Phytochemistry, Vol. 28, No. 2, pp. 393–397, 1989.
Printed in Great Britain.

0031-9422/89 $3.00 + 0.00

DEACYLATION AND CLEAVAGE OF CELL WALLS OF PANGOLA GRASS WITH BOROHYDRIDE

CLIVE W. FORD

Division of Tropical Crops and Pastures, CSIRO, Cunningham Laboratory, St Lucia, Queensland 4067, Australia

(*Received in revised form* 3 *August* 1988)

Key Word Index—*Digitaria decumbens*; Gramineae; tropical grass; cell walls; borohydride; deacylation; acetate; *p*-coumaric acid; lignin; hemicellulose; gel filtration.

Abstract—Aqueous borohydride (pH 9) removed up to 97% of acetyl groups from glucose pentaacetate, *myo*-inositol hexaacetate and cellulose acetate in 48 hr. When cellulase-digested pangola grass stem cell walls, which were resistant to further degradation with fresh 'cellulase' (Onozuka P-1500), were stirred with borohydride under the same conditions, 30% of the cell walls were solubilized. The cell wall residue could now be further degraded (36%) with 'cellulase'. The residue retained 45% of the original esterified *trans*-*p*-coumaric acid and 20% of the acetate. The borohydride soluble fraction, which contained *ca* half of the original *p*-coumaric acid still esterified, on neutralization gave a solution containing mainly hemicellulosic carbohydrates and negligible *p*-coumaric acid, and a precipitate consisting of lignin (75%), esterified *p*-coumaric acid (5%) and carbohydrate (20%) comprising mainly pentoses. The neutral soluble material was fractionated by gel filtration to yield polymeric and oligomeric carbohydrate. Substances retarded on the column consisted of aromatic residues spectroscopically similar to lignin and contained a small amount of bound carbohydrate, of which the major components were glycerol, arabinose, xylose, glucose and xylitol. The results indicated that part of the cell walls were bound together by many borohydride-labile linkages, probably aliphatic esters. The variety of structures isolated from the borohydride soluble material, some still containing aromatic and carbohydrate residues bound together, suggests that borohydride may be a useful reagent in studies aimed at elucidation of *in situ* cell wall structures which limit biodegradability.

INTRODUCTION

Graminaceous cell walls consist mainly of polysaccharides which, particularly in mature tropical grasses, are largely resistant to degradation by rumen microflora [1] or fungal cellulase [2]. The low biodegradability is attributable to association between macromolecules, involving polyaromatic lignin, producing structures into which microorganisms or enzymes can only partially penetrate. Other constituents which could contribute to resistance of cell walls to enzyme attack are acyl groups such as acetate, *p*-coumarate and ferulate, which can amount to 5–7% of the material [1]. The latter two phenolic acids have also been identified in grass cell walls combined as dimers [3, 4], which can potentially increase cell wall stability by forming ester cross-linkages between hydroxy groups of macromolecules [4].

The nature of bonding between lignin and cell wall polysaccharides is not yet established. However, studies have been made on isolated lignin fractions in which borohydride was used to differentiate between postulated structures containing carbonyl groups [5, 6]. Although these reports were not in total agreement, the results suggested that there were structures, present in the cell wall complexes, labile to borohydride, and others stable to borohydride but subsequently labile to alkali. Esters of *p*-hydroxybenzoic acid were reported to survive borohydride treatment in 0.01 M sodium hydroxide (pH 12) [7].

Borohydride is a well-established reducing agent in carbohydrate chemistry. Sugars are reduced to the corresponding alditols, and esterified uronic acids are converted to the corresponding monosaccharides [8]. Aliphatic carboxylic acid esters are generally reduced to primary alcohols. There is therefore a variety of reactions which may occur, in addition to possible differential cleavage of aromatic and aliphatic acyl groups, when cell wall complexes are treated with borohydride. Analysis of liberated products may lead to elucidation of structural features involved in the binding together of cell wall macromolecules. This paper investigates the composition of cell wall fragments produced by cleavage of chemical linkages when a cell wall preparation from pangola grass stem (*Digitaria decumbens*), resistant to degradation by fungal 'cellulase', was treated with aqueous potassium borohydride at pH 9. The effectiveness of borohydride as a deacetylation agent was studied using acetylated reference compounds derived from D-glucose, *myo*-inositol and cellulose.

