APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1994, p. 2985–2989
0099-2240/94/$04.00+0

Vol. 60, No. 8

Solubilization of Lignin by the Ruminal Anaerobic Fungus *Neocallimastix patriciarum*

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Received 16 February 1994/Accepted 25 May 1994

The ability of the ruminal anaerobic phycomycete *Neocallimastix patriciarum* to digest model lignin compounds and lignified structures in plant material was studied in batch culture. The fungus did not degrade or transform model lignin compounds that were representative of the predominant intermonomer linkages in lignin, nor did it solubilize acid detergent lignin that had been isolated from spear grass. In a stem fraction of sorghum, 33.6% of lignin was apparently solubilized by the fungus. Solubilization of ester- and ether-linked phenolics accounted for 9.2% of the lignin released. The amounts of free phenolic acids detected in culture fluid were equivalent to the apparent loss of ester-linked phenolics from the sorghum substrate. However, the fungus was unable to cleave the ether bond in hydroxycinnamic acid bridges that cross-link lignin and polysaccharide. It is suggested that the majority of the solubilized lignin fraction was a lignin carbohydrate complex containing ether-linked hydroxycinnamic acids. The lignin carbohydrate complex was probably solubilized through dissolution of xylan in the lignin-xylan matrix rather than by lignin depolymerization.

Ruminal fungi preferentially colonize lignocellulosic tissue and produce an array of extracellular enzymes that are capable of digesting the major structural carbohydrates of plant cell walls (11). However, their role in the degradation of lignin is not clear (36). Although Windham and Akin (41) were unable to demonstrate any loss of lignin by mixed fungal rumen populations, other studies with plant fragments and radiolabeled lignocellulose indicated that ruminal fungi are able to solubilize or degrade lignin; however, the mechanism was not determined (1, 14, 35). Although ruminal fungi may solubilize lignin, there is no evidence that they are able either to cleave the intermonomer bonds in lignin or to utilize lignin as a carbon source. Lignin is a polymer of phenylpropanoid units interlinked through a variety of nonhydrolyzable C—C and C—O—C bonds. Polysaccharide and lignin are linked by hydroxycinnamic acid bridges that are attached through their carboxyl or phenolic groups to form ester and ether bonds, respectively (18, 26, 29, 30). Hydroxycinnamic acids are also esterified to hemicellulose or etherified to lignin without forming bridges (15, 37). Ruminal fungi produce extracellular feruloyl and *p*-coumaroyl esterase enzymes which release hydroxycinnamic acids from arabinoxylan (3, 4, 5). However, the ability of ruminal fungi to cleave the ether bond in hydroxycinnamic acid bridges has not previously been investigated. The current study was designed to determine whether the anaerobic fungus *Neocallimastix patriciarum* could cleave lignin bonds and linkages between lignin and polysaccharide. This was investigated by monitoring the degradation of model lignin compounds and defined plant substrates in batch cultures of the fungus.

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MATERIALS AND METHODS

Organism, anaerobic techniques, and medium preparation. The ruminal anaerobic phycomycete *N. patriciarum* was a gift from C. G. Orpin, CSIRO Division of Tropical Crops and Pastures, Indooroopilly, Queensland, Australia. The anaerobic techniques of Hungate (17) as modified by Bryant (6) were used for medium preparation, storage, and transfer. Basal media containing 20% (vol/vol) clarified rumen fluid, 3.75% (vol/vol) minerals 1 and 2 (7), 0.1% (vol/vol) Pfennigs' trace minerals, 0.4% (wt/vol) Na_2CO_3, 0.05% (wt/vol) cysteine hydrochloride, and 0.001% (wt/vol) resazurin were prepared with (medium +C) or without (medium −C) a carbon source of 1% (wt/vol) Casitone (Difco Laboratories, Detroit, Mich.), 0.25% (wt/vol) yeast extract (Difco Laboratories), and 0.5% (wt/vol) cellobiose. The media were gassed with CO_2, dispensed into 25-ml Balch tubes (18 by 250 mm), stoppered, and autoclaved for 15 min at 15 lb/in^2. B vitamins (31) were added to each tube of medium just prior to inoculation, and incubations were carried out at 39°C in the dark. Media (9.5 ml) were inoculated with 0.5 ml of *N. patriciarum* which had been grown for 1 day in the same medium. Uninoculated, incubated controls were included consistently throughout the study. Model lignin compounds were filter sterilized and added to individual culture tubes just prior to inoculation. Plant material used in digestibility studies was weighed into individual culture tubes and was autoclaved with the media.

