



FERULATE CROSS-LINKING IN CELL WALLS ISOLATED FROM MAIZE CELL SUSPENSIONS

JOHN H. GRABBER,* RONALD D. HATFIELD,* JOHN RALPH,*† JERZY ZON‡ and NIKOLAUS AMRHEIN§

*U.S. Dairy Forage Research Center, USDA-Agricultural Research Service, Madison, WI 53706, U.S.A.; †Department of Forestry, University of Wisconsin, Madison, WI 53706, U.S.A.; ‡Institute of Organic Chemistry, Biochemistry and Biotechnology, Technical University of Wrocław, Wrocław, Poland; §Institut für Pflanzenwissenschaften, ETH-Zentrum, Zürich, Switzerland

(Received in revised form 1 May 1995)

Key Word Index—*Zea mays*; Gramineae; cell suspension culture; ferulic acid; dehydrodiferulic acids; lignin; arabinoxylan; peroxidase; cross-linking; phenylalanine ammonia-lyase; 2-aminoindan-2-phosphonic acid.

Abstract—Cross-linking of arabinoxylans by ferulate dehydrodimers and incorporation of feruloylated arabinoxylans into lignin were modelled with maize walls (*Zea mays* cv Black Mexican) containing 5.3–18.0 mg g⁻¹ of total ferulates. The proportion of dehydrodimers to total ferulates increased from ca 20 to 45% when dilute hydrogen peroxide was added to walls containing bound peroxidase. About 45% of the dehydrodimers were coupled by 8-5 linkages, with 8-8, 8-0-4 and 5-5 coupled dehydrodimers each comprising 10–25% of the total. The quantity of ferulates released by saponification were reduced by 83–95% when exogenously supplied hydroxycinnamyl alcohols were polymerized into walls by wall-bound peroxidase and *in situ* generated hydrogen peroxide. Only 40% of the ferulates incorporated into lignin were recovered following hydrolysis of ether linkages. These results indicate that primary walls in grasses become extensively cross-linked by ferulic and dehydrodiferulic acids during lignification, and that only a portion of ferulates in lignified tissues are measurable by current solvolytic methods.

INTRODUCTION

Ferulic acid is attached as an ester (1) (Fig. 1) to the C-5-hydroxyl of α -L-arabinose moieties of grass xylans [1]. It has long been presumed that arabinoxylans are cross-linked to a limited extent by a 5-5 coupled dehydrodimer (2) of ferulic acid [2–4]. Recently, our group [5] demonstrated that oxidative coupling mechanisms (oxidases and one-electron metal oxidants) form substantial amounts of 8-0-4, 8-5, and 8-8 coupled dehydrodimers (3–7) in addition to the 5-5 coupled product. The 5-5 coupled dehydrodimer accounted for only a fraction (5–15%) of all dehydrodimers released by saponification of cell walls from C₃ and C₄ grasses [5]. Therefore, previous studies have probably underestimated the importance of dehydrodimers as cross-linking molecules.

Ferulates are also implicated in the cross-linking of arabinoxylans to lignin. Experiments with lignin models indicate that ferulates are attached to lignin by 4-0- α' ether linkages (15) (formed by nucleophilic trapping of quinone methide intermediates, Fig. 2) [6], or by 4-0- β' ether (9), 8-0-4' styryl ether (10) and C-C linked structures (11–14) (formed by oxidative coupling mechanisms, Fig. 1) [7]. (When labelling cross-links, e.g. 4-0- α' , the

first term indicates the coupling site on the ferulate ester, and the primed term indicates the coupling site on the lignin moiety.) Most of the ferulate is probably attached to lignin by linkages that resist acid or alkaline hydrolysis [7]. As a result, the extent of ferulate cross-linking in lignified grasses cannot be estimated by solvolytic methods.

Our objective was to model how extensively wall polymers in grasses become cross-linked by oxidative coupling of ferulate esters. Cell walls from maize cell suspensions (*Zea mays* cv Black Mexican) are feruloylated; the quantity of ferulates available for cross-linking can be reduced by growing cell suspensions with 2-aminoindan-2-phosphonic acid (AIP), a specific inhibitor of phenylalanine ammonia-lyase (EC 4.3.1.5) [8, 9]. These cultures also contain wall-bound peroxidases (EC 1.11.1.7) that are capable of coupling ferulate monomers into dehydrodimers [5, 10] and of polymerizing lignin precursors into a wall-bound polymer that closely resembles lignin [10]. This paper describes how ferulate cross-linking was affected by ferulate concentrations, peroxidase activity, and lignification of cell walls isolated from maize cell suspensions.

