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Biodegradation of lignin-carbohydrate complexes

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Abstract

Covalent lignin-carbohydrate (LC) linkages exist in lignocellulose from wood and groups herbaceous plants. In wood, they consist of ester and ether linkages through sugar hydroxyl to the α-carbanol of phenylpropane subunits in lignin. In grasses, ferulic and *p*-coumaric acids are esterified to hemicelluloses and lignin, respectively. Hemicelluloses also contain substituents and side groups that restrict enzymatic attack. Water-soluble lignin-carbohydrate complexes (LCCs) often precipitate during digestion with polysaccharidases, and the residual sugars are more diverse than the bulk hemicellulose. A number of microbial esterases and hemicellulose polysaccharidases including acetyl xylan esterase, ferulic acid esterase, and *p*-coumaric esterase attack hemicellulose side chains. Accessory hemicellulases include α-L-arabinofuranosidase and α-methyl-glucuranosidase. Both of these side chains are involved in LC bonds. β-Glucosidase will attach sugar residues to lignin degradation products and when carbohydrate is attached to lignin, lignin peroxidase will depolymerize the lignin more readily.

Abbreviations: APPL – acid precipitable polymeric lignin; CBQase – cellobioquinone oxidoreductase; LC – lignincarbohydrate; LCC(s) – lignin-carbohydrate complex; DHP – Dehydrogenative polymerisate; DMSO – dimethylsulfoxide; DP – degree of polymerisation; MWEL – milled wood enzyme lignin; MWL – milled wood lignin (not digested with carbohydrases)

Introduction

This review explores the characteristics and biodegradation of bonds between lignin and carbohydrate. Lignin-carbohydrate complexes (LCCs) can be isolated as water-soluble entities from the walls of gymnosperms, angiosperms, and graminaceous plants (Azuma & Koshijima 1988), and they can be separated by gel filtration into three fractions. The component of lowest molecular weight consists mostly of carbohydrate, the two larger components mostly of lignin. Lignin-carbohydrate bonds are presumed to exist in higher molecular weight lignin fractions that are water insoluble. Softwood LCCs are distinct in that their carbohydrate portions consist of galactomannan, arabino-4-*O*-methylglucuronoxylan, and arabinogalactan linked to lignin at benzyl positions (Azuma et al. 1981; Mukoyoshi et al. 1981). In contrast, carbohydrate portions of hardwood and grass LCCs are composed exclusively of 4-*O*-methylglucuronoxylan and arabino-4-*O*-methylglucuronoxylan, respectively (Azuma & Koshijima 1988). *Trans-p*-coumaric and *p*-hydroxybenzoic acid are esterified to bamboo and poplar lignins, respectively (Shimada et al. 1971), and *trans*-ferulic acid is ether linked to lignin (Scalbert et al. 1985). Many different types of LC bonds have been proposed, but most evidence exists for ether and ester linkages.

Relatively little attention has been given to en-

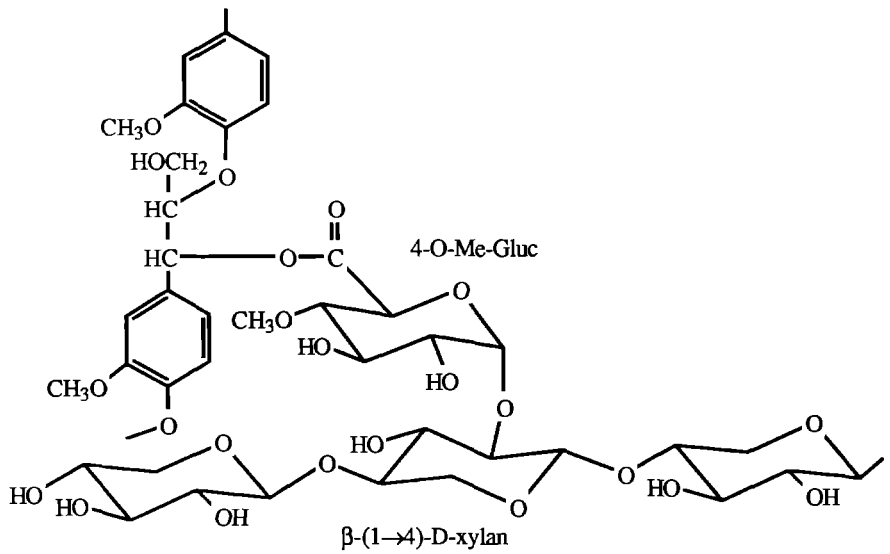

Fig. 1. Proposed structure of ester linkage between lignin and 4-*O*-methylglucuronoxylan in pine (after Watanabe & Koshijima 1988)

zymes capable of cleaving the chemical linkages between lignin and carbohydrate, the LC bonds. Such bonds occur in low frequency. They are heterogeneous; many are easily disrupted by acid or alkali during isolation, and they are still poorly defined. Although many sorts of linkages have been proposed, two have some substantive evidence. They link the α position of the phenyl propane lignin moiety to either carboxyl or free hydroxyls of hemicellulose through ester or ether linkages, respectively. Various chemical and enzymatic procedures have been used to isolate LC complexes, and a few biological systems have been shown to solubilize lignin preparations. No enzymes specific to LC bond cleavage have been described. The objective of this review is to focus our knowledge of the heterogeneous structures that comprise LC bonds, and to sort out the enzymes that attack related structures. A recent review of LCCs has been completed by Koshijima et al. (1989).

