence of some whole cell-walls but only traces of cell contents were visible.

Preparation of 'purified' cell-walls. Cell-walls (50 mg each time) were ground in a glass homogenizer with water, to pass a No. 1 glass sinter. The suspension was frozen to coagulate the cell-walls, melted, and filtered (No. 3 glass sinter). The residue was washed successively with H_2O, Me_2CO and Et_2O (5 ml of each), dried, and submitted to the neutral detergent-solvent extraction procedure as above. The yield of purified cell-walls was 17 mg (34%). This low yield was due to loss of colloidal material during filtrations. Electron microscopy of the purified cell-walls showed that no whole cell-walls were present.

Preparation of 'acetone powder'. Dried ground leaf blades (1·00 g) were soaked in Me_2CO (20 ml) overnight at 20°. The mixture was filtered (No. 1 glass sinter), and the residue washed with Me_2CO (100 ml), followed by Et_2O (150 ml) and dried over silica gel (yield of acetone powder 0·78 g).

Digestion of plant material. Cell-walls (30 mg) were incubated at 37° for 16 hr with cellulase plus 0·2 M NaOH–HOAc buffer (pH 4·8, 2·0 ml) containing 0·02% NaN_3. When 5·0 mg cellulase/30 mg cell-walls were employed, filtration (No. 1 glass sinter) gave Solution 1. For TLC examination, the filtrate was treated with Zeo-Karb 225 resin (H form) to remove Na ions. All UV absorption measurements were corrected for A due to cellulase. Acetone powder (30 mg) was incubated by the same method using 2·5 mg cellulase.

Separation of Solution 1 on Sephadex gels. G10, G15, G25, G50 and G75 gels were employed using the NaOH–HOAc buffer (containing NaN_3) as eluant.

Preparation of Substance A. 10 filtrates were prepared each of which was similar to Solution 1 except that the buffer was replaced by H_2O (pH 5·5) and the incubation time was increased to 7 days. The combined filtrates were concentrated and separated on Sephadex G25 with H_2O as eluant. The separation was monitored

[8] E. W. THOMPSON and R. D. PRESTON, *J. Exptl Bot.* **19**, 690 (1968).
[9] I. RIDGE and D. J. OSBORNE, *J. Exptl Bot.* **21**, 843 (1970).
[10] I. M. MORRISON and R. WAITE, *Hannah Dairy Res. Inst. Rep.* 63 (1968–1971).
[11] T. M. WOOD, *Biochem. J.* **109**, 217 (1968).
[12] P. J. VAN SOEST and R. H. WINE, *J. Ass. Offic. Agric, Chem.* **50**, 50 (1967).

by scanning between 260 and 400 nm. Substance A gave an elution peak at $K_{av} = 1{\cdot}76$; it was purified by repeating the separation on G25.

Hydrolysis of Substance A. Substance A (1 mg) was added to N NaOH (2·0 ml), shaken, and left for 30 min at 20°. A sample of this mixture was treated with Zeo-Karb 225 resin (H form) and used for TLC. A further sample of this mixture was hydrolysed with excess N HCl for 15 hr in a sealed tube at 80°; the product was treated to remove Na^+ and Cl^- (the latter with Dowex 1 X8, acetate form) and examined by TLC and GLC.

TLC of plant extracts. Cellulose plates (Schleicher & Schull, F 1440) were used with the following: Solvent 1, *n*-BuOH–HOAc–H_2O (62:15:23); Solvent 2, $CHCl_3$–HOAc–H_2O (10:9:1)[13]; Solvent 3, aq. phenol (*ca.* 90%)–H_2O (89:11) + 0·002% oxine.[14] Plates were examined in UV light and sprayed with either *p*-anisidine phthalate reagent[15] to detect reducing sugars or with Fast Blue B salt[16] (a phenolic coupling reagent). Reference sugars and phenolic acids were employed and identifications of unknown compounds were confirmed by co-chromatography.

Treatment of cell-walls with N NaOH *and GLC estimation of PCA, FA and V in plant extracts.* These methods have been described earlier.[17]

GLC estimation of reducing sugars. The method of Cheshire[18] was employed which was similar to that of Albersheim *et al.*,[19] except that excess HOAc was removed by evaporation with MeOH followed by Et_2O extraction to remove last traces of the acid before GLC.

Treatment of cell-walls with pronase or dithiothreitol (Cleland's reagent). Cell-walls (10 mg) were incubated for 24 hr at 20° and 37° with pronase (2·5 mg) or dithiothreitol (100 mg) plus 0·05 M Tris-HCl buffer (2·0 ml), containing 0·02 M $CaCl_2$, pH 8·0. The mixtures were filtered (No. 1 glass sinter) and the UV spectra of the filtrates determined.

Treatment of cell-walls with NaCl. Cell-walls (10 mg) were incubated for 16 hr at 37° with 2 M NaCl (2·0 ml). The A of the filtrates were determined as above.

Acknowledgements—The author wishes to thank Messrs. M. A. Chaudhry and E. C. Jones for technical assistance, Dr. P. Jarvis for electron microscopy, Miss H. Buchan for GLC analysis of reducing sugars and Dr. L. H. P. Jones for his interest in the course of this work.

[13] K. Egger, *J. Chromatog.* **5**, 74 (1961).
[14] D. W. Vomhof and T. C. Tucker, *J. Chromatog.* **17**, 300 (1965).
[15] B. A. Lewis and F. Smith, in *Thin-Layer Chromatography* (edited by E. Stahl), 2nd Edn, p. 811, Springer, Berlin (1969).
[16] H. Jatzkewitz and U. Lenz, *Hoppe-Seylers Z. Physiol. Chem.* **305**, 53 (1956).
[17] R. D. Hartley, *J. Chromatog.* **54**, 335 (1971).
[18] M. V. Cheshire, unpublished work.
[19] P. Albersheim, D. J. Nevins, P. D. English and A. Karr, *Carbohyd. Res.* **5**, 340 (1967).