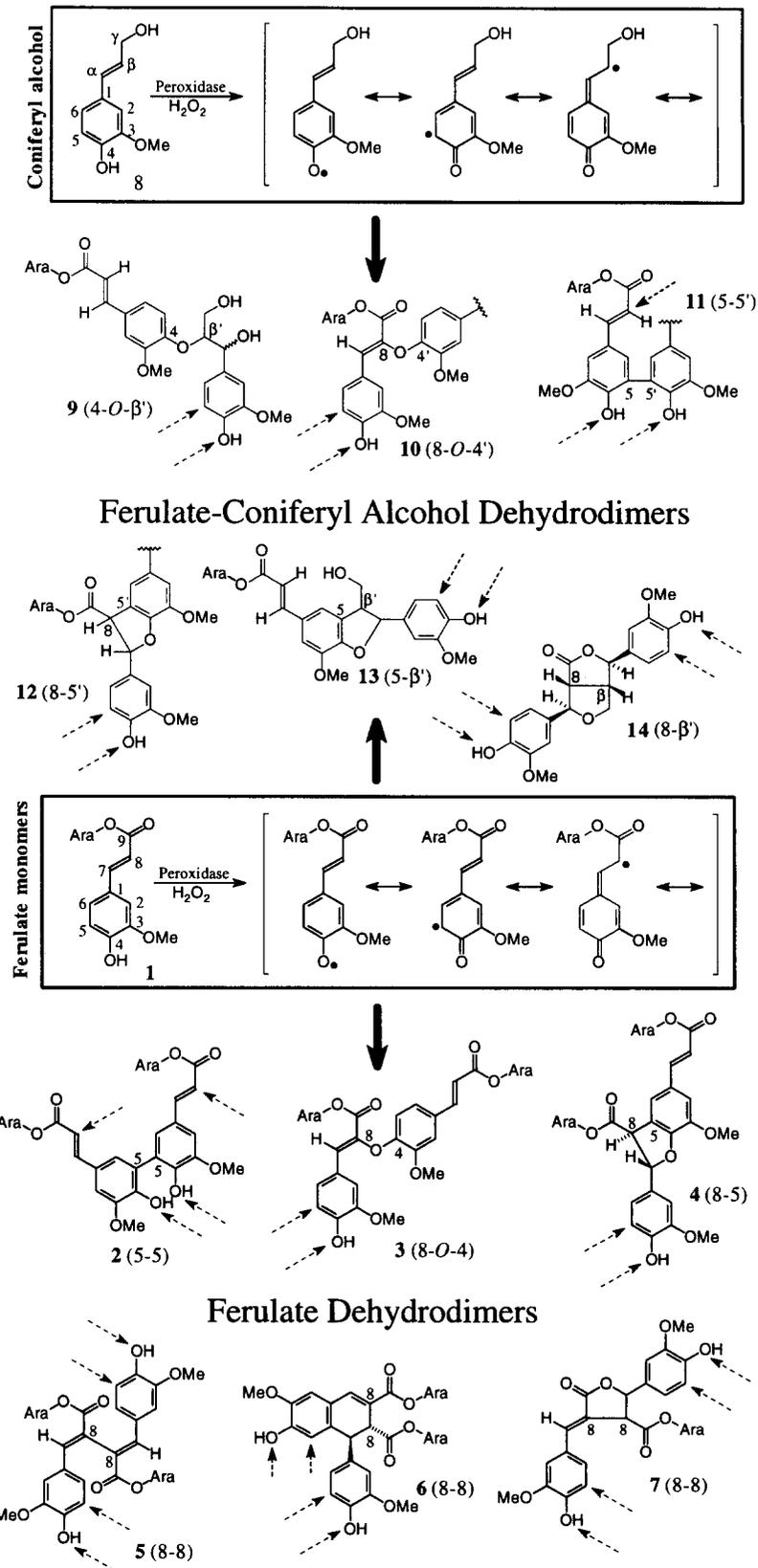


Fig. 1. Feruloylated arabinoxylan chains in grass walls become cross-linked by radical coupling of ferulate monomers (1) into ferulate dehydrodimers (e.g. 2–7). Dotted arrows indicate potential sites for further radical coupling with hydroxycinnamyl alcohols or lignin oligomers, resulting in cross-linking of arabinoxylans to lignin. Ferulate radicals can also couple with lignin monomer or oligomer radicals [e.g. from coniferyl alcohol [8]] to form ferulate–coniferyl alcohol dehydrodimers or oligomers (e.g. 9–14) that (with the exception of structure 14) cross-link arabinoxylans to lignin. ‘Ara’ is arabinofuranosyl residues on arabinoxylan.

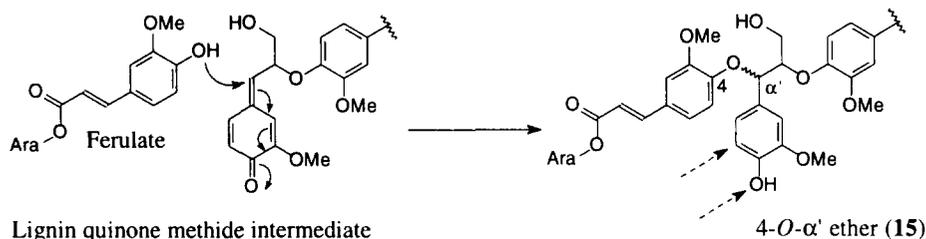


Fig. 2. Formation of 4-*O*- α' ethers (15) by nucleophilic addition of ferulate esters to lignin quinone methide intermediates. 'Ara' is arabinofuranosyl residues on arabinoxylan. Dotted arrows indicate potential sites for radical coupling with hydroxycinnamyl alcohols or lignin oligomers during lignification.

RESULTS AND DISCUSSION

Composition of cell walls

Maize cell suspensions consisted of small clusters of cells that undergo limited elongation. The packed cell volume increased from 2.5% at the initiation of cultures to ca 45% at the early stationary growth phase when cells were collected to prepare cell