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Cell Wall Structural Foundations: Molecular Basis for Improving Forage Digestibilities

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ABSTRACT

Forages play an important role in the world wide animal industry. The fiber (cell wall) portion makes up 300 to 800 μg g^{-1} of forage dry matter and represents a major source of nutritional energy for ruminants, but, unfortunately, less than 50% of this fraction is readily digested and utilized by the animal. Significant progress has been made in the past 30 to 40 yr towards understanding cell wall structure and function and developing mechanistic models that explain limitations to structural polysaccharide degradation and utilization by ruminants. In grasses, it is now clear that wall bound ferulates play a key role in cross-linking xylans to each other and to lignin, resulting in less degradable walls. Much has been accomplished in advancing our understanding of the lignification process in plants, particularly the genes and enzymes involved in the monolignol biosynthesis. The application of molecular techniques to this area has advanced our understanding of the metabolic process while providing tools for further exploration of wall structure and function and providing direction as to possible avenues to improve forage digestibility.

FORAGES PLAY an important role in the world wide animal industry. The fiber (cell wall) portion makes up 300 to 800 μg g^{-1} of forage dry matter and represents a major source of nutritional energy for ruminants, but, unfortunately less than 50% of this fraction is readily digested and utilized by the animal. Hence, cell walls are a controlling factor in determining the quality of forages. If a greater percentage of this potential energy was made available to the animal (i.e., increase the digestibility of the cell wall fraction), there would be a considerable positive economic impact. For example, in the U.S. dairy industry, a 10% increase in wall digestion would result in an additional $380 million in milk and meat sales while reducing manure solids by 2.3 million megagrams and grain input into the diet by 3.0 million megagrams. In this section, we will review research conducted in the past 40 to 50 yr on the chemistry and biochemistry of forage cell walls that impacts forage digestibility.

The structural and functional roles of plant cell walls (including utilization of wall polysaccharides by ruminants) are controlled by the composition and organization of individual wall components. Cell walls are composed of structural polysaccharides with varying compositions and structures, hydroxycinnamic acids, lignin, protein (both metabolic and structural), ions, and water. Component interactions, especially specific types of covalent linkages, control cell wall organization and structural integrity but may also control wall expansion during growth and degradation for herbivore utilization.

Milestones of Forage Cell Wall Chemistry and Biochemistry

When it comes to milestones in forage cell wall chemistry and biochemistry, it is a bit difficult to pinpoint critical events. R.L. Reid (1994) in discussing milestones in forage research states, "The incidence of research milestones, considered as discrete and revolutionary events, may be about as frequent as the diminishing occurrence of physical milestones on the American landscape. More often, advances in research occur in small and incremental steps, sometimes with obscure or unknown origins, but generally as variations on a

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preceding theme." This is a fitting description for forage wall chemistry and biochemistry in that progress is often incremental but may at times be exponential, with breakthroughs coming in rapid succession, greatly advancing the whole field. Is the milestone the exponential events or an incremental event that may have started the whole process? Although the leaps forward seem highly significant, they did not happen independently and indeed required the incremental advancements that preceded them. Rather than trying to make a judgment as to what events have been most critical in wall chemistry and biochemistry, we will look at a few critical aspects of cell wall chemistry and biochemistry and focus on a major theme that has dominated wall research.

Forage quality is dependent upon several factors and their interactions (see Fahey and Hussein in this review series). Progress has been made in removing or minimizing anti-quality factors found in some forages [e.g., formononetin in red clover (*Trifolium partense* L.), endophyte toxicity in fescue (*Festuca arundinacea* Schreb.); for other examples, see Casler and Vogel in this review series]. However, it is obvious that an overriding factor in improving forage quality requires altering the cell wall fraction of the total plant to provide more energy to the animal. Typical approaches have been to decrease the cell wall concentration while maintaining total biomass production. This has not always been an obtainable goal (Casler and Vogel, this series). The alternative is to alter the digestibility of the structural polysaccharides that make up forage walls, providing a more efficiently utilized nutrient source. Searching for the molecular mechanisms controlling wall degradation has been the main focus of chemical and biochemical studies over the past 40 yr.

Cell Wall Components

Carbohydrates

Structural polysaccharides can have quite complex molecular structures, yet if removed from the wall they are readily degraded to their component monosaccharides by microbial enzymes. However, when left within the wall matrix polysaccharide degradation is restricted and in some cases, such as the xylans in legumes, nearly escape ruminal degradation (Buxton and Brasche, 1991; Hatfield and Weimer, 1995). An exception is the pectic polysaccharides that are rapidly and extensively degraded from wall matrices, including heavily lignified stems (Chesson and Monro, 1982; Dehority et al., 1962; Hatfield and Weimer, 1995). Degradation is not only rapid but does not result in a decrease in ruminal pH that is often seen with rapidly degraded starch (Hatfield and Weimer, 1995). Pectic polysaccharides form a significant portion of alfalfa (*Medicago sativa* L.) cell walls (100–300 $\mu g\ g^{-1}$) (Hatfield, 1992) but from a nutritional point of view have been lumped in with the soluble fraction of forages. This is unfortunate because it has resulted in this wall fraction being ignored as a possible means of improving wall digestibility. Unlike cytoplasmic carbohydrates that are subject to metabolic turnover, pectic polysaccharides remain constant once incorporated into the wall. A small sampling of alfalfa and red clover cultivars revealed a reasonable level of variability for total pectin in both stems and leaves, suggesting genetic selection for increased pectins should be feasible (Hatfield and Smith, 1995). If one selects for additional pectic polysaccharides, there may be a better chance of maintaining high total biomass production. Unfortunately, this selection strategy will only be fruitful for legume forages, since grasses typically have only 10 $\mu g\ g^{-1}$ or less of their dry matter composed of pectic polysaccharides.