RESULTS AND DISCUSSION

Deacetylation of reference carbohydrates

Efficiency of deacetylation was measured as the rate of loss of acetate relative to the original acetate present in the compound. Formic acid was preferred to acetic acid for decomposition of excess borohydride to avoid introducing acetate into the system. Preliminary tests showed that no acetate was lost from the chosen substrates during evaporation of formic acid. Borohydride was found to deacetylate selected carbohydrates at different rates and

to different degrees (Fig. 1). Glucose pentaacetate was deacetylated the most efficiently, and after 24 hr, 95% of the original acetate had been cleaved. *myo*-Inositol was deacetylated more slowly, and although the reaction was also in effect complete after 24 hr, there remained bound acetate corresponding to one acetate group per molecule. Cellulose acetate was insoluble and required constant stirring to allow deacetylation to proceed. This deacetylation process was the slowest of the three reactions during the first 24 hr, but after 48 hr only 10% of the original acetate remained. The cellulose acetate reaction, being heterogeneous, was more comparable to treatment of cell wall material, which was accordingly stirred with borohydride for 48 hr.

Degradation of pangola cell walls

Pangola grass stem cell walls were pre-digested with 'cellulase' (Onozuka P-1500), a broad-spectrum enzyme mixture which contains hemicellulase and glycosidase activities. After borohydride treatment of this enzyme-degraded cell wall preparation (CCW-CR), a residue (CCW-CR-B) amounting to 70% of the starting material (CCW-CR) was recovered (Table 1). Incubation of CCW-CR-B with 'cellulase' solubilized a further 36% of solids. Thus a cell wall residue, formerly totally resistant to degradation with 'cellulase', had 30% of its solids dissolved by borohydride, and the residual cell walls could now be further degraded with 'cellulase'. It was clear from the appreciable amount of material dissolved and the degradability of the cell wall residue, that borohydride had caused considerably more structural changes in the cell walls than simple deacetylation. Analysis of CCW-CR-B indicated that *ca* 50% of the original *trans-p*-coumaric acid (tCA) had been removed by borohydride along with 80% of the acetate (Table 1). However, further studies on the borohydride solution detected only a trace of free tCA (*ca* 1% of original tCA). No coumaryl alcohol or dihydro derivatives of CA or the alcohol were found. Therefore the phenolic acid appeared to have been solubilized from the cell walls still largely in bound form. This was confirmed by alkali treatment of the borohydride solution which yielded tCA equivalent to 45% of the original amount in CCW-CR. Thus, over 90% of the original tCA in CCW-CR could be accounted for. The resistance of *p*-coumarate to cleavage by borohydride could be due to the stabilising effect of the conjugated double bond system extending from the aromatic ring through the ethylenic double bond to the carbonyl of the carboxy group. Carbohydrate in solution [9] and uronic acid [10] amounted to 10.1 and 2.1% of the CCW-CR respectively. There were negligible free sugars, and the monosaccharide components, produced by acid hydrolysis, were mainly xylose and arabinose with smaller proportions of xylitol, galactose and glucose. No arabinitol was detected, which indicated that only xylose reducing groups, which had been produced during hydrolysis of cell wall polysaccharides with 'cellulase', were still bound in the cell wall macromolecules. The 'cellulase', which solubilized 49% of the finely ground cell walls (CCW), had hemicellulase and a broad glycosidase activity, as indicated by free xylose and arabinose in addition to glucose in the enzyme digest.