Chemical characteristics of LC bonds

Ester linkages (CO-O-C) occur between the free carboxy group of uronic acids in hemicellulose and the benzyl groups in lignin. Some are present as acetyl side groups on hemicellulose, others are between uronic acids and lignin, and still others occur between hemicellulose chains. Monomeric side chains in wood xylans consist of 4-*O*-methylglucuronic acid units, and some 40% of the uronic acid groups in birch are esterified. In beech, one-third of the glucuronic acids present in LCCs are involved in an ester linkage between lignin and glucuronoxylan (Takahishi & Koshijima 1988b). However, many glucuronic acid groups may be esterified within the xylan polymer (Wang et al. 1967).

Direct evidence for the chemical nature of ester linkages between lignin and carbohydrate in pine has been obtained through the selective oxidation of carbonyls in lignin. Watanabe & Koshijima (1988) proposed that the 4-*O*-methylglucuronic acid residue in arabinoglucuronoxylan binds to lignin by an ester linkage in *Pinus densiflora* wood. The linkage position is probably the α or conjugated γ position of guaiacylalkane units (Fig. 1). Watanabe et al. (1989) found that mannose, galactose, and glucose are *O*-6 ether linked and xylose is *O*-2 or *O*-3 ether linked to the a benzyl hydroxyl in a neutral fraction of pine LCC.

Watanabe et al. (1989) also studied alkali-stable LC linkages. The alkali-stable linkages in LCC prepared from *Pinus densiflora* consist of acetyl glucomannan and β-(1→ 4) galactan bound to the

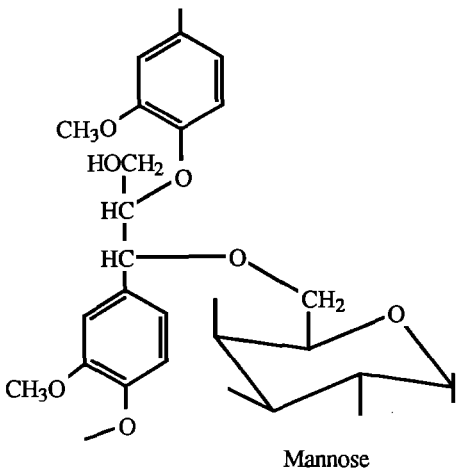

Fig. 2. Proposed structure for ether linkages between lignin and glucomannan of pine (after Watanabe et al. 1989).

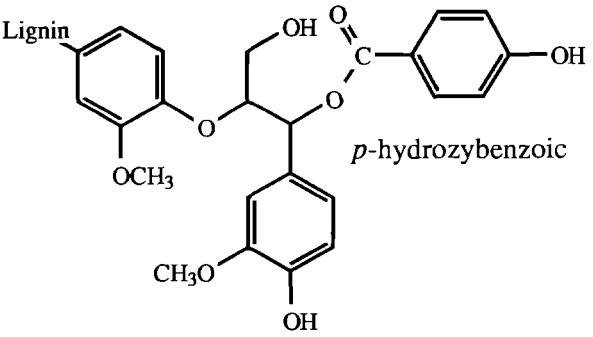

Fig. 3. Proposed structure for lignin-*p*-hydroxybenzoic acid ester in aspen.

lignin at the *O*-6 position of the hexoses (Fig. 2). The GC-MS analysis of methylated sugar derivatives led Watanabe et al. (1989) to conclude that the arabinoglucuronoxylan is bound to the lignin at the *O*-2 and *O*-3 positions of the xylose units, and that the linkage position in the lignin subunits is in the α or conjugated β positions of phenyl propane or propene units.

Minor (1982) investigated the LC bonds of loblolly pine MWEL to determine the positions of linkages in carbohydrates. The carbohydrates exist as oligomeric chains with degrees of polymerization of 7 to 14. Hexose units are bonded at *O*-6; L-arabinose is bonded exclusively at *O*-5. Galactan and arabinan are structurally of the 1→4 and 1→5 type, respectively, characteristic of the neutral substituents of pectins. In spruce, lignin is linked by ester bonds to 4-*O*-methylglucuronic acid, and arabinoxylan is linked through ether bonds (C-O-C) to the *O*-2 or *O*-3 positions of L-arabinose (Ericksson et al. 1980). For galactoglucomannan, ether bonds to position 3 of galactose have been indicated.

Esters of *p*-coumaric acid in sugar cane lignin and of *p*-hydroxybenzoic acid in aspen lignins were first demonstrated by Smith (1955) who proposed that the carboxy group of *p*-hydroxybenzoic acid is esterified to lignin in the α-position of the phenyl propane (Fig. 3).

Pectic substances may play an important role in binding lignin to the hemicellulose. Pectins are able to form both ester and ether linkages with lignin. LCC from birch contains about 7% galacturonic and 4% glucuronic acid, and a small amount of galacturonic acid is found in the LCC of spruce (Meshitsuka et al. 1983). Pectins are abundant in some fiberous plant materials such as mitsumata (*Edgeworthia papyrifera*). Fiber bundles are held together by pectic substances, and the pectins are aggregated with LCCs. Aggregates between lignin and pectins are particularly present in bast fibers, and *endo*-pectin and *endo*-pectate lyases from the soft-rot bacterium *Erwinia carotovora* release pectic fragments from this substrate (Tanabe & Kobayashi 1988). Alkaline presoaking accelerates biochemical pulping of mitsumata by pectinolytic enzymes (Tanabe & Kobayashi 1986, 1987).