Model compounds and plant material used as substrates. The chemical structures of the lignin model compounds used in this study are shown in Fig. 1 and are representative of the predominant intermonomer linkages in lignin. They include diaryl ether linkages and the two aryl-alkyl linkages found in lignin, involving the alpha (benzyl) and beta carbons. Some compounds incorporated 4-methylumbelliferone as part of the ether linkage, to facilitate detection of cleavage by fluorescence. The following lignin model compounds were synthesized as described in the literature: compound 1, guaiacylglycerol-β-guaiacyl ether (16); compound 2, guaiacylglycerol-β-*O*-(β-methylumbelliferyl)ether (38); compound 3, α-*O*-(β-methylumbelliferyl)-β-(hydroxy)propiovanillone (38); compound 4, *O*-benzyl-α-*O*-(β-methylumbelliferyl)-β-(hydroxy)propiovanil-

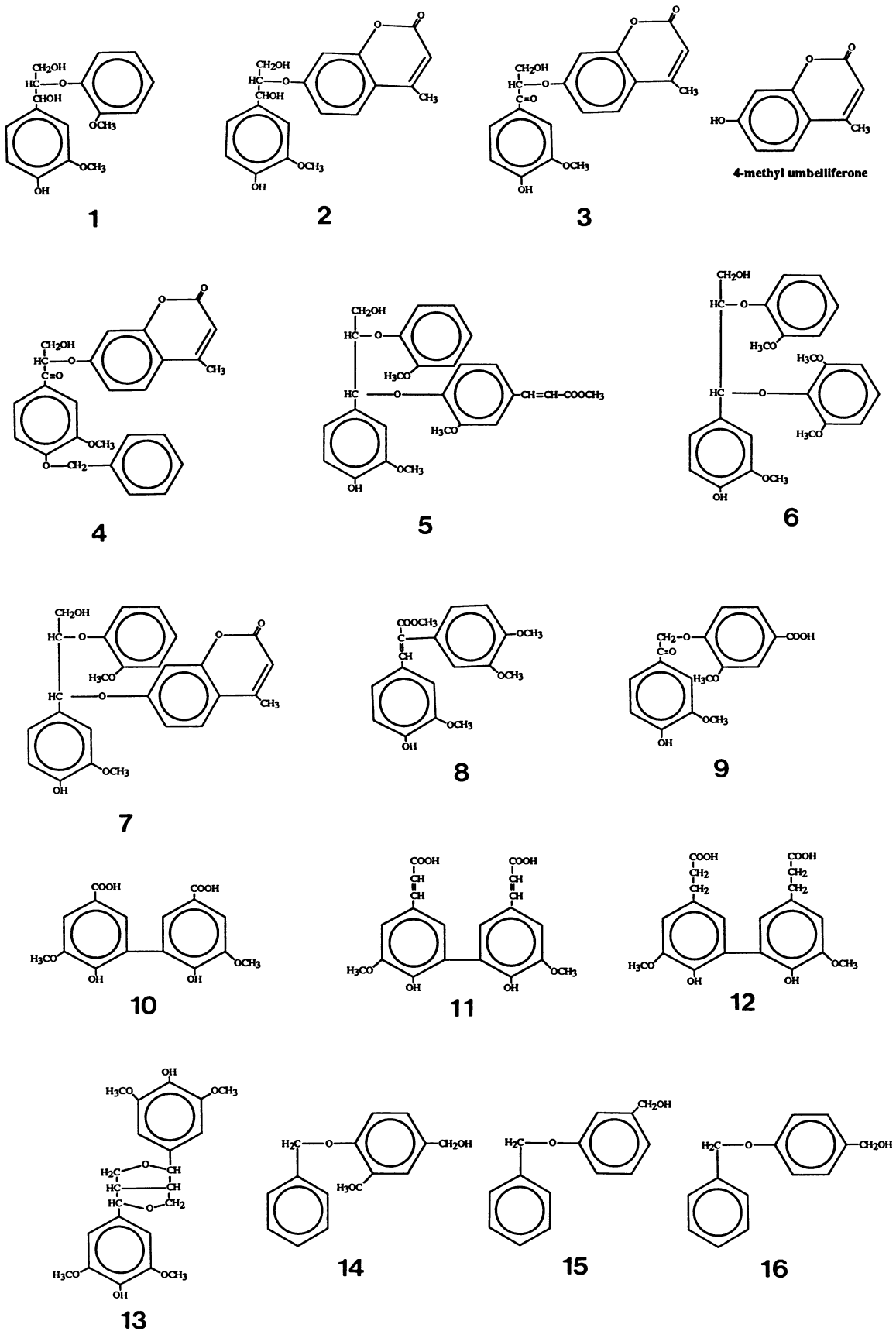

FIG. 1. Chemical structures of lignin model compounds and 4-methyl umbelliferone.