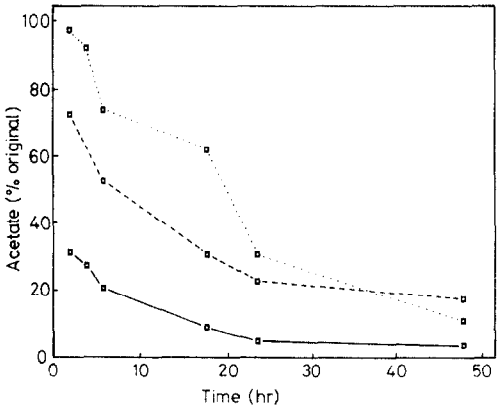

Fig. 1. Deacetylation of carbohydrate acetates with borohydride. ——, glucose pentaacetate; ---, myo-inositol hexaacetate; . . . , cellulose acetate.

Table 1. Cellulase digestibility (CD), and concentration of *trans-p*-coumaric acid (*t*CA), and acetate (Ac) in cellulase-degraded pangola grass stem cell walls before (CCW-CR) and after (CCW-CR-B) treatment with borohydride (% dry residue)

	CD	*t*CA	Ac
CCW-CR (100)*	0	4.1	2.5
CCW-CR-B (70)	36	2.6 (46)	0.7 (20)

*Figures in parenthesis are recoveries (%) relative to CCW-CR.

The precipitate BS2 (Table 2), produced on neutralization of the borohydride solution, amounted to 23% of CCW-CR, leaving 7% to account for in the neutral soluble fraction BS1 (Table 2). The borohydride soluble carbohydrate was distributed fairly evenly between the two fractions, and was qualitatively similar. Both fractions had a similar low xylose to arabinose ratio (2.5), but a notable difference was the higher proportion of xylitol present in BS1. Xylitol probably resulted from reduction of reducing xylose units produced by cellulase degradation of cell wall hemicellulosic pentosans. Therefore fraction BS1 appeared to contain a higher proportion of short chain pentosans than BS2. Carbohydrate amounted to over 70% of the solids in BS1, and to 20% in BS2. BS1 contained no uronic acid, which may have been due to reduction of uronic acid esters to the corresponding monosaccharides. This remains to be confirmed using borodeuteride and MS to differentiate the source of the monosaccharides. The major differences between the two fractions were the total partitioning of tCA into BS2, from which it could be liberated with alkali, and the high proportion of undefined solids also in the insoluble fraction. BS2 was a brown residue, insoluble in neutral or acidic aqueous solution, but soluble in alkali and dimethyl sulphoxide. It thus had properties commonly associated with a highly lignified plant residue. The presence of aromatic compounds as major components of BS2 was confirmed by pyrolysis-mass spectrometry [Ford, C. W. and Boon, J. J., unpublished results], which gave spectral patterns similar to lignin preparations from other sources [11]. The mass ions present indicated guaiacyl residues to be the main structural features, with lesser amounts of hydroxyphenyl and syringyl residues. Small amounts of

Table 2. Monomer components of material, soluble (BS1) and insoluble (BS2) in neutral aqueous solution, dissolved during borohydride treatment of cellulase-degraded pangola grass stem cell walls (molar ratios)

Fraction	tCA	Ara	Xyl	Gal	Glu	Xylitol	Xyl/Ara
BS1	0	22.7	56.0	5.7	7.1	8.5	2.5
BS2	19.3	20.5	49.1	2.5	4.3	4.3	2.4

pentosan and less of hexosan were also indicated. These results therefore established that a lignin fraction had been solubilized by borohydride, which, based on previous analyses [12], amounted to about half of the lignin originally present in the cell walls. The material BS2 may be analogous to the alkali-soluble lignin fraction from cell walls of pangola and other tropical grasses which gave a high negative correlation with rumen degradability of pentosans [1].

Solution BS1 was separated into two distinct carbohydrate fractions by gel filtration (Fig. 2). The higher M_r fraction 1 (73% carbohydrate), which eluted at the exclusion volume of the column, included strong UV absorbance which matched the elution profile of the carbohydrate part. The lower M_r fraction 2 (66% carbohydrate) eluted mainly within the fractionation range of the column, and the associated UV absorbance was much weaker than that in 1. The UV spectrum of 1, which had two maxima (285 and 317 nm) differed from 2 which had a single maximum at 276 nm, but had a similar shape to that previously reported for a lignin-carbohydrate complex esterified with *p*-coumaric acid (λ_{max} 283, 314 nm) [13]. However, the λ_{max} of the first UV absorbance band of the individual fractions which were combined to produce 1 decreased steadily with time of elution from 288 to 279 nm. There was, therefore, considerable heterogeneity in the UV absorbing components, which would have produced a decrease in the observed absorbance maxima. The results thus suggested that 1 contained polysaccharide esterified with a small amount of a phenolic acid, probably not tCA, previously undetected in the dilute solution BS1. Fraction 2 appeared to be a

