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Pectin degradation during colonization of wood by brown-rot fungi

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ABSTRACT

Brown-rot decay results in rapid reduction in degree of polymerization of holocellulose, with concomitant strength loss without removing lignin. Development of new methods of wood protection will require focusing on early events in the sequence of fungal attack during colonization. Pit membranes (sapwood) of wood cell walls represent a readily available source of non-lignified carbohydrate, i.e., pectin and cellulose. Commercial pectinases (Pectinol) and Trichoderma sp. have been shown to degrade pit membranes and increase penetration of preservatives. Plant pathogens have been shown to degrade pectin by the synergistic action of oxalic acid and polygalacturonase (PG). Brown-rot fungi have also been shown to produce oxalic acid and pectinase during the decay process. The oxalic acid solubilizes the pectin by chelating the Ca$^{2+}$ and the PG hydrolyses the β−1,4 linkages. We have demonstrated the ability of Postia placenta, Gloeophyllum trabeum, and Serpula incrassata to use pectin as a sole carbon source and to produce oxalic acid and PG on both liquid media and wood. Aspergillus niger and Trichoderma sp. also produce PG on wood but no oxalic acid or weight loss.

One key to pectin hydrolysis by plant pathogens has been shown to be fungal production of oxalic acid, which lowers the pH of the substrate and chelates calcium ions. Production of oxalic acid may serve a similar role during incipient wood decay as calcium oxalate has been found by scanning electron microscopy during both brown-rot and white-rot decay. Therefore, we hypothesized that in situ precipitation of existing calcium ions in wood may prevent the cascade of biochemical events involved in colonization of wood by brown-rot fungi, especially hydrolysis of pit membranes. Preliminary experiments in our laboratory have shown that brown-rot fungi, white-rot fungi, and termites are inhibited from effecting weight loss of wood following pretreatment of wood blocks with the selective water-soluble calcium-precipitating agent N. N-naphthalamide (NHA). We hypothesize that pectin utilization is an essential step during incipient brown-rot decay that helps to initiate fungal metabolism and promote the spread of fungal hyphae through wood. Our long-term goal is to stop the spread of decay fungi by treating wood with calcium-trapping agents such as NHA, with the ultimate goal of developing targeted strategies for environmentally benign decay prevention.

INTRODUCTION

Brown-rot decay is the most destructive and costly form of decay of softwoods in service. The decay mechanism can be characterized by diffusion of low molecular weight agents into the wood cell wall, causing extensive oxidative depolymerization of polymeric polysaccharides accompanied by measurable strength loss of wood prior to weight loss (1,2). Brown-rot fungi normally colonize softwoods via rays and axial resin canals and from there develop via penetration of window pit membranes into axial traeheids (3). In order for fungal hyphae to colonize the lumen of every cell, either penetration of pit membranes or formation of bore holes must provide access to adjacent cells. Increasing the permeability of the wood by degrading the pit membranes serves to disseminate both hyphae and decay agents. The precise mechanism by which brown-rot fungi initiate and sustain this biochemical alteration remains a mystery. The purpose of this chapter is to discuss the involvement of pectin degradation in the initiation of decay by brown-rot fungi.

PECTIN AND CALCIUM IN WOOD

Studies of pectin-hydrolyzing enzymes in wood-decay fungi are scarce, probably because of the relatively low content (trace to 4%) of pectin in solid wood (4). The low pectin content may be misleading in terms of importance during early decay. Anatomically, most of the pectin is located in the central region or torus of the pit membranes, ray cell wall, and the compound middle lamellae of the wood cell wall (Fig. 1) (5-7).

Pectins are composed mainly of linearly connected β−1,4-D-galacturonic acid units and their methyl esters,
interrupted in places by 1,2-linked L-rhamnose units. In wood cells, a major part of the pectic substance occurs as polygalacturonic acid in the middle lamella. Part of the polygalacturonic acid may be esterified or neutralized with other bases and thus exist as insoluble salts of calcium, magnesium, and iron (8).