Proteins

Proteins generally make up less than 50 $\mu g\ g^{-1}$ of the wall depending on the tissue type and maturity. As with polysaccharides, proteins outside the wall matrix are susceptible to degradation, but as a part of the total wall structure they may be completely resistant to degradation and pass intact through the digestive tract. The role of proteins bridges both metabolic and structural functions. Key enzymes are critical in the lignification process. For example, one-electron oxidation of not only monolignols but also ferulates (formation of radicals) is controlled within the wall by peroxidases and/or oxidases (O'Malley et al., 1993). For processes in which there is a need to tightly control radical formation (i.e., cross-coupling to form diferulates or coupling ferulates to monolignols), peroxidases offer plants considerable control. It is most likely that peroxidases are incorporated into the wall matrix and positioned within general areas critical for cross-linking. No reaction will occur until hydrogen peroxide (co-substrate) is supplied to initiate the one-electron oxidation of the phenol substrate, thus controlling the cross-coupling reaction until the appropriate time in wall development. Structural proteins, found in both monocot and dicot walls, appear to play critical roles in cross-linking wall components, particularly in primary walls. It is also possible that metabolic proteins become cross-linked to lignin resulting in a more structural function once wall development has reached a specific stage (Evans and Himmelsbach, 1991).

Lignin and Degradation

Although the wall is the sum of its parts and the interaction of individual components dictates structure and function, we can identify key elements having a pivotal role in regulating wall degradation. The culprit is lignin, with some assistance from molecules that cross-link lignin to other wall components. This is not to say that other components do not influence wall degradation. Indeed, the interactions among polysaccharides and proteins can impact degradation rates and extents of cell wall degradation (Hatfield, 1993). In the early 1900s, Waksman and Cordon (1938) recognized the inhibitory effect of lignin on cellulose degradation by soil microbes. Research establishing the negative correlation of lignin content in forage walls with in vitro wall degradation was not published until some 30 to 40 yr later (Johnson et al., 1962; Kamstra et al., 1958; Van

Soest et al., 1966). Since that time, researchers have pursued the underlying molecular mechanism restricting wall polysaccharide degradation. We will develop the lignin and cross-linking story in more detail.

What is Lignin?

Lignin is defined as "polymeric natural products arising from an enzyme-initiated dehydrogenative polymerization of three primary precursors" (Sarkanen and Ludwig, 1971). Peroxidases and/or oxidases react with lignin precursors (i.e., coniferyl, sinapyl, and *p*-coumaryl alcohols, Fig. 1) forming one-electron oxidized products that undergo radical coupling reactions to produce a growing polymer of lignin. In the past, the terms "core" and "non-core lignin" (Jung and Deetz, 1993) have been used to describe phenolic-based materials in forage plants. The term non-core lignin should not be used as it really applies only to the ester-linked hydroxycinnamates that are releasable with low temperature alkaline treatment. The hydroxycinnamates are structurally related to lignin precursors (Fig. 1) and they may be attached to lignin, playing critical roles in regulating wall matrix organization. Indeed, the strict definition for lignin given above may not be appropriate (only three precursors) as other components can become incorporated into the polymer we call "lignin." It would be better to define "lignin" with more of a functional definition as opposed to one strictly dependent on chemical composition; i.e., a phenolic-derived macromolecule (polymer) that interacts with other wall polymers to provide structural integrity, resistance to degradation, and water impermeability.

In the past few years as researchers delved into the structure of lignin and its cross-linking to wall polysaccharides, it has become clear that lignin's composition is more complex than a simplistic view of variable ratios of coniferyl alcohol (referred to as guaiacyl units) to sinapyl alcohol (referred to as syringyl units) with a few *p*-coumaryl alcohol units thrown in, particularly in grass lignins. Our understanding of lignin structure, not just composition, has greatly advanced in this decade. The importance of understanding the chemical structure of a molecule is that it provides us with clues to its biosynthesis. For example, it is generally accepted that *p*-coumarates are mainly esterified to monolignol units (coniferyl and sinapyl alcohols) within lignin though there is a bit of controversy as to their site of specific attachment. It is commonly thought that the ester linkage is on the α-carbon (C7 of the propanoid side chain Fig. 2) of lignin monomers (Iiyama et al., 1993). This is despite earlier work (Shimada et al., 1971) that estimated the majority of *p*CA units were attached to γ-carbon (C9) with the remainder attached at α-carbon of lignin units (Fig. 2). Ralph et al. (1994a) using ^{13}C-^{1}H NMR clearly showed that all (within detectable limits) were attached at the γ position. The importance of the α vs. δ attachment is that each has different biosynthetic implications. "Believing" that *p*CA is attached at the α-carbon suggests that the reaction scheme involves quinone methides, intermediate products of radical coupling. Therefore, attachment occurs during the formation of lignin and is an opportunistic reaction; i.e., if free *p*CA is available in the apoplastic space during lignin formation it could become ester-linked to the growing lignin molecule. The plant has no control over the process other than dumping *p*CA into the wall space; *p*CA would be in competition with other nucleophiles in the wall, including water. On the other hand, attachment to the γ-carbon can occur only through an enzyme-mediated

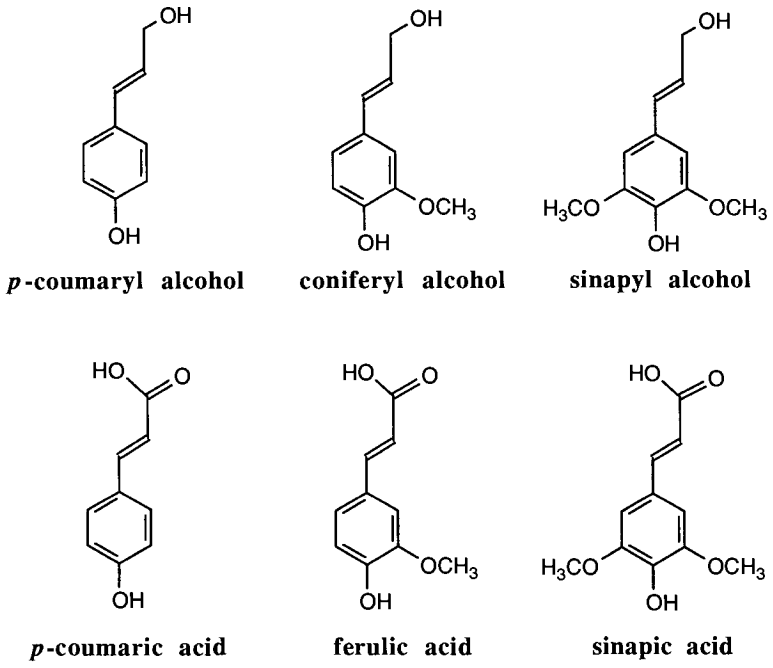

Fig. 1. Chemical structural characteristics of typical lignin precursors (coniferyl, sinapyl and *p*-coumaryl alcohols) and hydroxycinnamic acids (ferulate and *p*-coumarate) found in forage cell walls.