lone; compounds 5 to 7 were prepared by reaction of the quinone methide of guaiacylglycerol β-*O*-guaiacyl ether with methyl ferulate, 4-methylumbelliferone, and syringol, respectively, and were purified as previously indicated (24, 34); compound 8, stilbenecarboxylic acid methyl ester (13); compound 9, 3-methoxy-4-hydroxy-α-(2-methoxyphenoxy)-acetophenone (16); compound 10, 2,2′-dihydroxy-3,3′-dimethoxy-5,5′ dicarboxybiphenyl (10); compound 11, 5,5′-dehydrodiferulic acid; compound 12, 5,5′-dehydrodi-dihydroferulic acid (12); compound 13, syringaresinol (23). Compounds 14 to 16, used to test the lability of a simple benzyl ether linkage, were obtained commercially (Sigma-Aldrich). Compounds 1 to 4 and 8 to 13 were isolated by silica gel column chromatography and were identified by gas chromatography-mass spectrometry (for trimethylsilyl derivatives) and by proton nuclear magnetic resonance (for acetates). Compounds 5 to 7 were prepared from compound 1, which had been identified previously. These compounds were isolated as the mixture of erythro and threo forms by silica gel column chromatography. Formation of α-*O*-ether linkage in compounds 5 to 7 was confirmed by nuclear magnetic resonance analysis. Lignin model compounds 11 to 16 were dissolved in methanol (50 mM), while the remaining compounds were dissolved in dimethyl sulfoxide (50 to 100 mM) and were filter sterilized before being added to medium +C and medium −C at 0.25 to 0.5 mM.

Plant material from spear grass (*Heteropogon contortus*), Mitchell grass (*Astrebla* sp.) and sorghum (*Sorghum* sp.) that contained relatively high concentrations of cell wall phenolic acids (33) were chosen for digestibility studies. All plant material was oven dried (90°C) and ground through a 1-mm screen. Sorghum stem (internode 8) was dissected to produce a rind fraction (epidermis, sclerenchyma, and vascular bundle zone), which was cut into 1.5-mm lengths and digested in rumen fluid (in vitro) for 3 weeks (39, 40). The digested rind was included in medium −C at 10 g/liter to study the solubilization of phenolic acids by the fungus. In a second experiment, alkali-extracted plant material was used to study the solubilization of etherified phenolics by the fungus. Spear grass, Mitchell grass, and digested sorghum rind were extracted with 2 N NaOH to remove esterified phenolics (33), washed several times with distilled water, dried, and included in medium −C at 10 g/liter. Acid detergent lignin was also isolated from spear grass as described by Lowry et al. (32) and was included in medium −C and medium +C at 10 g/liter.

Chemical analyses. Cultures containing plant material were centrifuged at 3,000 × *g* for 15 min to precipitate insoluble material following incubation. The supernatant which contained solubilized plant components was aspirated by vacuum, and the residue was oven dried at 90° to determine dry matter (DM) loss. Lignin content of plant substrates and culture residues was determined by the acetyl bromide technique (19, 20). Ester-linked ferulic acid (FA) and *p*-coumaric acid (PCA) were extracted from plant samples and culture residues according to the technique of Lowry et al. (33) except that 2 N NaOH was used instead of 1 N NaOH. The plant residue and filtrate from the NaOH extraction were analyzed for ether-linked phenolics after hydrolysis with dioxane–2 N HCl (27). Supernatant from batch cultures of *N. patriciarum* was subsampled and analyzed for free, esterified, and etherified phenolic acids and phenols, as described previously.