The primary location of calcium in wood is in the pectin (9, 10). The effects of treating conifer wood with commercial pectinases to improve penetration of preservatives have been thoroughly studied. Tschernitz (11) demonstrated that the permeability of Douglas-fir sapwood increased following commercial pectinase treatment (Fig. 2) as long as the treatment was combined with either low pH or a calcium chelator (ammonium oxalate or sodium hexametaphosphate). The use of chelating agents to remove tissue calcium and thereby increase the volatility of pectic materials has long been practiced; ammonium oxalate was one of the first chelating agents used. In later years, ethylene diamine tetraacetate (EDTA), cyclohexane diamine tetraacetate (CDTA), and sodium hexametaphosphate (calgon) were used (12).

Calcium is the most abundant trace element in sound blocks of southern pine (Table 1) and black spruce (10). During decay by the brown-rot fungus Postia placenta (MD-698), calcium content rose two- to threefold in 4 weeks (Table 1). Calcium is considered an important agent in regulating plant cell wall hydrolysis (13). Calcium ions are intercalated between the polygalacturonic chains in an “egg-box” system, binding to carboxyl groups between opposing chains (14).

Calcium ions are intercalated between the polygalacturonic chains in an “egg-box” system, binding to carboxyl groups between opposing chains (14). Pectin is a good chelator of Ca$^{++}$ and acts as a selective binder for Ca$^{++}$ ions in undignified tissues (10). In parenchymatous plant tissue, the middle lamella is thought to consist principally of calcium salts of pectic substances. Cell wall separation can be effected with calcium-chelating agents or pectolytic enzymes. Extraction with chelating agents (ammonium oxalate, sodium hexametaphosphate, EDTA, CDTA) generally yields pectins with a relatively high degree of methylation. Removal of Ca$^{++}$ ions is critical for hydrolysis of pectic acid by polygalacturonase (PG) since these enzymes split glycosidic linkages adjacent to free carboxyl groups (15).

Whewellite and weddellite crystals, the mono- and dihydrate forms of calcium oxalate, respectively, may have a great effect on the biological and biochemical processes of wood decay because (a) the crystals are a reactive reservoir of calcium, (b) even small amounts of oxalate in solution increase the volatility of iron and aluminum, and (c) oxalate affects the pH of a solution by being both the anion of a weak acid and chelator of iron and aluminum (16).
Table 1—Elemental analysis of sound and brown-rotted southern pine

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total N (%)</th>
<th>P (ppm)</th>
<th>K (ppm)</th>
<th>Ca (ppm)</th>
<th>Mg (ppm)</th>
<th>S (ppm)</th>
<th>Zn (ppm)</th>
<th>B (ppm)</th>
<th>Mn (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.03</td>
<td>&lt;18.0</td>
<td>276.3</td>
<td>548</td>
<td>133.0</td>
<td>27.20</td>
<td>5.74</td>
<td>&lt;2.43</td>
<td>120.4</td>
</tr>
<tr>
<td>MAD-698</td>
<td>0.17</td>
<td>64.8</td>
<td>178.4</td>
<td>1,860</td>
<td>572.7</td>
<td>128.2</td>
<td>0.99</td>
<td>&lt;2.52</td>
<td>275.9</td>
</tr>
<tr>
<td>ME-20</td>
<td>0.11</td>
<td>&lt;16.9</td>
<td>79.57</td>
<td>1,076</td>
<td>411.3</td>
<td>83.57</td>
<td>4.87</td>
<td>2.88</td>
<td>179.9</td>
</tr>
</tbody>
</table>