walls. Cell walls isolated from maize cells suspensions contained 17.2 mg g⁻¹ ferulate esters, 0.5 mg g⁻¹ ferulate ethers, 0.6 mg g⁻¹ *p*-coumarate esters, 3.6 mg g⁻¹ Klason lignin and only trace quantities of ester-linked truxillic acids. Monosaccharide analysis revealed that cell walls contained 194 mg g⁻¹ arabinose, 169 mg g⁻¹ xylose, 9 mg g⁻¹ rhamnose, 8 mg g⁻¹ mannose, 78 mg g⁻¹ galactose, 316 mg g⁻¹ glucose and 126 mg g⁻¹ uronic acid. If we assume that 90% of the xylosyl residues were derived from xylans [11], then one per 11 xylosyl residues in xylan were substituted with feruloylated arabinose. Growing cultures with 40 μ M AIP reduced the concentration of total ferulates by 70% (Table 1) and the estimated substitution of xylans by feruloylated arabinose to one per 35 xylosyl residues. The concentration of minor phenolic components (e.g. *p*-coumarate esters and

Klason lignin) were also reduced by 45–70% by AIP treatment. AIP treatment reduced the concentration of arabinose and xylose by 6% (due perhaps to reduced feruloylation of arabinoxylans) and increased the concentration of glucose by 5%. Other work indicates that structural protein and cellulose deposition and cell wall development are not affected by AIP [12,13]. Overall, the composition of cell walls was characteristic of non-lignified primary walls of maize [11, 14].