Phenolic monomers were analyzed by the high-performance liquid chromatography (HPLC) procedure of Jung et al. (22). Thirty microliters of sample was injected into a reverse-phase analytical column (8 by 100 mm, Waters Nova-Pak C_{18}, Radial-Pak cartridge, 4-μm particle size) with a mobile phase of water-glacial acetic acid-butanol (350:1:7) that was eluted isocratically at 2 ml/min.

TABLE 1. Amounts of ester- and ether-linked phenolic acids solubilized from sorghum rind by *N. patriciarum* during 6-day incubation[a]

Parameter	Amt of phenolic acid (mmol/g of sorghum rind)	
	FA	PCA
Amt in substrate:		
Ester linked	0.025	0.098
Ether linked	0.015	0.016
Apparent loss from substrate:		
Ester linked	0.012	0.026
Ether linked	0.006	0.000
Amt in culture supernatant solubilized by fungus:		
Free acid	0.012	0.027
Soluble ether linked	0.006	0.003

[a] DM loss: 35.5 ± 0.9%.

Model lignin compounds 1 to 4 and 8 to 16 were analyzed quantitatively by HPLC. Culture fluid was subsampled, extracted with ethyl acetate (1:2 [vol:vol]) by being mixed for 30 min, and centrifuged. Nonextracted samples containing compounds 14 to 16 and extracts containing compounds 1 to 4, 8 to 10, and 13 to 16 were injected (30 μl) into a reverse-phase column (3.9 by 300 mm, Waters μBondapak C_{18} steel column, 10-μm particle size) with a mobile phase of methanol-water (1:1) pumped isocratically at 1 ml/min, except for analysis of compound 4, which was separated by using a mobile phase containing 70% methanol. Compounds 11 and 12 were analyzed by using the HPLC system described above for phenolic monomers. A long-wave UV lamp was used to detect the light blue fluorescence of 4-methylumbelliferone released from compounds 2, 3, 4, and 7 in cultures to a lower limit of detection of 0.005 mM and was indicative of etherase activity (38). However, subsequent experiments showed that the fungus can slowly degrade 4-methyl umbelliferone to nonfluorescent products and thus may have reduced the sensitivity of the assay.

Model lignin compounds 14, 15, and 16 were analyzed by thin-layer chromatography. Culture fluid was extracted with ethyl acetate as described previously and was concentrated by lyophilization. The dried residue (lignin compound) was resuspended in ethyl acetate at 50 times the original concentration and spotted on precoated thin-layer chromatography plates with silica gel 60F 254 (particle size, 250 μm). The plates were developed once with solvent system 1 (chloroform-ethyl acetate, 9:1) and once with solvent system 2 (ethyl acetate 100%). The compounds were detected as dark spots when they were illuminated with UV light.

All analyses were done in duplicate or triplicate.

RESULTS

N. patriciarum did not degrade or transform the model lignin compounds used in this study, which represented the predominant C—C and C—O—C intermonomer linkages in lignin. Lignin isolated from spear grass was not solubilized by the fungus.

There was an apparent loss of ester-linked phenolics (48.0% FA and 26.5% PCA) and ether-linked phenolics (40% FA and 0% PCA) from sorghum rind incubated with the fungus for 6 days (Table 1). Ester-linked FA and PCA were solubilized

TABLE 2. Amounts of ether-linked phenolic acids solubilized from NaOH-extracted sorghum rind, Mitchell grass, and spear grass by *N. patriciarum* during 6-day incubation[a]

Parameter	Amt of phenolic acid (mmol/g of plant material[b] or mmol/g of substrate[c] in:					
	Sorghum rind		Mitchell grass		Spear grass	
	FA	PCA	FA	PCA	FA	PCA
In substrate:						
Ester linked	0	0	0	0	0	0
Ether linked	0.0148	0.0102	0.0065	0.0058	0.0061	0.0078
In culture supernatant (solubilized by fungus):						
Free acid	0.0005	0.0017	0.000	0.0003	0.0001	0.0001
Soluble ether linked	0.0032	−0.0008	0.002	0.0005	0.0002	0.0009

[a] DM loss: sorghum rind, 39.4%; Mitchell grass, 45.3%; Spear grass, 37.7%.
[b] For acids measured in substrate.
[c] For acids measured in culture supernatant.

from sorghum rind as free acids at maximum linear rates ($r^2 > 0.92$) of 0.004 and 0.01 mmol/g of sorghum per day, respectively, during the first 4 days of incubation. The amounts of free phenolic acids detected in culture fluid were equivalent to the apparent loss of ester-linked phenolics in sorghum rind (Table 1). Soluble ester-linked phenolic acids and lignin-derived phenolics were not detected in culture supernatant fluids.