Esterified *p*-coumaric acid can comprise 5% to 10% of the total weight of isolated grass or bamboo lignin (Shimada et al. 1971), but *p*-hydroxyphenyl glycerol-β-aryl ether structures are of minor importance (Higuchi et al. 1967). The majority of *p*-coumaric acid molecules in bamboo and grass lignins are ester-linked to the terminal γ carbon of the side chain of the lignin molecule (Shimada et al. 1971) (Fig. 4). The *p*-coumaric ester linkages are extremely stable.

p-Coumaric and ferulic acids are bifunctional, they are able to form ester or ether linkages by reaction of their carboxyl or phenolic groups, respectively. *p*-Coumaric is mainly associated with lignin; ferulic acid, on the other hand, is mainly esterified with hemicellulose (Scalbert et al. 1985;

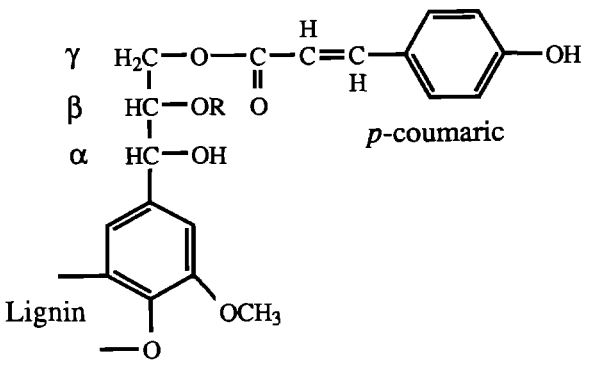

Fig. 4. Proposed *p*-coumaric ester linkage in grass lignins (after Shimada et al. 1971).

Atushi et al. 1984). Ferulic acid ethers might form cross links between lignin and hemicelluloses by the simultaneous esterification of their carboxyl group to arabinose substituents of arabinoglucuronxylan and etherification of their hydroxyl group to phenyl hydroxyls of lignin. Diferulic acids can be formed by polymerization with peroxidase/water, and studies suggest that these acids can crosslink hemicellulosic chains (Markwalder & Neukom 1976; Morrison 1974) (Fig. 5). Feruloylated arabinoxylans have been isolated from the LCC of bagasse (Kato et al. 1987), bamboo shoot cell walls (Ishii & Hiroi 1990), and pangola grass (*Digitaria decumber*) (Ford 1989) (Fig. 6).

The amount of carbohydrate remaining on lignin following exhaustive digestion with cellulases and hemicellulases can be measured by sugar analysis following acid hydrolysis. Obst (1982) found 10.8% carbohydrate in a MWEL from loblolly pine. A fraction of this amount (11%) was removed by dilute alkali. Obst estimated that there are approximately three LC bonds for every 100 phenylpropane units in lignin. The length of the side chains varies with the method by which MWEL is prepared. If extensive alkaline hydrolysis is performed prior to enzymatic digestion, a larger fraction of the residual carbohydrate is removed.

Biodegradation of LCCs

Enzymatic digestion of LCCs

Most carbohydrate chains or side groups appear to be attached to lignin through the non-reducing moieties. Because *exo*-splitting enzymes generally attack a substrate from the nonreducing end of a polysaccharide, removing substituents progressively toward the reducing end of the molecule, complete degradation is not possible. Even when carbohydrates are attached to the lignin by the *O*-1 hydroxyl, a single sugar residue could remain attached even after complete attack by *exo*-splitting glycosidases.

The action of *endo*-splitting glycanases is even more constrained. The binding sites of most *endo*-splitting polysaccharidases have not yet been well characterized. However, from transferase activities and other kinetic studies, Biely et al. (1981) showed that the substrate binding site of the *endo*-xylanase from *Cryptococcus* has eight to ten subsites for binding pyranose rings and the catalytic groups that make up the active site are located in the center. It is not surprising, therefore, that digestion of LCCs with *endo*-xylanases and *endo*-cellulases leaves residual polysaccharide oligomers with a degree of polymerization (DP) of 4 or more attached to the lignin moiety.

The action of an *endo*-xylanase purified from a commercial preparation from *Myrothecium verrucaria* illustrates the effects of substrate binding (Comtat et al. 1974). This preparation will cleave xylose residues from the nonreducing end of aspen 4-*O*-methylglucuronoxylan until it leaves a 4-*O*-methyl-α-D-glucuronic acid substituent linked 1→ 2 to a β-(1→ 4)-xylotriose. The enzyme cannot cleave the two β-(1→ 4)-D-xylan linkages immediately to the right of the 4-*O*-methylglucuronic acid side chain (in the direction of the reducing end). The residual xylotriose represents that portion of the substrate that is bound but that the enzyme cannot cleave. Presumably, other *endo*-splitting enzymes encounter even more extreme difficulties when carbohydrate is chemically linked adjacent to the lignin polymer because the side chains are generally greater than four residues. This could also be