Dehydrodimer formation in nonlignified walls

Dehydrodimers comprised 15% of total ferulates in normal cultures when cell walls were isolated in the presence of an oxidase inhibitor (mercaptoethanol). The proportion of dehydrodimers increased to 27% when feruloylation of arabinoxylans was reduced by AIP treatment of cultures (Table 1). This suggests that cells compensated for reduced ferulate deposition by increasing the extent of dehydrodimer formation. The proportion of dehydrodimers to total ferulate increased to ca 45% when dilute H₂O₂ was added to cell walls containing bound peroxidase. Aeration of wall suspensions (without added H₂O₂) did not increase dehydrodiferulate concen-

Table 1. Formation of ferulate dehydrodimers in cell walls with normal or reduced feruloylation (*n* = 2). Dimerization of ferulates by wall-bound peroxidase was stimulated by slowly adding dilute H₂O₂ to a suspension of cell walls isolated from maize cell suspensions. Wall suspensions not receiving H₂O₂ were isolated and incubated with mercaptoethanol (10 mM) to block extraneous coupling of ferulates. The quantity of ferulates available for forming dehydrodimers was reduced by growing cultures with 2-aminoindan-2-phosphonic acid (AIP), a specific inhibitor of phenylalanine ammonia lyase

AIP (μ M)	H ₂ O ₂ (mmol)	Ferulate monomers (mg g ⁻¹ cell wall)	Ferulate dehydrodimers (mg g ⁻¹ cell wall)				Total ferulates (mg g ⁻¹ cell wall)
			8-8	8-5	8- <i>O</i> -4	5-5	
0	0	14.60	0.57	1.22	0.34	0.45	17.20
0	0.4	8.83	0.88	2.78	1.25	1.13	14.87
40	0	3.84	0.33	0.66	0.17	0.27	5.27
40	0.4	2.26	0.33	1.09	0.33	0.35	4.37
Analysis of variance							
AIP		*	*	*	*	*	*
H ₂ O ₂		*	NS	*	*	*	NS
AIP \times H ₂ O ₂		*	NS	NS	NS	NS	NS

*, NS: significant at the 0.05 level of probability and not significant, respectively.

trations, indicating that laccases (EC 1.10.3.1) were not involved in oxidative coupling of ferulates (Grabber, J.H., unpublished results). Most studies suggest that dehydrodimer formation is much lower in graminaceous cell walls [15–17]. This discrepancy may be attributed to physiological factors that affect ferulate deposition and dehydrodimer formation [18], incorporation of dehydrodimers into lignin [19] or incomplete analysis of dehydrodimers [5]. In all treatments, the most abundant dehydrodimer was coupled by an 8-5 linkage (*ca* 45%), and 8-8, 8-*O*-4 and 5-5 couplings each comprised 10–25% of the total. A similar distribution of linkage types was also observed with mesophyll cell walls isolated from *Panicum virgatum* and *Dactylis glomerata* [5].

The extent of dehydrodimer formation in H₂O₂-treated cell walls is remarkable considering the low concentration of ferulates, especially in cultures grown with AIP. Our results suggest that the spatial distribution of ferulate monomers is regulated in maize walls to maximize the potential for cross-linking of arabinoxylans by dehydrodimer formation. Apparently, dehydrodimer formation in maize cultures was limited by the availability of H₂O₂ rather than by the abundance of wall-bound peroxidases or wall feruloylation. Oxidative conditions in cell walls may be limited by the presence of antioxidants like ascorbate [20], or by inadequate levels of precursors or oxidases required for H₂O₂ production [21, 22]. Extensive dehydrodiferulate formation probably occurs at the onset of lignification when the concentration of ferulates and the activity of peroxidases reach their maximal levels [17, 22, 23].

Incorporation of primary-wall ferulates into lignin

Cell walls were synthetically lignified by peroxidase-mediated polymerization of hydroxycinnamyl alcohols.

H₂O₂ was generated *in vitro* from glucose by glucose oxidase [24]. Oxidative coupling of ferulate esters to lignin by ether and C-C linkages prevents the release of parent acids upon saponification. Therefore, the quantity of ferulate cross-links between arabinoxylans and lignin was estimated by the difference in alkali-labile acids released from non-lignified and lignified cell walls. This approach must be tempered with some caution because a small proportion of ferulate monomers will form an 8-β' coupled product (14) whose formation involves cleavage of the ester linkage to eliminate the carbohydrate moiety [7]. It is also probable that 8-β' coupling of the 5-5 linked dehydrodimer would form a similarly de-esterified product [25]. Formation of these products would result in a slight overestimation of ferulate mediated cross-links between arabinoxylans and lignin. Saponification of cell walls from normal cultures indicated that 83% of ferulate esters were incorporated into lignin (Table 2). In cell walls from AIP treated cultures, an even higher proportion of ferulates (*ca* 95%) were incorporated into lignin.

