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PHENOLIC COMPONENTS AND DEGRADABILITY OF CELL WALLS OF GRASS AND LEGUME SPECIES*

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Key Word Index—*Lolium multiflorum*; *Lolium perenne*; *Phleum pratense*; Gramineae; *Trifolium pratense*; *Trifolium repens*; *Medicago sativa*; *Onobrychis viciifolia*; Leguminosae; cell walls; lignin-carbohydrate linkages; phenolic acids.

Abstract—Cell walls separated from the aerial parts of *Lolium multiflorum*, *Lolium perenne* and *Phleum pratense* contained bound *cis* and *trans* ferulic and *p*-coumaric acids and diferulic acid which were released from the walls by treatment with sodium hydroxide. The total content of these acids in *L. multiflorum* ranged from 5 to 16.8 mg/g of wall, the *trans*-ferulic acid content varying between 2.8 and 8.9 mg/g of wall. In addition, small amounts of *p*-hydroxybenzoic acid were released from senescent leaf blade plus sheath parts. Cell walls from legume species gave much smaller amounts of the acids, the total content of aerial parts of *Trifolium pratense* being <0.8 mg/g of wall. The degradability of the cell walls with a commercial cellulase preparation was determined and the water-soluble phenolic compounds released were estimated by UV absorption spectroscopy.

INTRODUCTION

Several workers [1–4] have shown that ferulic and *p*-coumaric acids are released from the cell walls of Gramineae by treatment with alkali. It has also been shown that treatment of cell walls of *Lolium multiflorum* with a crude cellulase containing hemicellulase activity, released a series of water-soluble carbohydrate esters of phenolic acids, including ferulic and *p*-coumaric [5, 6]. The phenolic acids were esterified to the cell wall carbohydrates through their carboxyl groups. Both ferulic and *p*-coumaric acids were present mainly in the *trans*-form [7, 8]. Very recently we have reported the release of diferulic acid, mainly the *trans,trans* isomer, from similar cell walls by treatment with NaOH [8]. This acid was also apparently esterified to the cell-wall carbohydrates.

It is well established that, as a plant ages, its lignin content increases and that this is highly correlated with decreasing degradability of the cell wall carbohydrates either in the rumen or with cellulase [9]. The lignin contents of Gramineae and Leguminosae vary between 2–15% of the dry wt. It seems likely that phenolic acids bound to cell walls are lignin precursors [8, 10, 11] and if so are of considerable importance in determining the digestibility of forage plants by ruminants.

The aim of the present work was to identify and estimate the phenolic components released from the cell walls of grass and legume species by treatment with NaOH or cellulase and to measure the degradability of the walls by treatment with cellulase.

RESULTS AND DISCUSSIONS

Treatment of cell walls of *L. multiflorum* with a similar cellulase preparation to that used previously [5] showed that leaf blades, which had the lowest cell wall contents, had the most degradable walls and stem the least degradable (Table 1). The amount of water-soluble carbohydrate esters of phenolic acids released by the cellulase treatment was estimated by measurement of the UV absorption of the filtrate at λ_{max} 324 nm [5, 6]: the highest value was found in the flag leaf blade and the lowest one with stem (Table 1). A second peak occurred at 290 nm. Senescent fractions gave higher ratios of A at 290 to 324 nm, suggesting that their filtrates might contain a higher proportion of esters of phenolic acids having less conjugation than substituted cinnamic acids. acids. Treatment of cell walls from parts of *L. perenne* and *Phleum pratense* showed similar behaviour to those of *L. multiflorum*.

Several of the parts of *Trifolium pratense* had lower cell wall contents than those of the Gramineae examined (Table 1) possibly due, at least in part, to removal of pectin by the method of cell wall separation [12] which involved extraction with a neutral solution containing SDS. In contrast to the *Lolium* species examined, only trace amounts of phenolic esters were detected in the filtrate obtained by cellulase treatment of cell walls of any of the parts of *T. pratense*. *T. repens*, *Medicago sativa* and *Onobrychis viciifolia* behaved similarly to *T. pratense*. These results agreed with those from UV fluorescence microscopy which indicated the presence of bound ferulic acid in cell walls of Gramineae but not in Leguminosae [11].

The phenolic acid components of the water-soluble carbohydrate esters from plant parts of *L. multiflorum*, *L. perenne* or *P. pratense* were released from the esters by treatment with NaOH. TLC indicated that *trans*-ferulic acid was the main component with some *cis*-ferulic, *cis* and *trans*-*p*-coumaric and diferulic acids. Small amounts of other unknown phenols were present:

*Part 5 in the series 'Lignin-carbohydrate linkages in plant cell walls'. For Part 4 see ref. [8].