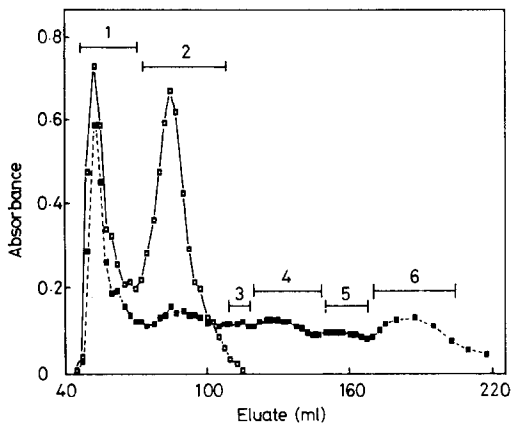

Fig. 2. Fractionation on Bio-Gel P-2 of neutral aqueous soluble material (BS1) extracted from pangola stem cell walls with borohydride. □——□, carbohydrate; ■----■, A_{280}. V_0 = 50 ml, V_i = 105 ml.

mixture of oligosaccharides which included some UV absorbing substances not linked to the carbohydrate. Fractions 1 and 2 contained 95% of the carbohydrate applied to the column [9] and 84% of the solids.

UV absorbing material which eluted after the inclusion volume of the column (Fig. 2, fractions 3–6) accounted for a further 13% of solids and 1.7% carbohydrate. Thus excellent recovery of material (97%) from the gel was obtained. Fractions 3–5 had single UV maxima (Table 3) in spectra typical for lignin [14], and IR absorbance bands corresponding to aromatic skeletal vibrations (1605, 1515 cm^{-1}). A small amount of free tCA, too low to quantify in dilute solution BS1, was concentrated in fraction 6, and probably masked the lignin absorbance because of its high molar absorptivity. Hence the fractions which were retarded on the column were possibly small aromatic molecules related to lignin, to which the associated carbohydrate was probably covalently bound. GC analysis detected only traces of free monosaccharides (in 3), but after acid hydrolysis arabinose, xylose, glucose, xylitol and glycerol were liberated (Table 3). The monosaccharide identities were confirmed by GC/MS analysis of 4. Methylation of 4 and GC analysis of the partially methylated alditol acetates [15] identified major structural features of the carbohydrate as end group arabinose and glucose, 3,4- and 2,4-linked xylose, and 4- or 2-linked xylose. Although alditol acetates of the last mentioned are identical, 4-linked xylose would be expected to predominate. These results suggested the presence of single glycosidically linked glucose residues and possibly glucose–xylose linkages in relatively short chains which were glycosidically linked to other, possibly non-carbohydrate, structures. No end-group xylose was detected. The high glycerol content of 4–6 may point to glycerides playing a role in the resistance of some cell wall structures to degradation with 'cellulase'. Since fatty acid esters should have been cleaved during borohydride treatment, the glycerol component was probably still bound in the UV absorbing structures. Two major components in the acid hydrolysis of 3–5 had GC RR_t values of 0.70 and 1.71 (glucitol = 1.00) and mass spectra with base peaks at *m/z* 127 and 129 respectively. The former appeared to be a TMSi derivative (*m/z* 73(45%)) and also had *m/z* 259 (65%). The structure and significance of these compounds remain to be determined.