PECTIN DEGRADATION

Role of Oxalic Acid

Production of oxalic acid has been proposed to be important in the mechanism of brown-rot fungi (17,18). It appears to be involved in many degradative processes simultaneously. Oxalate is reported to be integrally involved in the formation of hydroxyl radicals from H<sub>2</sub>O<sub>2</sub> and iron. Oxalate would act as a reducing agent for the conversion of Fe<sup>+++</sup> to Fe<sup>++</sup> required for the Fenton chemistry, which depolymerizes polysaccharides (19,20), or as a source of H<sub>2</sub>O<sub>2</sub> (21,22). Oxalic acid is also a moderately strong iron chelator and is the only chelator found universally in brown-rot fungi (23). Viikari and Ritschkoff (24) prevented brown-rot decay with both an organic (EDTA) and inorganic (tripolyphosphate) iron chelator. Oxalic acid is also involved in pH reduction and direct acid-catalyzed hydrolysis of the wood substrate (18,25,26). Oxalic acid is also involved in chelation of other cations, i.e., Ca<sup>++</sup>, especially from the calcium pectate in pit membranes and the compound middle lamellae and ray cells (17,27–30) and in the metal complexation of zinc (31). Production of oxalic acid would therefore enable fungi to weaken the wood structure, thus increasing the pore size to permit penetration by lignocellulolytic enzymes (32).

The least understood of these observations is the role of oxalic acid in calcium chelation and the formation of calcium oxalate crystals. Calcium oxalate frequently forms crystals that can be readily visualized by scanning electron microscopy in fungi, wood, and soil, which may serve to sequester and detoxify excess calcium (16,32–35). Brown-rot fungi may conserve the functions of oxalic acid shown to predominate among plant pathogenic fungi; i.e., the depolymerization of pectin by (a) direct disintegration of pectic substances, (b) synergistic action with PG activity (36,34), and (c) lowering of pH and chelation of calcium from calcium pectate (37). Pectin has been shown to induce PG and oxalic acid in brown-rot fungi grown in vitro (30,38). In addition to providing a calcium sink for wood-derived calcium, calcium oxalate may form from translocated calcium in order to buffer the wood and provide regulation of pH during decay (17,39,40).

Microbial Pectin-Degrading Enzymes

Recently, we reported the in vitro induction of PG and formation of oxalic acid to pectin in brown-rot fungi (1,30). Experiments were undertaken to confirm relationships between brown-rot decay, oxalic acid, and bordered pit degradation during brown-rot decay of southern pine. Extracellular PG was estimated in vitro using liquid cultures of three brown-rot species (P. placenta, Gloeophyllum trabeum, and Serpula incrassata) by cup-plate assay (Fig. 3), reducing sugars, and decrease in viscosity. The genera of brown-rot fungi used in the study were selected to represent the three different physiological types of brown-rot fungi. Our results indicate that PG and oxalic acid function in concert to degrade pectic substances, especially those in the torus of bordered pit membranes.

Tschemnitz and Sachs (7) provided the first experimental evidence to support the suggestion that fungal decomposition of pectin is of importance during incipient wood decay by brown-rot fungi (25,28,32,41). Three studies have addressed the presence of pectinase enzymes in brown-rot fungi (42–44). The capacity to solubilize and hydrolyze pectic substances might provide brown-rot fungi with a competitive advantage over other fungi. Solubilization of the bordered pit membranes of tracheids during incipient brown-rot decay would provide not only nonlignified carbon sources (pectin, hemicellulose, and cellulose) for growth but also access to adjacent tracheids (Fig. 1). Cowling (45) and Wilcox (46) reported that during early brown-rot decay by Postia sp., the hyphae ramified through the entire wood block prior to 5% weight loss, largely by penetration of simple and bordered pits. Enzymatic degradation of pit membranes was also observed in white-rot fungi (47). In addition to the pit membranes, the pectin in the compound middle lamellae (Fig. 1) may also be solubilized, thus weakening the structural cement between tracheids. Meier and Wilkie (48) reported cellulose (36%), pectin (20%), and glucomannan (8%) in the compound middle lamellae of pine tracheids. Large gaps or pockets were observed during ultrastructural studies of brown-rot decay (49).

The confirmation of endo-polygalacturonase in P. placenta (30) and the likely synergistic role played by
oxalic acid in hydrolyzing the membranes of bordered pits raised the issue of whether brown-rot fungi increase the permeability of wood during incipient decay (29,50). Preliminary experiments demonstrated that the permeability of southern pine cores increased to maximum during colonization by three brown-rot fungi (14 days). Increased permeability paralleled weight loss (Fig. 4). However, *P. placenta* ME-20, a nondecay isolate unable to accumulate oxalate or effect weight loss of wood, was also unable to hydrolyze the pit membranes, which underscores the important role of oxalic acid in calcium chelation and penetration of pit membranes during incipient decay (Fig. 5) (30). These data suggest the possibility that precipitation of calcium in situ might prevent decay by preventing (a) chelation of calcium from pectins by oxalate, (b) enzymatic hydrolysis of bordered pit membranes, (c) colonization of wood through pit apertures, and (d) decay and weight loss of wood.