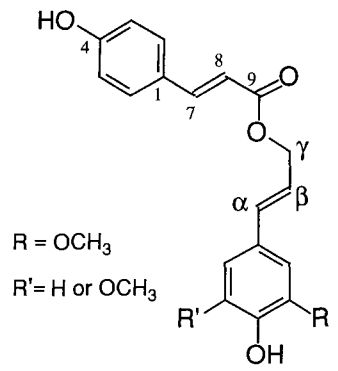

Fig. 2. Attachment of *p*-coumaric acid (*p*CA) via an ester linkage to the primary alcohol on the γ-carbon of lignin units. In grasses such as maize *p*CA is mainly ester linked to sinapyl alcohol units although attachment can occur on coniferyl alcohol units. For coniferyl alcohol R = OCH_3 and R′ = H and for sinapyl alcohol R = R′ = OCH_3.

reaction. The plant probably forms coniferyl or sinapyl *p*-coumarate as a unit for export to the wall and incorporation into lignin, providing the plant with complete control over the process. Knowing the specific bonding pattern tells us what biosynthetic process must have been involved in the formation of the molecule or, in this case, the esterified conjugate.

As we explore lignin structure with new and advanced analytical techniques, we realize that the lignification process is much more plastic than ever imagined. Lignins are not necessarily the product of radical coupling reactions involving coniferyl, sinapyl, and *p*-coumaryl alcohols. For example, consider the maize (Zea maize L.) brown midrib mutant bmr_3. It is known that this mutant contains a lower syringl/guaiacyl ratio because of reduced activity of an *O*-methyl transferase (Grand et al., 1985) involved in sinapyl alcohol formation. Lapierre et al. (1988) demonstrated that bmr_3 incorporates 5-hydroxyconiferyl alcohol (the intermediate in sinapyl alcohol biosynthesis) into its lignin. The mutation leading to reduced *O*-methyl transferase activity may not result in reduced lignin content but it does alter its composition. The plant is utilizing an available molecule that polymerizes into lignin efficiently even though it is not one of the accepted lignin building blocks. In addition, this structural work clearly indicates that the normal monolignol pathway is not as linear as we thought. It is obvious that acid intermediates (e.g., 5-hydroxyferulic acid) can be reduced to the corresponding alcohol (see biochemistry section). There are other examples of plasticity in the lignification process that have recently come to light. Coniferylaldehyde appears to be readily incorporated into lignins and is incorporated at increasing levels within plants that have reduced cinnamyl alcohol:$NADP^+$ oxidoreductase (CAD) activity as found in natural mutants of maize bmr_1 or pine, *Pinus taeda* L. (Ralph et al., 1997) or in molecularly altered plants (Baucher et al., 1996; Halpin et al., 1994a; Higuchi et al., 1994b). The pine CAD mutant also had a large amount of dihydroconiferyl alcohol indicating a significant divergence from the current monolignol biosynthetic pathway. The total impact of these changes on the properties of lignin is not known at this time, nor is their impact on wall cross-linking or ultimately on wall degradation known. What it does indicate is a greater latitude for potential schemes to alter lignin structure.

Role of Hydroxycinnamates within Wall Matrices

A related topic that is key to understanding restrictions on cell wall degradation is the role of hydroxycinnamates (see structures in Fig. 1). Early work demonstrated a negative correlation of wall hydroxycinnamates to wall degradation (reviewed by Jung and Deetz, 1993). From this has emerged the concept of wall cross-linking and its impact on wall utilization. Although there are several potential forms of cross-linking that can occur among wall components (Hatfield, 1993), we will discuss only the hydroxycinnamate interactions that covalently link polysaccharides to lignin. Much of the work presented will deal with chemical structure of grass walls simply because of the greater advancements made in understanding the molecular mechanisms involved in grass cell wall structure and function. This is also due to the unique chemical structure of grass walls which utilize bifunctional molecules allowing convenient cross coupling of wall components; i.e., the hydroxycinnamic acids (Fig. 1). Whether similar mechanisms occur in forage legumes remains to be demonstrated.

Ferulate Attachment to Polysaccharides

Ferulic acid is primarily attached to the walls of grasses, but it has also been found in spinach (*Spinacia oleracea* L.) cell cultures (Fry, 1983), sugar beets (*Beta vulgaris* L.) (Colquhoun et al., 1994), and most recently in pine (*Pinus pinaster* Aiton) hypocotyls (Sánchez et al., 1996) and water chestnuts [*Eleocharis dulcis* (Burman f.) Trin. ex Henschel] (Parr et al., 1996). The attachment of ferulic acid has been carefully analyzed in only a few plant species (Ishii, 1997). For grasses, the mode of attachment seems to be highly conserved, with the acid group esterified to the primary hydroxyl formed at the C5 position when arabinose is in the furanose form (Fig. 3). Structural characterization of wall fragments released through partial enzymatic hydrolysis of total cell walls revealed ferulated oligosaccharides with structures composed of FA-Ara-Xyl, FA-Ara-$(Xyl)_2$, or FA-Ara-Xyl_3. Mueller-Harvey et al. (1986) estimated that ferulic acid was substituted on one in 15 arabinose units in barley (*Hordeum vulgare* L.) straw. This level of substitution is based on extractable ferulates and probably underestimates the total amount of feruloylation within the grass (see section cross-linking polysaccharides). Dicots contain feruloylated pectic polysaccharides. Detailed structural work has been carried out on spinach culture cells (Fry, 1983) and sugar beet pulp (Colquhoun et al., 1994). In both cases, the feruloylation occurs on the arabinose or galactose side chains of pectic polysaccharides. Although there are increasing accounts of feruloylated wall polysaccharides, the detailed structural work is far behind the quantitation work; therefore, the specific attachment to polysaccharides is unknown in these plants. Ishii et al. (1990) has recently shown that ferulate attachment in bamboo [*Phyllosta-*