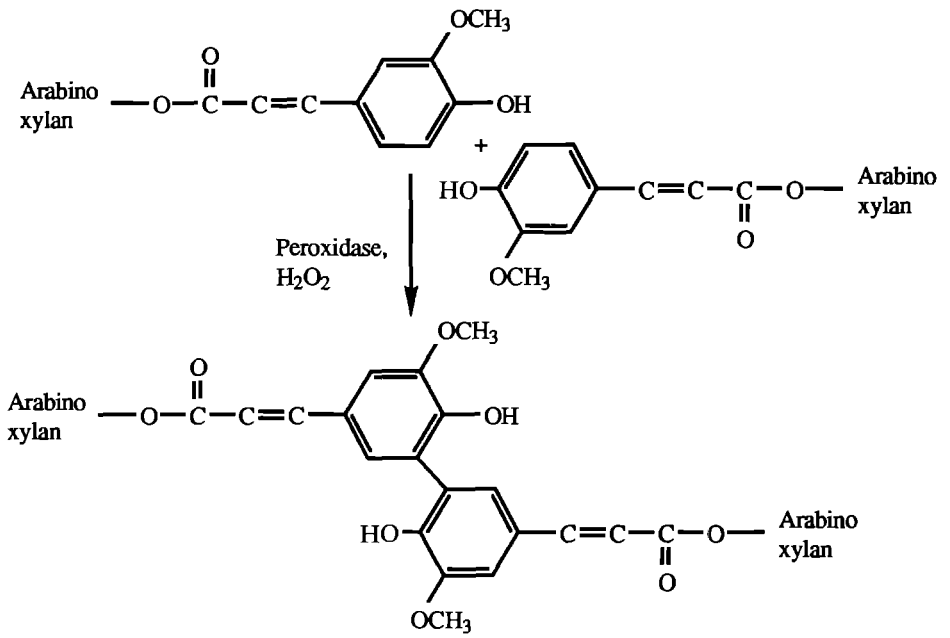

Fig. 5. Formation of diferulic acid in grasses (after Markwalder & Neukom 1975).

attributable to steric hindrance or to the presence of multiple cross-links between the lignin and carbohydrate polymers.

If there is low enzymatic activity for a particular side chain, longer hemicellulosic branches are produced. This was evidenced with the apparent enrichment of galactomannan during enzymatic digestion of Norway spruce (Iversen et al. 1987). The low activity of β-(1→ 4)-D-galactanase in enzyme preparations from *Trichoderma reesei* and *Aspergillus niger* led to the enrichment of galactomannan in the MWEL. One must be on guard against these possibilities when studying the structures of lignin complexes prepared by enzymatic digestion.

Through selective enzymatic degradation, it should be possible to identify which carbohydrates form LC linkages. Joseleau & Gancet (1981) took such an approach in characterizing residual carbohydrate after enzymatic digestion, alkaline hydrolysis (0.5 M NaOH), and mild acid hydrolysis (0.01 M oxalic) of an LCC isolated from aspen by dimethylsulphoxide (DMSO) extraction. The original LCC contained mostly carbohydrate, and the final material was predominantly lignin. Enzyme treatments and alkali extraction removed the bulk of the xylose and glucose while increasing the relative portions of arabinose and galactose. Rhamnose was also very persistent; its fraction relative to other sugars increased appreciably, indicating that rhamnose might be involved in an LC bond. Uronic acid likewise was significantly represented in the enzyme-, alkali-, and acid-treated material. It is not known whether uronic acid remains associated because of cross-linkages between lignin and carbohydrate or simply because of the stability of glycosidic linkages between uronic acids and xylan.

Residual LC structures after exhaustive enzymatic digestion

The presence of lignin, aromatic acids, and other modifications of hemicellulose clearly retards digestion of cellulose and hemicellulose by ruminants. Phenolic acids associated with forage fiber are known to reduce fiber digestion when they are in the free state. *p*-Coumaric, ferulic, and sinapic acids inhibit the activity of rumen bacteria and anaerobic fungi (Akin & Rigsby 1985). It seems likely, however, that the cross-linkages that these acids establish between hemicellulose chains and the lignin polymer are more important than is the

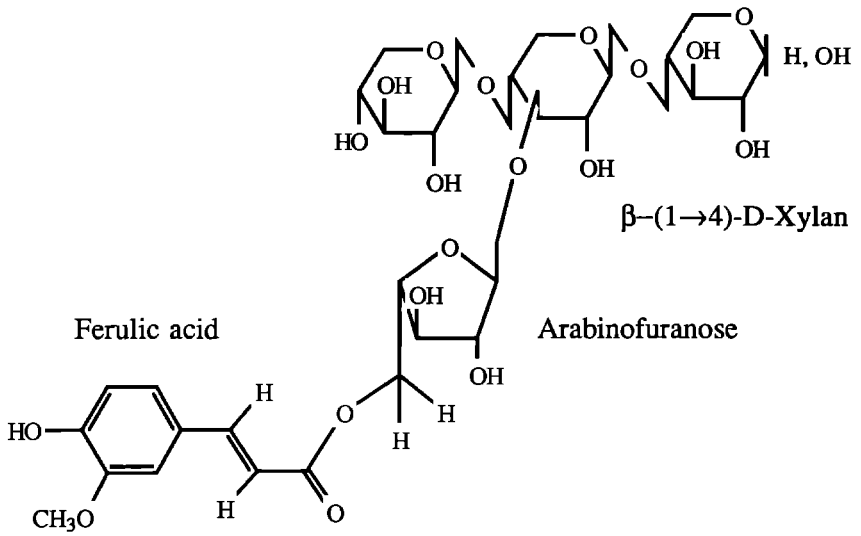

Fig. 6. Ferulic acid ester linkage to grass arabinoxylan (after Kato et al. 1987).

toxicity attributable to the monomers themselves because ruminant digestibility is greatly enhanced by alkali treatment, regardless of whether the phenolic acids are washed out of the residue.