This work has thus established that, in addition to deacetylation, borohydride treatment of grass cell walls caused cleavage of many other linkages between structures which had resisted 'cellulase' degradation. Borohydride soluble fractions rich in carbohydrate, comprising pentosan probably with bound phenolic acid and a mixture of pentose oligomers, were isolated. A highly lignified fraction, containing *ca* half of the original cell wall tCA still esterfied, was also solubilized. In other cell wall studies [16, 17], tFA and tCA have been reported to

Table 3. Absorbance maxima, and monomers released by acid hydrolysis of fractions retarded on Bio-Gel P-2 (molar ratios)

Fraction*	λ_{max} (nm)	Glyc	Ara	Xyl	Glu	Xylitol	Total sugars†
3	279	2.8	35.5	26.3	31.7	3.7	10
4	274	19.3	25.4	32.5	18.8	4.0	14
5	280	28.7	24.9	27.8	15.1	3.4	14
6	284	29.9	20.6	25.6	20.0	4.2	4

*See Fig. 2.
†% solids.

be esterfied to C-5 of terminal arabinofuranose residues. However, in fraction BS2, the relatively high proportion of tCA to pentoses suggests that in this material the phenolic acid may be more associated with aromatic nucleii than with carbohydrate. The wide variety of chemical structures liberated from the cell walls suggest that borohydride may be a useful reagent in studies aimed at elucidating *in situ* structural relationships between cell wall components.

EXPERIMENTAL

Origin of plant material. Crude cell wall residues prepared from pangola grass stems [12], were subsequently incubated with a commercial broad-spectrum fungal 'cellulase' (Onozuka P-1500), finely ground (<0.1 mm particles), and treated with DMSO [13]. The DMSO-treated cell wall preparation (CCW) was incubated with the same fungal 'cellulase' for 72 hr to produce a residue CCW-CR (51% CCW) resistant to further degradation with fresh enzymes.

General methods. Solutions were concd at <40° under red. pres. Phenolic acids were determined by GC of the TMSi derivatives [1]. Acetate was determined by GC as acetylpyrrolidine by a modification [1] of a method previously described [18]. Total carbohydrate in solution was estimated colorimetrically [9] and monosaccharide composition by GC of the TMSi ethers after hydrolysis with 2 M trifluoroacetic acid [19]. For GC/MS analysis a WCOT fused silica column (OV 101, 12 m × 0.2 mm) was used with a temperature program of 130° for 1 min, then 10°/min to 250°. Uronic acid concentration was determined colorimetrically with *m*-hydroxydiphenyl [10]. Carbohydrates were methylated, and the monomers analysed as the partially methylated alditol acetates by GC [15] using nickel columns (1800 × 2 mm) packed with (a) 0.4% OV 225 on surface-modified chromosorb [20] at 150°, or (b) 3% ECNSS on Gas-Chrom Q (100–200 mesh) at 170°.

Deacylation with borohydride. (a) *Deacetylation of reference compounds.* D-Glucose pentaacetate (10 mg), *myo*-inositol hexaacetate (10 mg) and cellulose acetate (15 mg) were each dissolved in Me_2CO (2 ml). Aliquots (7 × 0.1 ml) were subsampled from each soln into separate vials and the Me_2CO evapd in a stream of air. The residues from each compound were treated with aq. KBH_4 (0.5 ml, 0.2 M, pH 9) at 25° for times varying from 2 to 48 hr. Cellulose acetate was stirred during the procedure. The reactions were terminated by destroying excess borohydride with 90% HCO_2H. Solns were then air-dried, and the residues evapd with MeOH (6 × 0.5 ml). (b) *Deacylation of grass cell walls.* In preliminary analytical studies, cellulase-degraded cell walls (CCW-CR, 100 mg) were stirred with aq. KBH_4 (15 ml, 0.2 M) at 25° for 48 hr. Residual cell walls (CCW-CR-B) were centrifuged (2500 *g*), washed (3 × 5 ml H_2O, 2 × 5 ml EtOH) and dried at 55°. The supernatant soln and washings were combined (BS), filtered (sintered glass, porosity 2) and neutralized with HOAc (pH 6). A flocculent ppt. (BS2) was produced which was allowed to settle overnight at 2°, before centrifuging (2500 *g*) and decanting the clear, neutral soln (BS1). After washing twice with dil. HOAc, the precipitate BS2 was freeze-dried. The soln and washings (BS1) were combined, diluted to 50 ml and stored at 2° for further analysis.