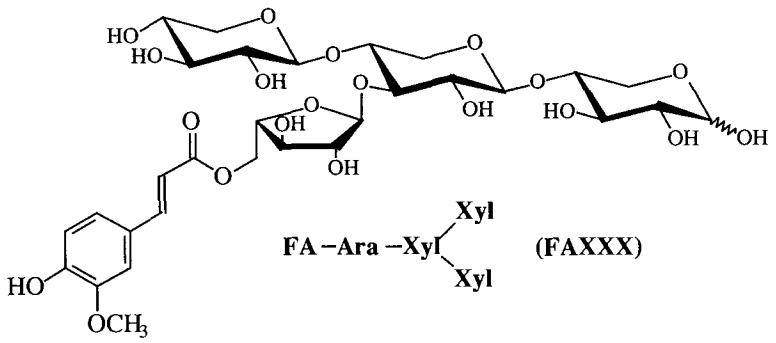

Fig. 3. Incorporation of ferulic acid into cell wall matrices is via attachment to structural polysaccharides. In grasses ferulic acid is covalently attached via an ester linkage formed between the carboxylic acid group of ferulic acid and the primary alcohol on C5 carbon of arabinose side chains of arabinoxylans.

chys edulis (Carriere) Houxeau de Lehaie] can also occur on secondary alcohol groups of xylose branch residues of xyloglucans. If ferulates are attached to xyloglucans of dicots, there could be the potential for significant cross-linking (dicots have 250 $\mu g\ g^{-1}$ xyloglucan as compared with 50 $\mu g\ g^{-1}$ in grass primary walls).

Formation of Dehydrodimers

Grissmann and Neukom (1971) demonstrated that oxidative cross-linking of feruloylated arabinoxylan from wheat (*Triticum aestivum* L.) flour would lead to the formation of polysaccharide gels. Hartley and Jones (1976) isolated dehydrodiferulic acid (see Structure 1, Fig. 4) from ryegrass (*Lolium multiflorum* Lam.) and speculated that these dimers may be a critical mechanism for cross-linking polysaccharides within wall matrices. Since that time, there have been numerous reports of 5–5 dehydrodiferulic acid isolated from plant walls (see references within Ishii, 1997). Ishii (1991) isolated a diferuloyl arabinoxylan hexasaccharide from bamboo shoots, providing definitive proof that oxidative cross coupling of ferulates cross-links polysaccharides.

It is interesting to note that only one dehydrodiferulate, the 5–5 coupled molecule, was identified, yet coupling must be mediated through a one-electron oxidative process, most likely via wall-bound peroxidase. In a review, Yamamoto et al. (1989) even stated that "no direct evidence has ever been obtained in vivo to prove that dehydrodiferulic acids are formed via free-radical coupling reactions." We have for some time been interested in wall cross-linking mechanisms, particularly those involving hydroxycinnamic acids. It seemed unusual to us that molecules like ferulic acid would undergo oxidative free-radical coupling to form a single

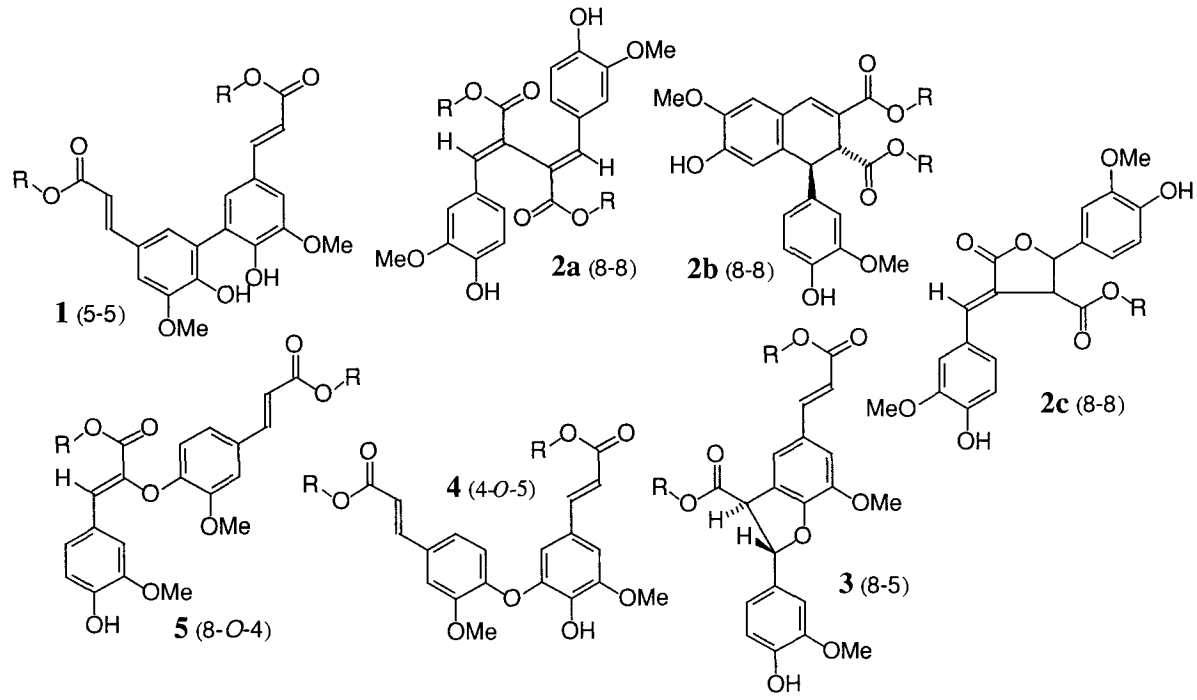

Fig. 4. Dehydrodiferulates are formed within wall matrices as the result of radical coupling reactions initiated by wall peroxidases and hydrogen peroxide. Potential radical coupling products that can be formed between two ferulate radicals. Structure 1 ferulates coupled C5 to C5; Structures 2a, 2b,2c ferulates coupled C8 to C8 showing different structural isomers that can form (Structures 2b and 2c) after the primary coupling reaction Structure 2a; Structure 3 ferulates coupled C8 to C5; Structure 4 ferulates coupled via ether linkage at C4 to C5; Structure 5 ferulates coupled via ether linkage at C4 to C8.