Table 1. Degradability and phenolic components of the cell walls of *Lolium multiflorum* and *Trifolium pratense*

Plant part	Date of harvest	Yield (% of total dry matter)	Cell walls (% dry matter of plant part)	Cell walls degraded by cellulase (%)	Phenolic components of cell walls: Filtrate from cellulase treatment*		Filtrate from NaOH treatment*	
					A at λ_{max} 324 nm	Ratio of A at λ_{max} 290 nm to 324 nm	A at λ_{max} 331 nm	*Trans*-ferulic acid equivalents† (mg/g cell wall)
Lolium multiflorum								
Flag leaf blade 1	May 10	11.3	40.1	75.9	0.68	0.77	1.10	16.1
2		20.1	33.0	68 7	0.46	0.76	0.87	12 7
3		23.5	30 5	68.0	0 43	0.77	0.84	12 3
4		13.0	34.4	72.0	0.43	0.81	0.75	11.0
Senescent leaf blade + sheath		6 6	50.5	40.6	0.27	0.93	0 80	11.7
Total leaf sheath‡ + immature blade		25.5	38.2	57 5	0.56	0 73	1 25	18 3
Total leaf blade + sheath§	June 5	26.2	45.5	42.8	0.31	0 83	1 00	14 7
Senescent leaf blade + sheath		9 6	61.1	52 9	0.32	1 00	1.48	21.7
Stem		36 9	50.5	20 1	0 23	0.71	0.82	12.0
Ear		27 3	54.8	28.3	0 56	0 76	1.74	25.5
Trifolium pratense								
Total leaf blade	May 16	58.6	24.5	51 7	Trace		Trace	Trace
Total petiole + leaf sheath		25.8	24.2	52 8	Trace	—	Trace	Trace
Stem		15.6	21 1	61.0	Trace	—	Trace	Trace
Total leaf blade	July 2	18 2	24.8	62.9	Trace	—	Trace	Trace
Total petiole + leaf sheath		9 0	42.2	38 8	Trace	—	Trace	Trace
Stem (top)‖		26.6	51 2	26.4	Trace	—	Trace	Trace
Stem (base)‖		30.7	56.8	27.4	Trace	—	Trace	Trace
Total flowers + growing points		15.5	32.9	38.4	Trace		Trace	Trace

Flag leaf blade was the highest expanded leaf blade. Leaf blade 2 was adjacent to flag leaf blade, etc. *Filtrate from treatment of cell walls (30 mg) diluted to 50 ml, 1 cm cells. †Calc. by comparison of A at λ_{max} 331 nm with ϵ of *trans* sodium ferulate at the same λ. ‡Excluding senescent sheath. §Excluding senescent leaf blade + sheath ‖Stem cut at mid-point and tops and bases analysed separately.

sinapic and caffeic acids, which are chemically closely related to ferulic and *p*-coumaric acids, were not detected. The same phenolic acids were released from the cell walls by direct treatment with NaOH. In addition, *p*-hydroxybenzoic acid was released from senescent leaf blade plus sheath parts. Treatment of cell walls of *T. pratense*, *T. repens*, *M. sativa* or *O. viciifolia* with NaOH gave only traces of *trans*-ferulic, *cis*-ferulic and *trans*-*p*-coumaric acids.

Phenolic acids present in cell walls of parts of *L. multiflorum* and *T. pratense* were examined in more detail by quantitative analysis after treatment of the walls with NaOH. The *cis* and *trans* isomers of ferulic and *p*-coumaric acids, together with *p*-hydroxybenzoic acid, were estimated by GLC and the total diferulic acid by TLC. The chromatographic solvent employed separated diferulic acid from the other acids but did not separate the three geometrical isomers of the acid [8].