**INHIBITION OF WOOD DECAY BY CALCIUM PRECIPITATION IN SITU**

Many physiological processes in eukaryotic organisms are under the control of calcium, such as enzyme secretion, metabolic regulation, and cytoplasmic transport (51). Calcium often mediates cellular processes through binding to specific proteins that serve as receptors. Of the calcium-binding proteins, calmodulin is the most widely distributed. Calmodulin antagonists bind with high affinity and thereby inhibit calmodulin-dependent enzymes. Hill and Waggener (52) reported that the calmodulin antagonists chlorpromazine and trifluoperazine blocked secretion of β−1,4−endoglucanase by *Trichoderma reesei*, indicating that
calmodulin may function as a regulatory agent in some critical stage in enzyme secretion.

The most popular calcium-trapping agents used in ultrahistochemistry (TEM) are oxalate and pyroantimonate anions (53). However, oxalate has a high selectivity but low sensitivity for calcium (54). Pyroantimonate also forms insoluble electron-dense products with the sodium and magnesium ions (53). For these reasons, both oxalate and pyroantimonate have been compared to N,N-naphthaloylhydroxylamine (NHA). NHA is a water-soluble heterocyclic calcium capture agent (mol wt 235), which was initially used for quantitative determination of the calcium content in serum (55) before being used to demonstrate cellular calcium in electron microscopy (56). NHA forms a very stable and very selective complex with Ca$^{++}$ that is insoluble in 100 mM ethylene glycol tetraacetic acid (EGTA) (57).

Sobota et al. (57) reported retention of 93% cellular calcium using NHA. The NHA procedure for immobilizing and visualizing cellular calcium possesses high sensitivity and selectivity, and it is much simpler than the conventionally used pyroantimonate technique (57,58).

We have been investigating the applicability of using Ca$^{++}$ precipitating agents to inhibit fungal degradation of wood. The immediate goal of this research is to determine the mechanism by which the calcium-trapping agent NHA prevents brown-rot decay in wood. These data will help to determine whether the inhibitory effects are direct toxicity on the fungi or indirect effects of calcium precipitation in wood that prevents depolymerization and subsequent decay. In preliminary tests, NHA did not inhibit mold and sapstain fungi on wood, suggesting that direct toxic effects do not apply to all types of fungi. NHA may interfere only with the brown-rot/white-rot decay mechanism via calcium binding and with calcium cycling. Our long-term goal is to develop new and more specific approaches to preventing and controlling decay by brown-rot fungi. Because present wood preservatives pose a threat to the environment during treatment and disposal, there is an urgent need for new, sharply targeted, and benign wood-preserving methods. In concert, these experiments may develop important information on what new substrate targets in wood can potentially inhibit wood decay and how chemicals precipitate from and stay bound to the wood. Treating wood with agents targeted specifically to the mechanism of brown-rot decay should provide environmentally friendly protection of wood.

**Results of NHA Inhibitory Studies**

*Growth inhibition of decay, stain, and mold fungi on agar*— Growth of decay, stain, and mold fungi on 2% agar plates was tested with three concentrations of NHA (Table 2).
Figure 5. Seating electron micrographs of southern pień bordered pits after brown-rot decay (14 days). (1) Control; (2) *P. placenta* MAD-698; (3,4) *G. trabeum* MAD-617; (5) *S. incrassata* MAD-563; (6) *P. placenta* ME-20. t is pit membrane torus; m, pit membrane; hs, hyphal sheath.
Table 2—NHA inhibition of fungi on malt agar