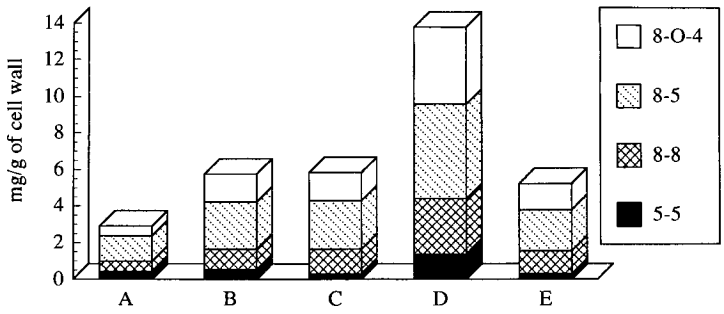

Fig. 5. Concentration μg g^{-1} cell wall material) of dehydrodiferulates derived from 5-5, 8-5, 8-8, and 8-*O*-4 coupling reactions for various plant cell wall samples. Data adapted from Ralph et al. 1994b.

dimer isomer while coniferyl alcohol (a main component of most lignins) undergoes oxidative free radical coupling to produce a variety of dimers with different molecular bonds. From model studies using FA-Ara and plant peroxidases, we could not duplicate the observations cited in the literature. Ferulic acid was easily oxidized by the peroxidase, and the radical coupling products represented the range of dehydrodiferulates predicted from free-radical coupling chemistry (Fig. 4). However, only trace amounts of the 5–5 dimer were produced. Producing the same results from a range of peroxidases isolated from maize walls ruled out the possibility of a specific peroxidase for 5–5-diferulate formation (Hatfield et al., 1995). Ferulate 5–5-dimers may not be favored structures and arise only under special conditions when the molecules are held in the proper spatial orientations. Analysis of extracts from saponified walls of grasses revealed the presence of the predicted range of dehydrodiferulate dimers (Fig. 4; Ralph et al., 1994b). Of critical importance is the fact that the total quantity of dehydrodiferulates was 20 times the level of the 5–5-dimer alone (Fig. 5), indicating that past compositional work had severely underestimated the level of polysaccharide cross-linking in walls.

Cyclodimers of Hydroxycinnamates

The identification of the photochemially derived phenolic acid cyclodimers, 4,4′-dihydroxy-α-truxillic acid (I), 4,4′-dihydroxy-3,3′-dimethoxy—truxillic acid (II), and 4,4′-dihydroxy-3-methoxy—truxillic acid (III) in grasses (Ford and Hartley, 1990; Hartley et al., 1990a) adds another possible mechanism for cross-linking polysacchrides. Wall polysaccharides cross-linked through photochemical dimerization of hydroxycinnamic acids could lead to a decrease in cell wall digestibility (Ford and Hartley, 1990; Hartley et al., 1990a,b). However, the level of these cyclodimers appears to be quite variable depending upon the plant species (Ford and Hartley, 1990; Hartley et al., 1990a,b; Ralph et al., 1994b). Because the formation of cyclodimers is a photochemical process, the plant does not have direct control over this type of cross-linking mechanism. The role of cyclodimer formation is not clear, yet it could have an additive impact when considered with all other types of cross-linking.

Cross-Linking Polysaccharides to Lignin

Early work in forages assumed that lignin was covalently bound to wall polysaccharides but direct chemical evidence (i.e., specific bonds between identified molecules) was lacking (Chesson, 1993 and ref within). Morrison (1974) provided evidence that at least three different types of cross-linking may occur in lignin-carbohydrate complexes from ryegrass, but the types of molecules involved were only speculated upon. Watanabe and coworkers (Watanabe and Koshijima, 1988; Watanabe et al., 1989) demonstrated a direct linkage of xylopyranosyl residues of glucuronoarabinoxylan, glucosyl residues of glucomannans, and galactosyl residues of β-(1-4)-galactans via α-ether bonds to lignin of *Pinus densiflora* Sieb.et Zucc. Similar work has not been done on forage materials, and linkages of this type and their role in cross-linking forage walls is unknown. The bifunctional nature of ferulic and *p*-coumaric acids has led to the natural speculation that they may act as cross-links between specific polysaccharides and lignin (Scalbert et al., 1985).

A high temperature alkaline hydrolysis procedure has been used to release some of the ether-linked ferulates, suggesting that hydroxycinnamic acids are bridging molecules between lignin and polysaccharides (Iiyama et al., 1990; Scalbert et al., 1985). Later, Lam et al. (1992) developed a novel analytical scheme that clearly demonstrated ferulic acid etherified to lignin was also esterified to arabinoxylans, thus demonstrating that ferulates were indeed cross-linking lignin and polysaccharides. However, in both cases the regiochemical relationship of ferulates to lignin was not determined although they assumed that all ferulate etherification was at the α-carbon position of the propanoid side chains of lignin monomers (Iiyama et al., 1993, 1994; Lam et al., 1992). Their work also demonstrated that *p*-coumarates were not acting as cross-linking agents within grass walls. This work from Stone's group renewed the speculation that ferulic acid may act as initiation sites for lignification (Chesson, 1988; Fry, 1986). Ralph et al. (1995), using NMR spectrometry, analyzed ryegrass walls that were grown in $^{13}CO_2$ (about 15% ^{13}C enrichment) and unambiguously demonstrated that ferulates (attached to C5 of arabinose units) do form covalent linkages to lignin monomers (both coniferyl and sinapyl alcohol residues). A critical aspect of this work is the definition of the