Studies with dilignol models and isolated lignins suggest that high temperature alkaline hydrolysis will release ferulates coupled with lignin by 4-*O*-α' and 4-*O*-β' ether linkages, whereas C-C and 8-*O*-4' styryl-ether linkages will remain intact (Grabber, J. H., unpublished results) [26]. Lignification significantly increased the quantity of ferulates coupled to lignin by hydrolysable ether linkages (Table 2), but the amount of parent acids released by hydrolysis accounted for only *ca* 40% of the ferulates incorporated into lignin. Partial recovery of ferulic and dehydrodiferulic acids indicated that a substantial proportion of the cross-links were C-C or 8-*O*-4' styryl-ether linkages formed by oxidative coupling mechanisms. It remains to be determined if the acids released by solvolysis were coupled to lignin by 4-*O*-α' ether linkages (formed by nucleophilic trapping of quinone methide

Table 2. Incorporation of ferulates (monomers plus dehydrodimers) into synthetic lignins formed within cell walls isolated from maize cell suspensions (*n* = 3). Cultures were grown with 2-aminoindan-2-phosphonic acid (AIP) to reduce the concentration of ferulates available for incorporation into lignin. Wall-bound peroxidase and *in vitro* generated H₂O₂ were used to polymerize hydroxycinnamyl alcohols (HCA) into lignin

Treatment		Klason lignin (mg g ⁻¹ cell wall)	Ferulates released by saponification of ester linkages (mg g ⁻¹ cell wall)	Ferulates released by hydrolysis of ether linkages (mg g ⁻¹ cell wall)
AIP (μM)	HCA (mg g ⁻¹ cell wall)			
0	0	10	18.04	0.49
0	94	113	3.02	6.80
10	0	8	8.07	0.24
10	97	105	0.38	3.16
Analysis of variance				
AIP		*	*	*
HCA		*	*	*
AIP × HCA		*	*	*

* Significant at the 0.05 level of probability.

intermediates) or by 4-*O*- β' ether linkages) formed by oxidative coupling mechanisms). Current work with this model system is focused on further delineating what types of cross-linked structures are formed between ferulates and lignin and how extensively various ferulate monomers and dehydrodimers become incorporated into lignin.

In conclusion, our results suggest that dehydrodimer formation in non-lignified primary cell walls is apparently limited by the production of H_2O_2 . During lignification, cell walls become extensively cross-linked by oxidative dehydrodimerization of esterified ferulate monomers and by incorporation of ferulate monomers and dehydrodimers into lignin. Only a fraction of the ferulates incorporated into lignin were releasable by solvolysis, indicating that the abundance of cross-linked structures in lignified tissues has been greatly underestimated in previous studies.

EXPERIMENTAL

In all studies, maize cell suspensions (*Z. mays* cv Black Mexican) were grown in 1 l flasks under standard conditions [27]. Growth of cultures was monitored by measuring the packed-cell vol. of cells following centrifugation at 250 *g* for 5 min. After 16 days of culture, cells were collected on a nylon mesh (20 μ m), suspended in ice-cold HEPES buffer (25 mM, pH 7.0) and ruptured by a probe-type sonicator or by several passages through a Parr Nitrogen Bomb maintained at 1500 psi. Wall fragments were collected on a nylon mesh and washed sequentially with HEPES buffer, 100 mM $CaCl_2$ and H_2O to remove cytoplasmic contaminants and peroxidases which were not tightly bound to cell walls.

Formation of dehydrodiferulates in nonlignified cell walls. Cells walls (ca 750 mg dry wt) from cultures grown with 0 or 40 μ M AIP were suspended in 75 ml PIPES buffer (10 mM, pH 6.5, with 2 mM $CaCl_2$). Cell walls for this experiment were isolated and suspended with solvents alone or solvents plus mercaptoethanol (10 mM) to inhibit peroxidase activity. Oxidative coupling of ferulate monomers was stimulated by adding 0.4 mmol H_2O_2 in 80 ml PIPES buffer to mercaptoethanol-free suspensions at a rate of 4 ml hr^{-1} . One hour after additions were completed, cell walls were collected on a nylon mesh, washed with H_2O , and dried with Me_2CO .