In a comparison of different ruminant feeds, an increase in the extent of substitution at the *O*-5 position of arabinose correlated with the amount of residual material in digested residues (Chesson et al. 1983). The influence of the lignin, however, was out of proportion to its presence in the feed (Chesson 1988). When a steer digests grass, LCCs are solubilized in the rumen. This dissolution accounts for about half the total lignin intake (Gaillard & Richards 1975). At least two studies of LCCs isolated from ruminants have been published (Neilson & Richards 1982; Conchie et al. 1988). The amounts of residual carbohydrate in the LCC isolated from ruminants are relatively low (7.7% from bovine; 5.5% from sheep), and the glycosidic chains are relatively short. Methylation analysis indicates that unlike MWEL, the predominant side chain on rumen LCC is a single glucose residue bound glycosidically to the residual polyphenol. It is unclear how such a structure might arise. Other branched chain structures are also observed, some of which are linked into the polyphenol at more than one location (Fig. 7). As in the case of MWEL, oligoxylosyl residues are also present, some of which are substituted with L-arabinose. Because of the anaerobic conditions that exist in the rumen, relatively little degradation of lignin is believed to occur.

The influence of LCCs on ruminant digestion was studied by examining the solubilization of LCCs using cell-free hemicellulase complexes from the rumen (Brice & Morrison 1982). LCCs from grasses of increasing maturity were isolated and treated with cell-free rumen hemicellulases. As the lignin content increased, the extent of degradation declined, indicating that the lignin content of the LC was the overriding factor in determining its digestibility.

Degradation of lignin does not seem to occur in the anaerobic environment of the rumen, even though substantial solubilization takes place. This observation was recently confirmed using an artificial rumen reactor (Kivaisi et al. 1990). The supernatant solutions of effluents contained lignin-derived compounds that were released by rumen microorganisms.

Solubilization of LCC by microbial activity

In recent years, studies on the solubilization of lignin from grasses or wood labeled with ^{14}C phenylalanine have proliferated (Crawford 1978; Reid

et al. 1982; McCarthy et al. 1984). The bulk of the radioactive label is incorporated into the lignin rather than into carbohydrate or protein of the plant, but it is clear that lignin purified from the labeled plant tissue contains significant amounts of carbohydrate. This material is probably closer to the structure of native lignin than is synthetic lignin prepared by in vitro dehydrogenative polymerization of coniferyl alcohol (Kirk et al. 1975).

Commonly, biodegradation of the MWL is followed by trapping the $^{14}CO_2$ respired from active cultures. Between 25% and 40% of the ^{14}C added to active cultures can be recovered as $^{14}CO_2$. Given that a fully aerobic organism will respire about half the carbon provided to it while incorporating the other half as cellular material, this represents the metabolism of 50% to 80% of the total lignin present. However, not all the ^{14}C lignin added to cultures is released as $^{14}CO_2$. A significant fraction of the total lignin – as much as 30% – can be recovered from solution as a polymer (Crawford et al. 1983). This material precipitates from culture filtrates following acidification to pH 3 to 5. In this characteristic, it is similar to the LCC solubilized in the rumen of cattle (Gaillard & Richards 1975). The solubilized acid precipitable polymeric lignin (APPL) has an apparent molecular weight of ≥ 20 kDa and shows signs of partial degradation. *Phanerochaete chrysosporium* will form water-soluble products from lignin isolated from aspen (Reid et al. 1982) or wheat seedlings (McCarthy et al. 1984), as will a number of actinomycetes. Organisms reported to solubilize grass lignins include *Streptomyces viridosporus, S. baddius* (Pometto & Crawford 1986), *S. cyanus, Thermomonospora mesophila,* and *Actinomadura* sp. (Mason et al. 1988; Zimmerman & Broda 1989; Mason et al. 1990).

Many different enzymatic activities from these organisms have been reported, including activities of *endo*-glucanase, xylanase, several esterases, and an extracellular peroxidase (Ramachandra et al. 1987). The roles that these enzymes play in lignin solubilization are not yet entirely clear, but various correlations have been made between the appearance of extracellular peroxidase activity and lignin solubilization or mineralization. The streptomycete lignin peroxidase has been purified and partially characterized (Ramachandra et al. 1988). It is a heme protein with an apparent molecular weight of 17.8 kDa. As such, it is appreciably smaller than the 42 kDa lignin peroxidase or the 46 kDa manganese peroxidase described in *P. chrysosporium* and other white-rot fungi (Tien & Kirk 1983; Tien & Kirk 1984; Paszczynski et al. 1985). The streptomycete enzyme has been reported to cleave a β-aryl ether model dimer in a manner similar to that observed for *P. chrysosporium*.