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Reduction in radial fungal growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001% NHA 0.01% NHA 0.1% NHA</td>
</tr>
<tr>
<td>Postia placenta MAD-698</td>
<td>72 100 100</td>
</tr>
<tr>
<td>Serpula incrassata MAD-563</td>
<td>74 100 100</td>
</tr>
<tr>
<td>Gloeophyllum trabeum MAD-617</td>
<td>18 66 100</td>
</tr>
<tr>
<td>Trametes versicolor MAD-697</td>
<td>6 59 100</td>
</tr>
<tr>
<td>Ceratocystis opistalni C-188</td>
<td>70 80 100</td>
</tr>
<tr>
<td>Trichoderma sp. P-71H</td>
<td>0 6 100</td>
</tr>
</tbody>
</table>

Table 3—Weight loss of southern pine in ASTM D 1413 soil–block test

<table>
<thead>
<tr>
<th>NHA (%)</th>
<th>S. incrassata MAD-563</th>
<th>G. trabeum MAD-617</th>
<th>P. placenta MAD-698</th>
<th>T. versicolor MAD-697</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>55.5 ± 5.6</td>
<td>55.1 ± 3.0</td>
<td>52.2 ± 2.9</td>
<td>33.8 ± 9.9</td>
</tr>
<tr>
<td>0.05</td>
<td>54.6 ± 2.1</td>
<td>49.1 ± 3.1</td>
<td>32.2 ± 14.0</td>
<td>33.2 ± 4.9</td>
</tr>
<tr>
<td>0.1</td>
<td>37.2 ± 7.4</td>
<td>46.8 ± 6.4</td>
<td>18.6 ± 17.2</td>
<td>10.9 ± 11.2</td>
</tr>
<tr>
<td>0.5</td>
<td>−1.5 ± 0.4</td>
<td>1.6 ± 1.4</td>
<td>−0.9 ± 0.1</td>
<td>−0.3 ± 0.3</td>
</tr>
</tbody>
</table>

*Mean and standard deviation of four replicates.

These results suggest that NHA is more inhibitory to brown-rot fungi previously shown to be oxalic acid accumulators (MAD-698 and MAD-563) and less inhibitory to oxalic acid non-accumulators (MAD-617) and molds like Trichoderma except at the highest concentration (0.1%), where all fungi were uniformly inhibited. Trametes versicolor MAD-697 is a white-rot fungus.

**Growth inhibition of sapstain fungi and mold fungi on wood** - Southern pine and maple wood blocks were pretreated with three concentrations of NHA (0.1%, 0.5%, 1.0%). No inhibition of sapstain fungi and mold fungi (Trichoderma, Aspergillus, and Penicillium sp.) was detected except for C. opistalni at the highest two NHA concentrations. These results appear to exclude broad-spectrum toxic inhibition of fungal growth.

**Decay resistance in soil–block test** — Decay resistance was demonstrated in a 6-week ASTM D 2017-81 (59) soil–block exposure test of southern pine wood blocks (48 test/16 control blocks) to three brown-rot and one white-rot fungi. When the wood was treated with 0.5% NHA, mean weight loss was negligible (<1.6%) (Table 3); mean weight loss in untreated controls was 49%. Ten percent aqueous NHA was ineffective, indicating minimal inhibitory concentration between 0.1% and 0.5% NHA.

In another test, decay resistance of southern pine sticks (15 × 2.0 × 0.3 cm) treated with 1% aqueous NHA was compared to that of untreated sticks in a modified ASTM soil–block test (Fig. 6). Weight loss of treated sticks was more severe than that in the standard soil–block test as a result of direct soil contact; ≤11% weight loss was measured for G. trabeum.

Leachability of NHA from standard wood blocks was determined using the AWPA standard E11-87 (60) (Table 4). This comparison of leached samples (water for 2 weeks) should be helpful in differentiating the indirect effects of calcium precipitation in wood from direct toxic effects of residual, unbound chemicals.