Incorporation of primary-wall ferulates into lignin. The potential extent and type of ferulate cross-links in lignified walls were investigated by using wall-bound peroxidases and *in situ* generated H_2O_2 to polymerize hydroxycinnamyl alcohols into a suspension of non-lignified walls from cultures grown with 0 or 10 μ M AIP. After draining excess of H_2O , cell walls (ca 2.8 g dry wt) were suspended in 170 ml PIPES buffer (25 mM, pH 6.5) with 2 mM $CaCl_2$ and 25 μ kat glucose oxidase (Sigma, Type VII-S). Coniferyl alcohol (120 mg, 0.67 mmol), sinapyl alcohol (140 mg, 0.67 mmol) and glucose (300 mg, 1.67 mmol) were dissolved in 200 ml PIPES buffer and added to stirred cell-wall suspensions with a peristaltic pump over a 25 hr period. Hydroxycinnamyl alcohols

were prepared according to the method of ref. [28]. After all additions were completed, suspensions were kept in the dark and mixed for an additional 45 hr. Cell walls in non-lignified controls were incubated with glucose oxidase. A few drops of toluene were added periodically to inhibit microbial growth. After incubations were completed, cell walls were collected on a nylon mesh and washed with H_2O and dried with Me_2CO .

Chemical analyses. The lignin content of cell walls was estimated by a modified Klason procedure [29]. Neutral sugars, released by H_2SO_4 hydrolysis of cell walls, were quantified by HPLC [30] and uronic acids were determined by a colorimetric procedure [31]. Ester- and ether-linked monomers and dimers of hydroxycinnamic acids were determined sequentially on cell wall samples. Samples (50 mg) were placed in translucent Teflon vials with 2 N NaOH (3 ml, degassed). 2-Hydroxycinnamic acid (0.1 mg) was added as an int. standard. Vials were flushed with N_2 , sealed with a Teflon cap, and incubated for 20 hr at room temp. Samples were acidified with 12 N HCl (0.55 ml) and extracted with Et_2O (2.5 ml, 3 \times) to isolate hydroxycinnamic acid monomers and dimers, which were ester-linked to arabinoxylans. Samples were then flushed with a stream of N_2 to remove residual Et_2O , and 12 N NaOH (1.75 ml, degassed) was added to make samples ca 4 N base. 3-Hydroxy-4-methoxycinnamic acid (0.1 mg) was added as an int. standard. Vials were flushed with N_2 , capped, and sealed in a Teflon bomb containing ca 20 ml of H_2O . Bombs were placed in a sand bath at 170 $^\circ$ for 2 hr. After cooling, samples were transferred to test tubes, acidified with 12 M HCl (1.85 ml) and extracted with Et_2O (4.0 ml, 2 \times) to isolate hydroxycinnamic acid monomers and dimers which were attached to lignin by hydrolysable ether linkages. Extracts were dried and hydroxycinnamic acids were silylated and analysed by GC [5]. Amounts of ester-linked acids were calculated according to GC response factors of ferulic, *p*-coumaric and dehydrodiferulic acids relative to 2-hydroxycinnamic acid [5]. Initial attempts to determine response factors for ether-linked acids failed due to extensive degradation of dehydrodimer model compounds during alkaline hydrolysis at 170 $^\circ$. We found, however, that response factors of acids run through the entire procedure with cellulose (Sigma, Sigmacell 100) were similar to GC response factors. In addition, relative peak areas of acids released from non-lignified cell walls by saponification were similar to those obtained after alkaline hydrolysis at 170 $^\circ$. Therefore, amounts of etherified acids were based on GC response factors relative to 3-hydroxy-4-methoxycinnamic acid.