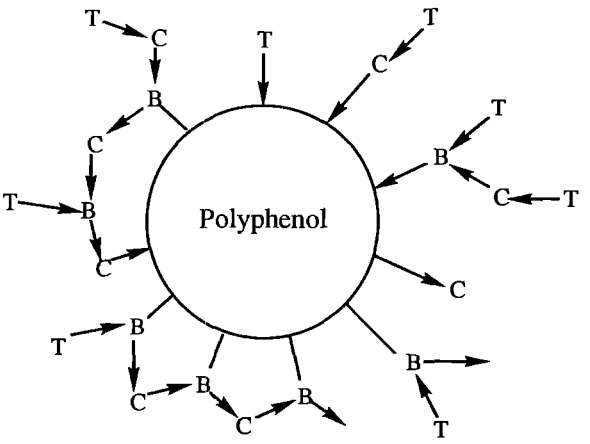

Fig. 7. Proposed structure for residual lignin carbohydrate complex from sheep rumen: T non-reducing terminal sugar residue; C chain residue; B branch point; → glycosidic linkage (arrow pointing away from C-1); — ether linkage; → free reducing group (after Conchie, Hay & Lomax 1988).

The enzyme (or enzymes) responsible for solubilizing lignin has not been fully characterized. Strains of streptomyces that produce significant quantities of APPL also produce peroxidases and cellulases (Adhi et al. 1989). With *T. mesophila*, the ability to solubilize labeled wheat lignocellulose is extracellular and inducible, but it does not correlate with xylanase or cellulase production (McCarthy et al. 1986).

Zimmerman and Broda (1989) recently reported the solubilization of lignocellulose from undigested ball-milled barley by the extracellular broth from *S. cyanus, T. mesophila,* and *Actinomadura* sp. Ground and ball-milled barley straw samples were incubated with cultures of these organisms, and weight losses were recorded. Such solubilization

might simply represent the release of low molecular weight ^{14}C-labeled lignin from the carbohydrate complex. This could occur if a portion of the lignin is released as the carbohydrate is removed. Mason et al. (1988), working in the same laboratory, described the production of extracellular proteins in the broths of these cultures. They assayed fractions from a sizing gel and found that the solubilizing activity had an apparent molecular weight of about 20,000 – similar to the peroxidase reported by Ramachandra et al. (1988). Because all the xylanases and cellulases present had an apparent molecular weight of about 45,000, the authors concluded that the solubilization activity is unlikely to be from a cellulase or xylanase. An international patent application for the use of a cell-free enzyme from *S. cyanus* for the solubilization of lignocellulose has been filed. (Broda et al. 1987).

Esterases from *S. viridosporus* were reported to release *p*-coumaric and vanillic acid into the medium when concentrated, extracellular enzyme was placed on appropriately labeled substrates (Donnelly & Crawford 1988). At least eight different esterases were described. When grown on lignocellulose from corn stover, *S. viridosporus* T7A and *S. badius* 252 produced endoglucanase, xylanase, and lignin peroxidase activity (Adhi et al. 1989). Since the *p*-coumaric acid is known to be esterified essentially only to the lignin, these organisms apparently attack that substrate. In the process, they produce an APPL that can be detected in supernatant solutions.

In summary, research on the solubilization of LCC by microbial activity has periodically shown that cellulases, hemicellulases, esterases, and perhaps peroxidases all correlate with lignin solubilization. The mineralization rates and extents reported for streptomyces are relatively low, and the solubilized lignin is not extensively modified. Lignin mineralization and solubilization could, therefore be attributable to two (or more) different enzymes. These studies require more rigorous clarification.

Accessory enzymes for hemicellulose utilization

Substantial attention has been given to the principal enzymes involved in lignocellulose utilization, particularly cellulases, xylanases, and, more recently, peroxidases. A number of enzymes, however, appear to be critical in the early steps of hemicellulose utilization. These include acetyl xylan esterases, ferulic and *p*-coumaric esterases, α-L-arabinofuranosidases, and α-4-*O*-methyl glucuranosidases.

Acetyl xylan esterase was first described by Biely et al. (1985) in several species of fungi known to degrade lignocellulose and most especially in *A. pullulans*. Acetyl xylan esterase was subsequently described in a number of different microbes including *Schizophylum commune* (MacKenzie & Bilous 1988), *Aspergillus niger* and *Trichoderma reesei* (Biely et al. 1985; Poutanen & Sundberg 1988), *Rhodotorula mucilaginosa* (Lee et al. 1987) and *Fibrobacter succinogenes* (McDermid et al. 1990), and various anaerobic fungi (Borneman et al. 1990). Acetyl xylan esterase acts in a cooperative manner with endoxylanase to degrade xylans (Biely et al. 1986). This enzyme is not involved in breaking LC bonds since the acetyl esters are terminal groups.

Many different esterase activities have been described, but it has not always been apparent that the assay employed was specific for physiologically important activities. Particular care must be taken in using 4-nitrophenyl acetate as an analog of acetylated xylan since activities against 4-nitrophenyl acetate and acetylated xylan may show no correlation (Khan et al. 1990).

Relatively little is known about enzymes that are capable of releasing aromatic acids from hemicellulose. The substrates are often poorly defined, and most enzymes have been obtained only in crude preparations. Ferulic and *p*-coumaric acid esterases have been identified in extracellular broths of *S. viridosporus* (Deobald & Crawford 1987), but the activities have not always been specific (Donnelly & Crawford 1988). MacKenzie et al. (1987) first assayed for ferulic acid esterase from *Streptomyces flavogrieseus* using native substrate. In this assay, the ferulic acid was identified by HPLC. Ferulic

acid esterase is produced along with α-L-arabinofuranosidase and α-4-*O*-methylglucuronidase by cells growing on xylan-containing media. With *S. flavogriseus*, oat spelts xylan was a much stronger inducer for these enzymes than cellulose. However, not all organisms respond in this manner. *Schizophyllum commune*, for example, produced more xylanase and acetyl xylan esterase when grown on avicel cellulose than when grown on xylan (MacKenzie & Bilous 1988).