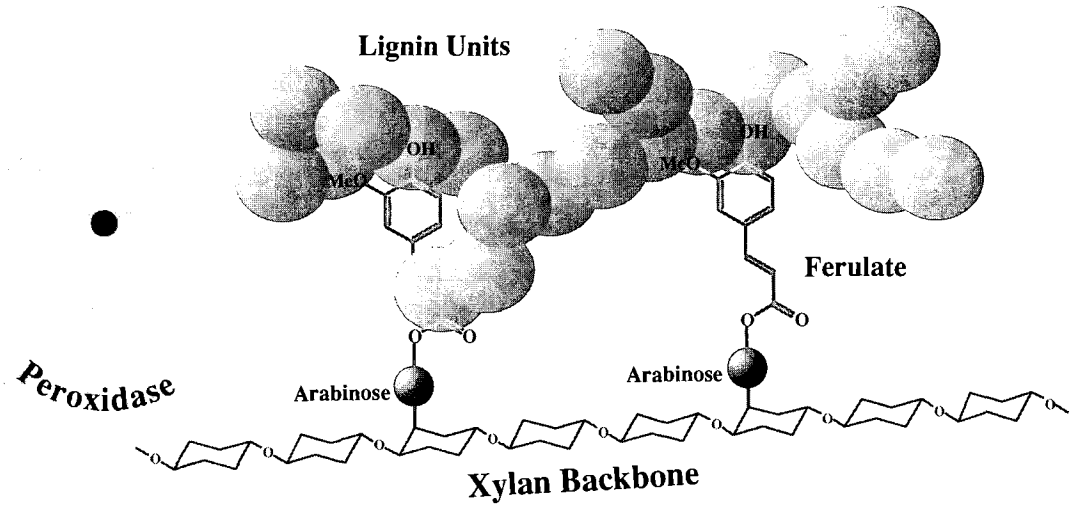

Fig. 6. Schematic diagram of ferulate radical coupling to lignin, both coniferyl and sinapyl alcohol residues become linked to accessible ferulates. Ferulates act as initiation sites for lignin formation.

regiochemical characteristics of the bonding patterns (not only what molecules but what carbons on the molecules are involved in the linkages). This work confirmed that ferulates on arabinoxylans do become covalently linked to lignin monomers by radical coupling reactions and that the types of linkages formed in some cases preclude their release from the wall by solvolytic techniques. In addition, the types of bonds formed can only occur if ferulates are reacting with monolignols (i.e., coniferyl or sinapyl alcohol monomers). This means that one of the first events of wall lignification is the reaction with ferulates; therefore, ferulates are functioning as initiation sites or, more correctly, nucleation sites for the lignification process (Fig. 6). Dehydrodiferulates are also incorporated into the newly forming lignin polymers in the same way as ferulate monomers (Quideau et al. 1997; Ralph et al., 1997). The positioning of ferulates within the wall may regulate lignin formation patterns and control cross-linking within wall matrices. Therefore, controlling the levels of total feruloylation should directly impact levels of cross-linking. The bad news is that there are no analytical tools available to quantify completely the ferulates in lignified grass walls. On the basis of model studies, it would appear that 50 to 60% of the total ferulates (monomers and dehydrodimers) are not releasable from lignified walls.

Impact of Cross-Linking on Wall Degradation

As already mentioned, the importance of cross-linking in suppressing wall degradation has been speculated upon for some time with correlative evidence to support this assumption (Chesson, 1988; Jung and Ralph, 1990). An in vitro wall model system has been developed by Grabber et al. (1995) that allows manipulation of ferulate concentration, formation of dehydrodiferulates, and lignin levels and composition. The model is based upon walls isolated from maize suspension culture cells that take advantage of the wall bound peroxidases to initiate radical coupling reactions (i.e., formation of dehydrodiferulates and lignin polymers) that mimic primary wall cross-linking and lignification. By means of this model system, it is possible to separate the cross-linking events and evaluate their individual and additive effects on wall polysaccharide degradation (Grabber et al., 1998a,b). Simple formation of dehydrodiferulates decreased the rate, and to a lesser degree, the extent of wall degradation (Fig. 7). The addition of lignin to the wall matrix (cross-linking polysaccharides to lignin via wall ferulates) depresses rate and extent of wall degradation with a more pronounced impact on the arabinoxylans than any other wall polysaccharide (Fig. 7). Model systems of this type allow selective manipulation of complex metabolic processes to identify the most critical and or limiting steps.

Lignin and Hydroxycinnamate Biochemistry

Every enzyme in the lignin pathway (phenyl propanoid, Fig. 8) has been studied in one plant system or another, and impacts of development and environmental conditions have been examined (the reader is directed to recent reviews for specific details; Boudet and Grima-Pettenati, 1996; Boudet et al., 1995; Higuchi, 1990; Iiyama et al., 1993; Whetten and Sederoff, 1995). Synthesis of *p*-coumaroylated and feruloylated molecules arise as branch points from the monolignol pathway. What is not well understood is the mechanism of attachment to the acceptor alcohol forming the necessary ester linkage (in the case of ferulates in grasses the C5 hydroxyl of arabinosyl substitutions on arabinoxylans). Fry and Miller (1989) fed ^{3}H arabinose to spinach cultures and monitored its incorporation into ferulated arabinosyl units (FA-Ara) on major wall polysaccharides. Similar experiments were conducted with cell cultures of tall fescue (Myton and Fry, 1994). They found that arabinosylation and feruloylation occurred intracellularly and co-synthetically. Alternatively, Yamamoto et al. (1989) suggested that feruloyl-CoA was incorporated in barley coleoptile walls in an extracellular fashion with the site of feruloylation being located within the wall matrix itself. From a metabolic point of view,