**Scanning Electron Microscopy of Crystals**

With the advent of environmental scanning electron microscopy (ESEM), it is now possible to examine unfixed and undried specimens under high resolution in the natural state (35,61,62). We used a Hitachi S-2360N ESEM to examine in the wet state southern pine wood blocks decayed by two strains of P. placenta. The most striking result was the presence of crystals inside the hyphal sheath of both P. placenta MAD-698 and ME-20 (Fig. 7). By means of x-ray analysis (EDS) and morphology, we concluded that the crystals observed were those of calcium oxalate monohydrate (unpublished results).
Figure 6. Comparison of percentage of weight loss caused by brown-rot decay of control and treated (1% NHA) southern pine sticks (Ref. 64)

Table 4—Weight loss of unleached and leached southern pine in AWPA soil–block test

<table>
<thead>
<tr>
<th>NHA (%)</th>
<th>S. incrassata (MAD-563)</th>
<th>G. trabeum (MAD-617)</th>
<th>P. placenta (MAD-698)</th>
<th>T. versicolor (MAD-697)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>53.6 ± 0.9</td>
<td>57.8 ± 4.5</td>
<td>49.0 ± 1.8</td>
<td>45.6 ± 5.1</td>
</tr>
<tr>
<td>0.5 unleached</td>
<td>1.5 ± 0.2</td>
<td>23.1 ± 1.8</td>
<td>15.9 ± 7.0</td>
<td>15.4 ± 2.5</td>
</tr>
<tr>
<td>0.5 leached</td>
<td>8.7 ± 9.8</td>
<td>20.4 ± 6.6</td>
<td>14.6 ± 8.6</td>
<td>15.5 ± 4.3</td>
</tr>
<tr>
<td>1.0 unleached</td>
<td>1.4 ± 0.1</td>
<td>10.0 ± 4.8</td>
<td>3.6 ± 1.3</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>1.0 leached</td>
<td>3.3 ± 2.6</td>
<td>5.4 ± 2.7</td>
<td>1.4 ± 0.1</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>2.0 unleached</td>
<td>1.5 ± 0.2</td>
<td>2.7 ± 0.6</td>
<td>1.6 ± 0.1</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>2.0 leached</td>
<td>1.1 ± 0.2</td>
<td>3.0 ± 0.6</td>
<td>1.2 ± 0.3</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

*Mean and standard deviation of four replicates.*
Figure 7. Electron micrographs of calcium oxalate crystals of *P. placenta* *in situ* (ESEM) and *in vitro* (SEM, lower right). Internal monohydrate (oxalic acid) crystals—upper right and left external dihydrate (oxalic acid) crystals—lower right and left.

Figure 8. Scanning electron micrographs of bordered pits of untreated (left) and pretreated (1% NHA; right) southern pine.
Southern pine wood blocks treated with 1% NHA were examined by scanning electron microscopy for evidence of calcium precipitation and localization. Cuboidal crystals of calcium-NHA were observed (42) clustered on the torus of the bordered pit membranes (Fig. 8). Militz and Homan (63) observed similar localization of calcium oxalate sealing the pores of the pit membranes of spruce sapwood following treatment with 1% oxalic acid.

CONCLUSIONS

Because present wood preservatives pose a threat to the environment during treatment and disposal, there is an urgent need for new, sharply targeted, and benign wood-preserving methods. Oxalic acid, pectinases, and calcium appear to be critical elements of brown-rot wood decay. Treatment of wood with materials that interfere with these important components of the decay process should provide a more specific means of preventing wood decay. The long-term goal of this research is to determine the mechanism by which the calcium trapping agent N,N-naphthaloylhydroxylamine (NHA) inhibits fungal decay in wood. This will determine whether the inhibitory effects are directly toxic to the fungi or if indirect effects of calcium precipitation in wood prevent depolymerization and subsequent decay. In preliminary tests, NHA did not inhibit mold and sapstain fungi on wood, suggesting that direct toxic effects do not apply to all types of fungi, but may interfere only with the brown-rot/white-rot decay mechanism via calcium binding and interference with calcium cycling. In concert, these experiments may develop important information on what new substrate targets in wood can potentially inhibit wood decay and how related chemicals precipitate in wood and resist leaching. Treating wood with agents targeted specifically to the mechanism of colonization should ultimately lead to environmentally friendly protection of our valuable wood resource.

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Pectin degradation in brown-rot decay

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