The ferulic acid esterase of *S. commune* exhibits specificity for its substrate, and it has been separated from other enzymes. Borneman et al. (1990) assayed feruloyl and *p*-coumaryl esterase activities from culture filtrates of anaerobic fungi using dried cell walls of Bermuda grass (*Cyndon dactylon* [L] Pers) as a substrate. The enzyme preparations released ferulic acid more readily than they released *p*-coumaric acid from plant cell walls. Assays using methyl ferulate or methyl *p*-coumarate as substrates in place of dried cell walls showed the presence of about five times as much enzyme activity. McDermid et al. (1990) employed ethyl esters of *p*-coumarate and ferulate as substrates for these activities.

The use of a realistic model substrate can greatly facilitate purification and kinetic studies. Recently, Hatfield, Helm & Rolph (pers. comm.) synthesized 5-*O*-trans-feruloyl-α-L-arabinofuranoside in gram quantities for use as an enzyme substrate. Progress of the reaction can be followed by HPLC or TLC. The substrate is soluble in water and can be readily used for kinetic studies. Much more work needs to be done in this area, particularly with the synthesis of lignin-hemicellulose esters as model substrates.

α-L-Arabinofuranosidase catalyzes the hydrolysis of nonreducing terminal α-L-arabinofuranoside linkages in L-arabinan or from D-xylan. This enzyme has been recognized for a number of years, and the characteristics of α-L-arabinosidases of microbial and plant origin have been reviewed (Kaji 1984). Since these enzymes act primarily on terminal arabinose side chains, they are probably not directly involved in removing carbohydrate from lignin. The α-(1→ 3) linkage is hydrolyzed relatively easily. An enzyme capable of acting on L-arabinofuran substituted at *O*-5 would be of potential interest.

The α-(1→2)-4-*O*-methylglucuronic acid sidechain resists hydrolysis by xylanases (Timell, 1962), and for that matter by acid. In fact, the presence of the 4-*O*-methylglucuronic acid group stabilizes nearby xylosidic bonds in the xylan main chain, and the substituent must be removed for xylan hydrolysis to proceed. Puls et al. (1987) described a-glucuranosidases from two basidiomycetes, *Agaricus bisporus* and *Pleurotus ostreatus*; the enzyme from *A. bisporus* was partially characterized. This enzyme is evidently not one of the first to attack the xylan polymer because it is relatively large (≈ 450 kDa). Activity was optimal at pH 3.3 and 52° C. α-Glucuronidase acts in synergism with xylanases and β-xylosidases to hydrolyze glucuronoxylan. The yield of xylose greatly increases in the presence of this enzyme.

Most filamentous fungi appear to be poor producers of α-methylglucuronidases. Ishihara and Shimizu (1988) systematically screened α-glucuronidase-producing fungi in order to identify other enzymes. Of nine *Trichoderma* and five basidiomycete species (including a strain of *A. bisporus*), *Tyromyces palustris* was the best producer. Concentrated protein precipitates from cell broths were screened against a model substrate of 2-*O*-(4-*O*-methyl-α-D-glucuronopyranose)-D-xylitol. This enabled the detection of reducing group production against a very low background. Even though *T. palustris* produced more α-glucuronidase than did other fungi, total activity amounted to < 0.1 unit/ml. Moreover, the activity was very labile, even in frozen storage. *Streptomyces flavogriesus* and *S. olivochromogenes* also formed α-*O*-methylglucuronidase at low titers (MacKenzie et al. 1987; Johnson et al. 1988).

Role of glycosides in lignin degradation

Several recent reports indicate that glycosides might be involved in lignin biodegradation. Kondo & Imamura (1987) first reported that when vanilyl alcohol or veratryl alcohol were included in glucose- or cellobiose-containing media that had been

inoculated with wood-rotting fungi, lignin glucosides were formed in the cellulose medium during the early phases of cultivation. Such glucosides could also be formed using a commercial β-glucosidase in place of the culture broth. β-Glucosidase biosynthesized six monomeric glucosides from lignin model compounds and cellobiose (Kondo et al. 1988). These compounds are linked through position *O*-1 of the sugar moiety to alcoholic (but not phenolic) hydroxyls of the lignin model.

In another study by Kondo & Imamura (1989a), three lignin model compounds, 4-*O*-ethylsyringylglycerol-β-syringyl ether, veratryl alcohol, and veratraldehyde, were degraded by *P. chrysosporium* and *Coriolus versicolor* in media containing either monosaccharides or polysaccharides as carbon sources. The authors found that the rate of consumption of the lignin models was much faster in polysaccharide than monosaccharide media. In media containing xylan or holocellulose, veratryl alcohol was transformed predominantly into veratryl-*O*-β-D-xyloside, which then disappeared rapidly from the medium. Veratraldehyde was first reduced to veratryl alcohol, then glycosylated, and finally consumed.

That carbohydrate is essential for the mineralization of lignin models (Kirk et al. 1976) and that the expression of lignin biodegradation is regulated by carbon catabolite repression (Jeffries et al. 1981) have been known for a long time. However, the role of carbohydrate in the assimilation of lignin degradation products has been demonstrated only recently.