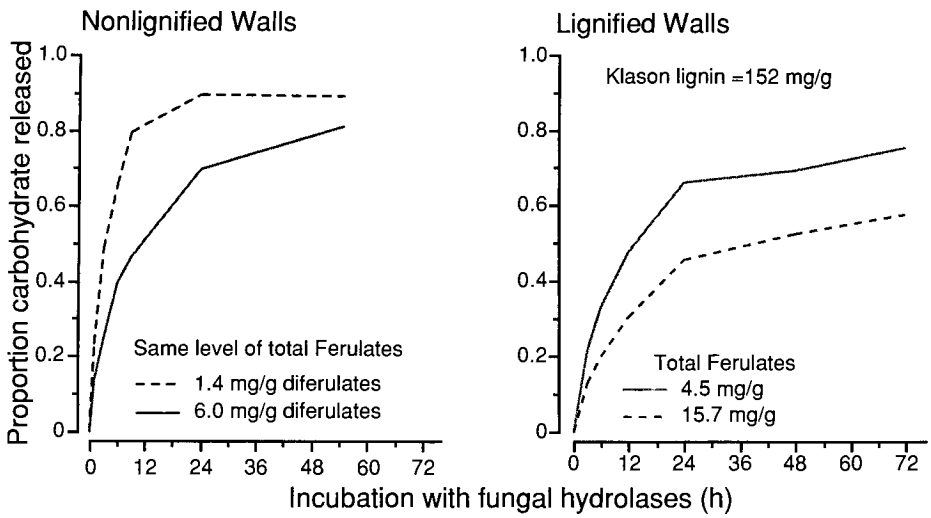

Fig. 7. Impact of diferulate formation (nonlignified walls) and lignification (lignified walls) upon wall structural polysaccharide degradation by fungal hydrolases. Walls were isolated from maize cell cultures and treated with hydrogen peroxide to stimulate ferulate cross-linking (nonlignified walls) or with hydrogen peroxide and coniferyl alcohol to simulate wall lignification along with ferulate dimerization. Both ferulate monomers and dimers are effectively incorporated into the lignin polymer.

this would not appear to be as likely because it would be difficult for the plant to recover the CoA product of the reaction (Iiyama et al., 1993). The most definitive work to date is that of Meyer et al. (1991) who have shown that a microsomal preparation from suspension-cultured parsley [*Petroselinum crispum* (Miller) Nyman ex A.W. Hill] cells can transfer ferulic acid from feruloyl-CoA to polysaccharide acceptors. No similar work has been done in grass species. At least in grasses, controlling the level of polysaccharide feruloylation would be key in regulating a major form of wall cross-linking.

The application of molecular genetics to the study of the monolignol pathway as well as the use of specifically labeled pathway intermediates has led to the revelation

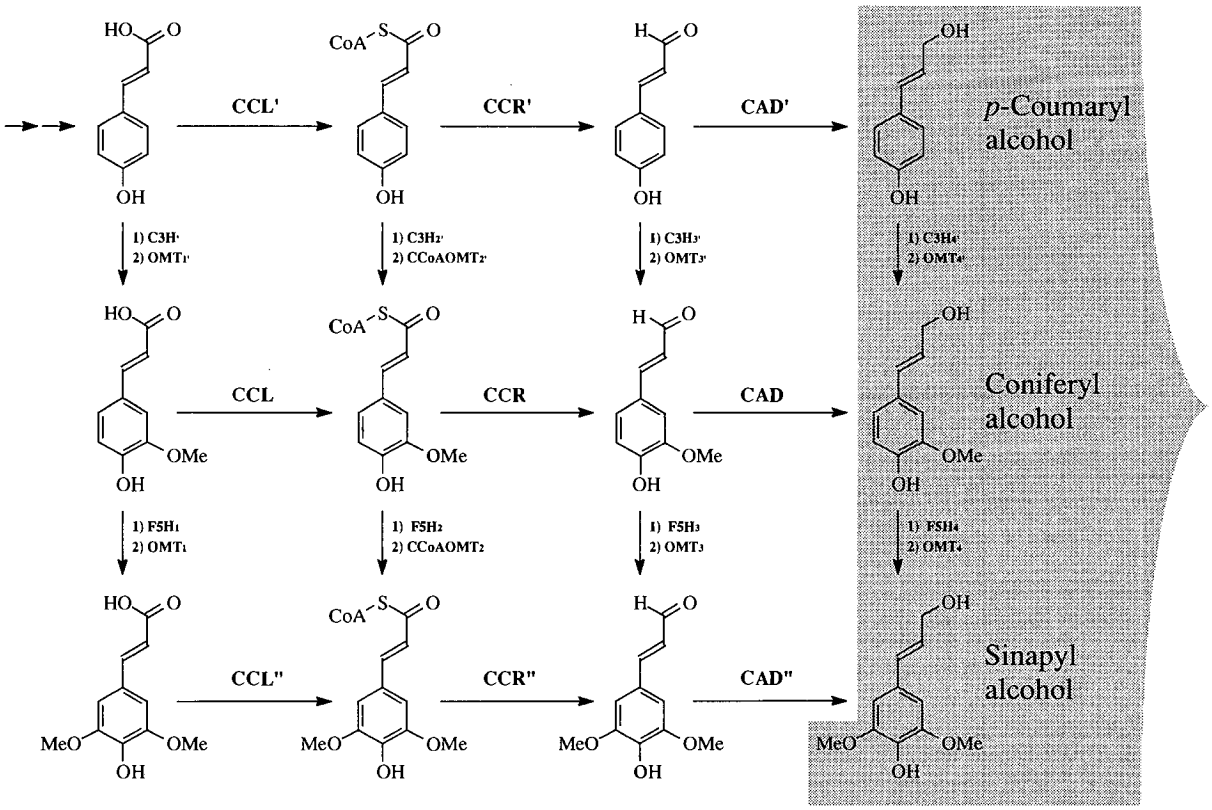

Fig. 8. Schematic representation of the monolignol biosynthesis pathway. The diagram illustrates the complex interactions of enzymes and alternate pathways to produce monolignols that are used to produce lignin.