The total content of phenolic acids of *L. multiflorum* cell walls estimated by chromatography (Table 2) can be compared with the yield of phenolic acids estimated by UV, calculated as *trans*-ferulic acid equivalents (Table 1). The largest difference between these two estimations was found for senescent leaf blade plus sheath, the

Table 2. Amounts (mg/g cell wall) of ferulic, *p*-coumaric, *p*-hydroxybenzoic and diferulic acids released from *Lolium multiflorum* and *Trifolium pratense* cell walls by treatment with sodium hydroxide

Plant part	Date of harvest	Ferulic acid		*p*-Coumaric acid		*p*-Hydroxybenzoic acid	Diferulic acid	Total content of phenolic acids*
		trans-	*cis*-	*trans*-	*cis*-			
Lolium multiflorum								
Flag leaf blade 1	May 10	5.5	1.7	2.7	0.6	n.d.	0.3	10.8
2		5.3	1.4	1.2	0.3	n.d.	0.3	8.5
3		5.3	1.6	0.8	0.2	n.d.	0.3	8.2
4		4.7	1.5	0.4	0.2	n.d.	0.4	7.2
Senescent leaf blade + sheath		3.5	0.9	0.6	0.2	0.2	0.2	5.6
Total leaf sheath† + immature blade		7.6	1.2	4.2	0.4	n.d.	0.3	13.7
Total leaf blade + sheath ‡	June 5	5.3	0.7	2.4	0.3	n.d.	0.3	9.0
Senescent leaf blade + sheath		2.8	0.7	0.9	0.2	0.2	0.1	4.9
Stem		5.2	0.7	4.9	0.4	n.d.	0.2	11.4
Ear		8.9	0.8	5.9	0.8	n.d.	0.3	16.7
Trifolium pratense								
Total leaf blade	May 16	0.5	<0.1	<0.1	<0.1	n.d.	n.d.	>0.5, <0.8
Petiole + leaf sheath		<0.1	<0.1	<0.1	<0.1	n.d.	n.d.	<0.4
Stem		<0.1	<0.1	<0.1	<0.1	n.d.	n.d.	<0.4

n.d. not detected. *Calc. by summation of individual amounts of detected acids. †Excluding senescent sheath. ‡Excluding senescent leaf blade + sheath.

Table 3. Degradability of cell walls of *Lolium multiflorum* and *Trifolium pratense* after treatment with sodium hydroxide

Plant part	Date of harvest	Cell walls degraded by cellulase (%)	Cell walls degraded by NaOH (%)	Cell walls degraded by NaOH followed by cellulase treatment (%)
Lolium multiflorum				
Total leaf blade + sheath*	June 5	42.8	36.4	85.3
Stem		20.1	36.1	71.3
Trifolium pratense				
Total leaf blade	July 2	62.9	30.8	76.3
Stem (base)†		27.4	22.6	47.6

*Excluding senescent leaf blade + sheath. †Stem cut at mid-point and lower half (base) analysed.

values from UV being more than twice those from chromatography. This difference is probably due to the presence of undetected phenolic compounds. Dimers and higher polymers of ferulic and *p*-coumaric acids, together with diferulic acid, might be expected to be present if it is assumed that the monomers are lignin precursor units which are converted into higher polymers by coupling reactions involving peroxide–peroxidase [8, 13, 14]. It is of interest to note that in earlier work [4] senescent blade plus sheath had more than twice the lignin content of the younger blade plus sheath.

Quantitative chromatography of the phenolic acids from cell walls of all parts of *L. multiflorum* showed that the amounts of the *trans* isomer of ferulic or *p*-coumaric acids were greater than those for the corresponding *cis* isomer (Table 2). The younger leaf blades contained more ferulic and *p*-coumaric acids than the older blades. Although all parts contained more ferulic than *p*-coumaric acid, the stem showed the smallest difference.

The amounts of phenolic acids in *T.pratense* cell walls were low compared with *L.multiflorum* (Table 2) as expected from the results of qualitative TLC. Only ferulic and *p*-coumaric acids were detected. The leaf blade contained more ferulic acid than the other parts although the concentration was only about 10% of that in cell walls of leaf blade of *L. multiflorum* (Table 2).

NaOH treatment of graminaceous plants causes a marked increase in their digestibility by ruminants but this treatment has much less effect on *M. sativa* [15, 16] suggesting the possibility that cell walls of the Gramineae and Leguminosae behave differently on alkali treatment. This possibility was examined by treating cell walls of parts of *L. multiflorum* and *T. pratense* with NaOH and measuring their degradability with cellulase. The results, summarised in Table 3, show that the alkali had a greater effect on stem than on leaf blade plus sheath and that the greatest increase in degradability occurred with stem of *L. multiflorum*. The greater effect of NaOH on the degradability of the cell walls of *L. multiflorum* compared with *T. pratense* could be due to the rupture of ester links between phenolic components and polysaccharides in the walls of the former. However, it is possible that other differences on cell wall composition such as polysaccharide constituents, could account for this effect.