Preparative scale studies. (a) *Isolation of borohydride fractions BS1 and BS2.* Cell walls (CCW-CR, 3.4 g) were stirred with aq. KBH_4 (370 ml, 0.2 M) at 25° for 48 hr. The cell wall residue was centrifuged, washed with H_2O (4 × 50 ml) followed by EtOH, Et_2O and finally drying at 55° (recovery 69.2%). The combined supernatant soln and aq. washings (BS) were neutralized (pH 6) with HOAc to produce a ppt. (BS2), which was centrifuged, washed with dil. HOAc (3 × 10 ml) and then freeze-dried to give a brown powder (646 mg, 19% CCW-CR). The soln and washings combined (BS1) were concd (25 ml) and passed slowly through a column of cation exchange resin [Amberlite IR 120 (H^+), 160 × 28 mm]. The eluate was collected until negative to tests for carbohydrate [9], concd to dryness, and then portions of MeOH added and evapd (6 × 10 ml). Redissolved in H_2O, BS1 was freeze-dried to yield 209 mg residue (6.1% CCW-CR, 97% recovery of carbohydrate from the column). (b) *Gel filtration of BS1.* Freeze-dried residue BS1 (209 mg) was dissolved as far as possible in H_2O (3 × 1 ml, 92% soluble). After centrifuging, the combined solns were applied to a column (250 × 25 mm) of Bio-Gel P-2 (−400 mesh) which was eluted with H_2O at 5.3 ml/hr. The eluate (2.5 ml fractions) was monitored for carbohydrates [9], absorbance at 280 nm and changes in the wavelength of the absorbance maximum (λ_{max}) near 285 nm.

Acknowledgements—The author thanks Mrs L. Howse for skilful technical assistance, and Mr K. Dobson (Dept of Chemical Engineering, University of Queensland) for help with the MS analysis.

REFERENCES

1. Ford, C. W. and Elliott, R. (1987) *J. Agric. Sci. Camb.* **108**, 201.
2. Ford, C. W., Elliott, R. and Maynard, P. J. (1987) *J. Agric. Sci. Camb.* **108**, 129.
3. Ford, C. W. and Hartley, R. D. (1988) *J. Chromatogr.* **436**, 484.
4. Ford, C. W. and Hartley, R. D. (1988) *J. Sci. Food Agric.* (in press).
5. Smith, D. C. C. (1955) *Nature* **176**, 927.
6. Hergert, H. L. (1960) *J. Org. Chem.* **25**, 405.
7. Smith, D. C. C. (1955) *J. Chem. Soc.* 2347.
8. Wolfrom, M. L. and Anno, K. (1952) *J. Am. Chem. Soc.* **74**, 5583.

9. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350.
10. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484.
11. Genuit, W., Boon, J. J. and Faix, O. (1987) *Anal. Chem.* **59**, 508.
12. Ford, C. W. (1978) *Aust. J. Agric. Res.* **29**, 1157.
13. Ford, C. W. (1986) *Carbohydr. Res.* **147**, 101.
14. Goldschmid, O. (1971) in *Lignins–Occurrence, Formation, Structure and Reactions* (Sarkanen, K. V. and Ludwig, C. H., eds) p. 245. Wiley– Interscience, New York.
15. Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B. and Lonngren, J. (1976) *Chem. Commun., Univ. Stockholm* No. 8, 2.
16. Kato, Y. and Nevins, D. J. (1985) *Carbohydr. Res.* **137**, 139.
17. Mueller-Harvey, I., Hartley, R. D., Harris, P. J. and Curzon, E. H. (1986) *Carbohydr. Res.* **148**, 71.
18. Mansson, R. and Samuelsson, B. (1981) *Sven. Papperstidn.* **84**, R15.
19. Ford, C. W. (1979) *J. Sci. Food Agric.* **30**, 853.
20. Akhrem, A. A., Avvakumov, G. V. and Strel'chyonok, O. A. (1979) *J. Chromatogr.* **176**, 207.