Acknowledgements—The authors are grateful to Stéphane Quideau (Currently at Department of Organic Chemistry, The Pennsylvania State University) for providing coniferyl and sinapyl alcohols. The work was supported in part by the USDA-ARS Administrator Funded Post Doc program and by USDA-NRI competitive grants #92-37304-8057 (Plant Growth and Development Section) and #94-37500-0580 (Enhancing Value and Use of Agricultural and Forest Products). Mention

of trade name, proprietary product or specific equipment does not constitute a guarantee of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

REFERENCES

1. Mueller-Harvey, I., Hartley, R. D., Harris, P. J. and Curzon, E. H. (1986) *Carbohydr. Res.* **148**, 71.
2. Markwalder, H. U. and Neukom, H. (1976) *Phytochemistry* **15**, 836.
3. Hartley, R. D. and Morrison, W. H., III (1991) *J. Sci. Food Agric.* **55**, 365.
4. Lam, T. B. T., Iiyama, K. and Stone, B. A. (1992) *Phytochemistry* **31**, 2655.
5. Ralph, J., Quideau, S., Grabber, J. H. and Hatfield, R. D. (1994) *J. Chem. Soc., Perkin Trans. I* 3485.
6. Scalbert, A., Monties, B., Rolando, C. and Sierra-Escudero, A. (1986) *Holzforschung* **40**, 191.
7. Ralph, J., Helm, R. F., Quideau, S. and Hatfield, R. D. (1992) *J. Chem. Soc., Perkin Trans. I* 2961.
8. Grabber, J. H., Amrhein, N., Mannisto, P. T. and Zon, J. (1991) *Proc. Int. Symp. on Forage Cell Wall Structure and Digestibility*, p. B10, Madison, WI, U.S.A.
9. Zon, J. and Amrhein, N. (1992) *Liebigs Ann. Chem.* **625**.
10. Grabber, J. H., Ralph, J., Quideau, S. and Hatfield, R. D. (1995) *J. Agric. Food Chem.* (in press).
11. Carpita, N. C. (1984) *Plant Physiol.* **76**, 205.
12. Keller, B., Nierhaus-Wunderwald, D. and Amrhein, N. (1990) *J. Struct. Biol.* **104**, 144.
13. Schmutz, A., Jenny, T., Amrhein, N. and Ryser, U. (1993) *Planta* **189**, 453.
14. Darvill, A. G., Smith, C. J. and Hall, M. A. (1978) *New Phytologist* **80**, 503.
15. Harris, P. J., Hartley, R. D. and Lowry, K. H. (1980) *J. Sci. Food Agric.* **31**, 959.
16. Shibuya, N. (1984) *Phytochemistry* **23**, 2233.
17. Carpita, N. C. (1986) *Plant Physiology* **80**, 660.
18. Kato, Y., Yamanouchi, H., Hinata, K., Ohsumi, C. and Hayashi, T. (1994) *Plant Physiol.* **104**, 147.
19. Lam, T. B. T., Iiyama, K. and Stone, B. A. (1992) *Phytochemistry* **31**, 1179.
20. Takahama, U. and Oniki, T. (1992) *Plant Cell Physiol.* **33**, 379.
21. Dean, J. F. D. and Eriksson, K.-E. (1992) *Holzfor-schung* **46**, 135.
22. McDougall, G. J. (1992) *Phytochemistry* **31**, 3385.
23. Nishitani, K. and Nevins, D. J. (1990) *Plant Physiol.* **93**, 396.
24. Tollier, M.-T., Lapierre, C., Monties, B., Francesch, C. and Rolando, C. (1991) *Proc. Sixth Int. Symp. of Wood and Pulping Chemistry*, p. 35 Melbourne, Australia.
25. Quideau, S. (1994) Ph.D. Thesis. University of Wisconsin-Madison, U.S.A. (University Microfilms International #9428350).
26. Sarkanen, K. V. and Ludwig, C. H. (1971) *Lignins, Occurrence, Formation, Structure and Reactions*. Wiley-Interscience, New York.
27. Kieliszewski, M. and Lamport, D. T. A. (1987) *Plant Physiol.* **85**, 823.
28. Quideau, S. and Ralph, J. (1992) *J. Agric. Food Chem.* **40**, 1108.
29. Theander, O. and Westerlund, E. A. (1986) *J. Agric. Food Chem.* **34**, 330.
30. Jones, B. A., Hatfield, R. D. and Muck, R. E. (1992) *J. Sci. Food Agric.* **60**, 147.
31. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Analyt. Biochem.* **54**, 484.