EXPERIMENTAL

All manipulations of solns or phenolic acids and their TMSi derivatives were carried out in 'white' fluorescent light to prevent isomerisation [17].

Plant material. Italian ryegrass (*Lolium multiflorum* Lam., cv Tetila Tetrone), perennial ryegrass (*Lolium perenne* L., cv Endura), timothy (*Phleum pratense* L., cv S352), red clover (*Trifolium pratense* L., cv Hungaropoly), lucerne (*Medicago sativa* L., cv Europa) and sainfoin (*Onobrychis viciifolia* Scop., cv Cotswold Common) were harvested from the field in 1973 on two separate occasions during primary growth to provide samples before and after the onset of flowering: white clover (*Trifolium repens* L., cv S100) was harvested only before flowering. Samples before flowering were harvested on May 10 (Gramineae) and May 16 (Leguminosae). Gramineae were also harvested just after ear emergence (*L. multiflorum* June 5, *P. pratense* June 12 and *L. perenne* June 26). *T. pratense*, *M. sativa* and *O. viciifolia* were also harvested after the onset of flowering, on July 2. The freshly-harvested samples were separated into various parts, freeze-dried and ground to a powder. Cell walls were separated as previously reported by extraction with a neutral detergent soln [9].

Treatment of cell walls with a commercial cellulase preparation (Oxyporus *sp.*). This method has been described previously using cell walls (30 mg), cellulase (2.5 mg) and buffer (pH 4.8, 2 ml). The filtrate was diluted for UV absorption spectroscopy [9].

Saponification of the water-soluble compounds from treatment of cell walls with cellulase, followed by TLC of the phenolic acids released. These methods have been described previously [8]. The solvents for TLC were the same as those described below.

Treatment of cell walls with NaOH. (i) TLC. Cell walls (30 mg) were shaken with N NaOH (2 ml) under N_2 (containing <5 ppm O_2) at 20° for 20 hr. The mixture was filtered (No. 1 porosity glass sinter) and the residue washed with H_2O. Phenolic acids in the filtrate were recovered by acidification with 6N HCl to pH 2.5 followed by extraction with Et_2O (3 × 5 ml). The combined Et_2O extracts were dried and the solvent evapd at ambient temp. The residue was dissolved in Me_2CO (200 μl) and qualitative TLC carried out using cellulose plates and HCO_2H–H_2O (1:24) and PhMe–HCO_2H–H_2O (8:9:3, upper phase) solvents as previously described [8]. Diferulic acid was estimated quantitatively by TLC (PhMe–HCO_2H–H_2O solvent) by comparison with reference *trans,trans*-diferulic acid. The reference acid was treated with NaOH, acidified and extracted as described above, before submitting to TLC. (ii) GLC. Cell walls were treated with NaOH as described above and 2,4-dihydroxybenzoic acid added as internal standard to the alkaline filtrate. The residue from Et_2O extraction was silylated by adding *N,O-bis* (trimethylsilyl) trifluoroacetamide (200 μl) plus THF (20 μl) and heating at 37° for 20 hr with occasional shaking. The *cis* and *trans* isomers of ferulic and *p*-coumaric acids were determined as described earlier using 5% OV-25 as stationary phase and a column temp. of 195° [17]. *p*-Hydroxybenzoic acid was determined in a similar manner except that the column

temp. was maintained at 160°: R_t of the TMSi ether was 14 min compared with 33.5 min for the fully silylated TMSi ether of 2,4-dihydroxybenzoic acid used as internal standard (at 195° the TMSi ether of *p*-hydroxybenzoic acid was eluted close to the solvent peak). A calibration graph of peak area vs wt was obtained for the TMSi derivative of each of the acids in the range 1–200 μg of acid: a linear relationship was found in each case. Reference mixtures containing similar amounts of *p*-hydroxybenzoic, and *cis* and *trans* ferulic and *p*-coumaric acids as were present in cell wall extracts, were treated with NaOH, acidified, extracted with Et_2O and chromatographed by the above methods. The amounts of the acids obtained from cell walls were then determined by comparison with these results. (iii) UV absorption spectroscopy. Cell walls were treated with NaOH as described above, filtered, and the filtrate and washings diluted to 50 ml with H_2O.

Sequential treatment of cell walls with NaOH and cellulase. Cell walls (30 mg) were treated with N NaOH (2 ml) followed by treatment of the residue with cellulase (2.5 mg) and buffer (pH 4.8, 2 ml) by the above methods.

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