Alkali-treated plant material contained 0.006 to 0.015 and 0.006 to 0.01 mmol of ether-linked FA and PCA per g, respectively, but no detectable ester-linked phenolics (Table 2). Negligible quantities of free phenolic acids were released from these plant substrates by the fungus, except for free PCA (0.002 mmol/g of substrate) released from sorghum rind. However, the negative value calculated for release of soluble ether-linked phenolics from this substrate indicates that the value for PCA release may be an overestimate. Furthermore, there was no evidence of release of ether-linked phenolics as free acids from untreated sorghum rind (Table 1).

Sorghum rind contained 0.255 g of lignin per g of DM, of which 33.3% was apparently solubilized during incubation (Table 3). Solubilization of ester- and ether-linked phenolics accounted for 9.4% of the lignin lost from sorghum rind. Alkali-extracted sorghum contained 33.3% less lignin, of which 21.2% was apparently solubilized. The ratio of the proportion of DM loss to the proportion of total lignin loss for sorghum rind incubated with the fungus was 1.07, compared with 1.97 for sodium hydroxide-treated material, even though DM losses for both substrates were similar in extent.

TABLE 3. Amounts of lignin solubilized from sorghum rind by *N. patriciarum* during 6-day incubation[a]

Parameter	Amt (g/g of dry matter) of lignin solubilized from:	
	Sorghum rind	NaOH-extracted sorghum rind
Lignin content	0.255	0.170
Apparent lignin loss from substrate	0.085	0.036
Apparent loss of ester-linked phenolic acids	0.006	0
Apparent loss of ether-linked phenolic acids	0.002	0.003

[a] All values were adjusted for nonspecific loss of lignin measured in uninoculated controls. DM loss: sorghum rind, 35.5%; NaOH-extracted sorghum rind, 41.9%.

DISCUSSION

The degree of cell wall lignification and cross-linking of lignin and polysaccharide through hydroxycinnamic acid bridges is thought to limit the degradation of fiber by ruminal microorganisms (2, 21). These studies indicate that *N. patriciarum* is unable to cleave the ether linkage in these bridges. The fungus solubilized phenolics as free acids when they were linked to fiber by ester bonds only, but we were unable to determine whether the ester bond in the hydroxycinnamic acid bridge was hydrolyzed. Quantitative analyses that distinguish ester-linked, ether-linked, and bridge-linked hydroxycinnamic acids may resolve this issue (30). There was no evidence of enzymatic depolymerization of lignin and subsequent release of phenols, but apparently 20 to 30% of lignin was solubilized by the fungus. The action on sorghum rind was remarkable, given the prior prolonged incubation in rumen fluid. Approximately 10% of the solubilized lignin was accounted for as ester- and ether-linked hydroxycinnamic acids, but the structural identity of the remainder was not determined. It is likely that the solubilized lignin fraction was a lignin carbohydrate complex (LCC) containing ether-linked hydroxycinnamic acids that became soluble through dissolution of xylan in the lignin-xylan matrix. LCCs extracted from grasses and legumes are rich in lignin and contain significant amounts of esterified and etherified phenolic acids (25, 28). In the current study, soluble lignin did not contain phenolics that were only ester linked, as these were released as free acids by the fungus. On the basis of studies with model lignin compounds, it has been suggested that cleavage of β-aryl ether linkages in polymeric lignin by ruminal microorganisms is the mechanism by which soluble LCCs are released (8, 28). Clearly, this is not the mechanism by which *N. patriciarum* solubilizes lignin.

Our results are in accord with the model of cell wall degradation proposed by Chesson (9). Chesson suggests that LCCs are released from the cell wall as a result of digestion of the surrounding carbohydrate and not from direct lignin degradation. Lignin in sodium-hydroxide-treated sorghum rind appeared more resistant to solubilization than did lignin in untreated plant material. This indicates that the lignin fraction extracted by sodium hydroxide, which includes ester-linked phenolics, is more amenable to release by the fungus.

This study supports the findings of Akin and Benner (1), that ruminal fungi are able to solubilize lignin but not to degrade it to end products of metabolism.

ACKNOWLEDGMENT

This work was partly funded by the Australian Meat Research Corporation, Project CS.143-Rumen Microbe Manipulation Program.

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