that the pathway is not as linear as previously thought. Ye et al. (1994) found an alternative pathway for the methylation of the 3- and 5-hydroxyls leading to feruloyl and 5-hydroxy feruloyl intermediates, respectively. They identified an *O*-methyltransferase (OMT) that has a high affinity for caffeoyl CoA and 5-hydroxyferuloyl CoA as opposed to OMTs that methylate caffeic acid and 5-hydroxyferulic acid. Both types of OMTs were detected in a range of plants and tissues types, suggesting that it may be widespread among plants. Using ^{3}H labeled coniferin (glucoside of coniferyl alcohol) fed to cultured poplar hybrid (*Populus alba* L. × *Populus tremula* L.) plantlets, Daubresse et al. (1995) labeled lignin polymers within the developing plants. They unexpectedly found that a substantial amount of the label was incorporated into syringyl units. This means that the hydroxylation and methylation steps can also occur on coniferin and/or coniferyl alcohol in addition to the normal acid precursors. This work supports earlier similar observations of Matsui et al. (1994). Taken together, the findings of these groups indicated that the monolignol pathway is more branched or grid-like than previously thought (Fig. 8).

Attention has recently focused on the genes encoding the various enzymes within the monolignol biosynthetic pathway with hopes of understanding their regulation (Boudet and Grima-Pettenati, 1996; Boudet et al., 1995; Whetten and Sederoff, 1995). The last two enzymes of the lignin pathway (cinnamyl CoA reductase, CCR and cinnamyl alcohol: $NADP^{+}$ oxidoreductase, CAD) are good candidates for genetic manipulation of lignin in that they are solely involved in monolignol biosynthesis and one does not have to be concerned about altering some other metabolic pathway (e.g., flavonoid or phytoalexins). Efforts to alter lignin through the down regulation of CAD have been successful to some degree (Baucher et al., 1996; Halpin et al., 1994b; Higuchi et al., 1994a). In all cases, there was a definite shift in the lignin composition towards higher levels of cinnamyl aldehyde units and lower coniferyl alcohol units. However, total lignin levels were virtually unchanged, though there was a change in the alkaline solubility of lignin. Recent ^{13}C-^{1}H NMR work analyzing ^{13}C labeled lignins isolated from tobacco (*Nicotiana tabacum* L.) lines with down regulated CAD (by antisense genes) and other tobacco lines with down regulated CCR (by antisense genes) revealed the incorporation of non-traditional components in their lignins (Ralph et al., 1999). The antisense CAD tobacco contained lower coniferyl alcohol and higher levels of cinnamaldehydes along with elevated levels of benzaldehydes with little change in Klason lignin levels. Tobacco plants with down regulated CCR had reduced Klason lignin levels (≈50%), lower coniferyl alcohol units and increased levels of tyramine ferulate incorporated into the lignin. The question of improved digestibility from these modifications has yet to be clearly demonstrated. There is some indication that antisense CAD and OMT plants have improved degradation (Bernard-Vailhe et al., 1996a,b). Using a wall model system, Grabber et al. (1998c) have shown that increased incorporation of cinnamyl aldehyde monomers into lignin actually decreased wall degradability. Even though genetically manipulated plants and some of the bmr mutants have increased cell wall digestibilities, the mechanism behind these improvements (relatively small in some cases) is not clear. The results from genetically manipulated plants and from the natural mutants (bmr and pine CAD deficient) clearly indicate the metabolic plasticity involved in the lignification process. There are many unanswered questions as to the impact of these alterations on wall degradation, but it indicates the wide potential for altering lignin structure.

Conclusions and Future Directions

Over the past 35 to 40 yr, a tremendous amount of knowledge has been gained in the area forage cell wall chemistry, structure, and function, particularly as these attributes relate to fiber utilization by ruminants. Much of this work was summarized in a recent publication that was the result of an International Symposium on Forage Cell Wall Structure and Digestibility (Jung et al., 1993). This accumulation of knowledge has allowed the development of molecular mechanistic models describing wall component interactions that limit wall degradation. Within the last decade, particular advances made in understanding metabolic processes involved in monolignol biosynthesis, lignin polymer formation, and structural analysis of lignin and its cross-linking to wall polysaccharides have revealed a great deal about the plasticity within the whole process. The cross-linking picture in grasses is becoming much clearer, confirming earlier speculation that ferulates play a key role. Targeting the transferase for feruloylation of polysaccharides may well be the key to controlling a major form of cross-linking in grasses. Genetic selection for altered feruloylation of polysaccharides is feasible but requires the development of an evaluation system that can readily handle thousands of plants. The most expedient approach may be to combine molecular approaches with traditional selection processes.

Although our understanding of grass walls has increased, we are well behind in forage legumes (i.e., alfalfa). In general, there has been less effort devoted to legumes like alfalfa and perhaps this is due to a feeling that they are as good as they need to be. This unfortunately is short sighted. Every increase in wall digestiblity will lead to increased profits not only from gains in net energy but also improved protein utilization. It seems reasonable that cross-linking mechanism(s) are playing a role in legume wall degradation; we just don't have a good handle on the molecules or processes involved. Certainly hydroxycinnamates must be more closely evaluated in legume walls to determine their role, if any, in matrix interactions. Although we are behind in understanding legume wall interactions there are other avenues available now to improve wall degradation. Structural polysaccharides that make up the pectic fraction of walls (120–220 $\mu g\ g^{-1}$ of alfalfa stem walls) are rapidly and completely degraded by ruminal microbes. Genetic selection for increased pectin content of walls should lead to improved digestibility.

As current analytical technologies (e.g., NMR, mass spectrometry, infrared spectrometry, etc.) continue to evolve, there will be new opportunities to elucidate fine structural details of wall polymers and their interactions, providing new insights into wall metabolism. Such information will provide the clues as to how we can manipulate the plant. The increased application of molecular approaches to cell wall chemistry (altering lignin concentration and composition, crosslinking, etc.) will provide us with a better picture of how these processes are regulated within the plant and how we can alter them to fit our needs. The recent revelations of plant responses to genetic manipulation of enzymes within the monolignol pathway can be viewed with disappointment (the plant may always respond to produce a functional lignin) or with excitement (there may be a tremendous opportunity to alter lignin and cross-linking through structural modifications). We prefer to look to the future with excitement.

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