One of the first enzymes implicated in lignin biodegradation was cellobiose : quinone oxidoreductase (CBQase). This enzyme catalyzes the reduction of a quinone and the simultaneous oxidation of cellobiose. Westermark & Ericksson (1974a,b) discovered this enzyme and proposed that its role might be to prevent repolymerization of lignin during degradation. More recent studies (Odier et al. 1987) have not borne this out, but the enzyme may be important nonetheless. The CBQase of *P. chrysosporium* binds very tightly to microcrystalline cellulose, but such binding does not block its ability to oxidize cellobiose, indicating that the binding and catalytic sites are in two different domains (Renganathan et al. 1990).

An essential feature of lignin biodegradation is that degradation products resulting from the activity of extracellular enzymes must be taken up by the mycelium; glycosylation by β-glucosidase seems to be an important part of this process. Whether or not sugars attached to lignin in the native substrate by nonglycosidic linkages play a similar role has not been addressed.

Glycosylation could also serve to detoxify lignin degradation products. Veratryl alcohol and vanilyl alcohol, for example, are toxic to the growth of *C. versicolor* and *T. palustris*, whereas the toxicity of the glycosides of these compounds is greatly reduced (Kondo & Imamura 1989b). The presence of a glycosyl group has also been shown to prevent polymerization of vanillyl alcohol by phenol oxidase.

Kondo et al. (1990) recently showed that glycosides can facilitate the depolymerization of dehydrogenative polymerisate (DHP) by lignin peroxidase of *P. chrysosporium* and reduce repolymerization by laccase III of *C. versicolor*. To clarify the role of glycosylation in lignin degradation, DHP and DHP-glucosides were treated with horseradish peroxidase, commercial laccase, laccase III of *C. versicolor*, and lignin peroxidase of *P. chrysosporium*. During oxidation, DHP changed color and precipitated whereas DHP glucoside only changed color. Moreover, molecular weight distribution studies showed that oxidation of DHP by enzyme preparations containing laccase or peroxidase resulted in polymerization rather than depolymerization. In contrast, enzymatically catalyzed depolymerization of DHP glucoside was observed. DHP-glucoside was also depolymerized more extensively by peroxidase than by laccase.

Enzymatic treatments of pulps

For many applications, residual lignin in kraft pulp must be removed by bleaching. Successive chlorination and alkali extraction remove the remaining lignin to leave a bright, strong pulp suitable for

printing papers and other consumer products. Although chlorine bleaching solves the immediate problem of residual lignin, the chlorinated aromatic hydrocarbons produced in the bleaching step are recalcitrant and toxic. These chlorinated products are hard to remove from waste streams and trace quantities are left in the paper, so other bleaching processes have been devised. One approach is to use hemicellulases to facilitate bleaching.

Several different research groups have found that the bleaching of hardwood and softwood kraft pulp can be enhanced by xylanase. The xylanase treatment reduces chemical consumption and kappa number and increases brightness. Pine kraft pulp was delignified $>50\%$ following hemicellulase treatment and oxygen bleaching (Kantelinen et al. 1988). Small amounts of lignin were released by the enzyme treatment alone. Fungal xylanase from *Sporotricum dimorphosum* lowered the lignin content of unbleached softwood and hardwood kraft pulps (Chauvet et al. 1987). Hemicellulases having different specificities for substrate DP and side groups have been used, but more data are needed on the effects of mannanases, cellulases, and other enzymes. Enzyme-treated paper sheets show slight decreases in interfiber bonding strength. The mechanical strength of fibers is not affected, but interfiber bonding decreases if cellulases are present. This has been confirmed with the pretreatment of pulp with a xylanase from the thermophilic actinomycete *Saccharomonospora viridis* (Roberts et al. 1990). Viscosity decreases with some enzyme treatments, but only limited hemicellulose hydrolysis is necessary to enhance lignin removal. In the absence of cellulase, xylanase treatment increases viscosity. Pulps treated with cloned xylanase from *Bacillus subtilis* have retained viscosity and strength properties while lignin removal has been facilitated (Jurasek & Paice 1988; Paice et al. 1988a,b).

Conclusions

Covalent lignin-carbohydrate linkages can be ester bonds through the free carboxy of uronic and aromatic acids or ether linkages through sugar hydroxyls. Sugar hydroxyls include the primary hydroxyl of L-arabinose (*O*-5) or of D-glucose or D-mannose (*O*-6) or the secondary hydroxyl, as in the case of the *O*-2 or *O*-3 of xylose. Some linkages through the glycosidic hydroxyl (*O*-1) also appear to exist. Ester linkages can occur through the carboxyl group of uronic, ferulic, *p*-coumaric or *p*-hydroxybutyric acids. In all cases, the α-carbon of the phenylpropane subunit in lignin appears to be involved in native lignin-carbohydrate bonds, but further study is needed. Many ester linkages are disrupted by mild alkali, but a significant number of alkali-stable bonds are present. Although some microbial esterases and other enzymes have been shown to attack and solubilize lignin from lignocellulose, their substrate specificities have not been fully characterized. The positive effect of glycosylation on assimilation and degradation of lignin model compounds is supported by long-standing evidence that carbohydrate is necessary for lignin mineralization. The role of carbohydrates linked by non-glycosidic bonds remains to be clarified. It is not clear what role, if any, uronic and aromatic acid esters might play in facilitating or hindering lignin biodegradation. To date, no enzyme has been shown to cleave any bond between polymeric lignin and carbohydrate.

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