Structural Characteristics of Cell Walls of Forage Grasses - Their Nutritional Evaluation for Ruminants* - - Review -

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ABSTRACT : The walls of all higher plants are organized as a cellulosic, fibrillar phase embedded in a matrix phase composed of non-cellulosic polysaccharides, some proteins and, in most secondary walls, lignin. At the effective utilization of plant biomass, qualitative and quantitative analyses of plant cell walls are essential. Structural features of individual components are being clarified using newly developed equipments and techniques. However, "empirical" procedures to elucidate plant cell walls, which are not due to scientific definition of components, are still applied in some fields. These procedures may give misunderstanding for the effective utilization of plant biomass. In addition, interesting the investigation of wall organization is moving towards not only qualitatively characterisation, but also quantitation of the associations between wall components. These involve polysaccharide-polysaccharide and polysaccharide-lignin cross-links. Investigation of the associations is being done in order to understand the chemical structure, organization and biosynthesis of the cell wall and physiology of the plants. Procedures for qualitative and quantitative analyses based on the definition of cell wall components are reviewed focussing in nutritional elucidation of forage grasses by ruminant microorganisms. *(Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 6 : 862-879)*

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INTRODUCTION

Due to the data for land use of FAOSTAT, area of grassland in the world is 3.4 billion ha, at least 10 billion tons (dry weight) of grasses are produced each year (Iiyama, 2000). Annual biomass production, that is, gross CO_2 fixation, in grassland (19 billion tons) (production of biomass at well managed farm is 23 t/ha/yr) (table 1) (Shinjo and Hoshino, 1989) is equal to those in forest (19 billion tons) and agricultural area (22 billion tons) (table 2). However only 20-30% of this biomass of grasses is utilized as nutrients, because of low digestibility. If the digestibility of forage grasses is able to increase even 1%, human would get more proteins, and it could strongly contribute to preserve the wild herbivorous mammals.

It is significantly important to understand factors affecting digestibility, especially the relationship between digestibility and structural feature of grass cell walls. Since Van Soest (1963, 1982), lignin is believed as the most regulating factor for digestibility. This knowledge should be re-considered from the viewpoint of a cell wall scientist. We have applied some procedures, which are used among cell wall scientists for cell walls of plants, and some of new procedures were developed to understand exact structural feature of plant cell walls.

Procedures to be applied to discuss structural feature of cell walls of forage plants and their nutritional evaluation for ruminants are reviewed in this paper.

SCHEMATIC PROCESS FOR STRUCTURAL ANALYSES OF GRASS CELL WALLS

The process for quantitative and structural analyses of cell wall components of forage plants is summarized in figure 1.

PRE-TREATMENT OF FORAGE PLANTS FOR QUANTITATIVE AND STRUCTURAL ANALYSES

Forage plant material to be subjected for quantity and structural analyses should be frozen immediately after harvesting, then freeze-dried to avoid post harvest modification. The dried sample is ground using a Wiley mill or equivalent equipments to pass 420 μm sieve. Ash content is determined by combustion for 3 hr at 700℃. The ash is treated with boiling 0.5 M HCl for 90 min, the residue collected by a fine glass-filter (G4) is weighed as silica after being oven dried.

The ground sample is extracted successively with boiling 80% (v/v) ethanol (1 hr×3) followed with hot

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Table 1. Dry matter yield of five tropical pasture grasses (kg/m^2)

Species	Year				Average
	1984	1985	1986	1987	
Rhodes grass					
Chloris gayana Kunth cv. Katambora	1.55	2.11	1.68	1.83	1.79
Guinea grass					
Panicum maximum Jacq cv. Gatton panic	1.09	1.73	1.68	2.24	1.69
Green panic					
Panicum maximum Jacq var. trichoglume	1.18	1.62	1.59	1.98	1.59
Setaria					
Setaria sphacelate Staf & Hubard cv. Kazungula	1.28	2.43	2.40	2.80	2.23
Buffel					
Cenchrus ciliaris L. cv. Biloela	0.99	1.50	1.47	1.67	1.41

(Shinjo and Hoshino, 1989)

Table 2. Fixation of carbon dioxide by plants

		Comment
Total land area	14.90×10^9 ha	
Forest area	4.14×10^9 ha	FAO Database
Grassland	3.40×10^9 ha	FAO Database
Agricultural area	1.45×10^9 ha	FAO Database
Forestry		
Unit storage of trees	100 m^3/ha	Tropical rain forest: 120-180 m^3/ha, S.G. 0.5 ton/m^3
Accumulation in forest area	207.0×10^9 ton	Oven dry weight
Gross annual CO_2 fixation	19.0×10^9 ton	Annual increase: 5.0%. (Tropical rain forest: 10-15%)
Grassland		
Annual productivity at grassland	3 ton /ha	Oven dry weight. Well managed grassland: 30 ton /ha
Annual production	10.2×10^9ton	Oven dry weight
Gross annual CO_2 fixation	18.7×10^9ton	
Agricultural area		
Crops*[1]	3.3×10^9ton	Oven dry weight. Major crops only.
Wastes	8.7×10^9ton	Straw, bagasse, & roots. Oven dry weight.
Annual production at agric. area	11.9×10^9ton	
Gross annual CO_2 fixation	22.0×10^9ton	
Total		
Annual increase of products	32.5×10^9ton	
Gross annual CO_2 fixation	60.0×10^9ton	

*[1] Major crops: rice (production of crop on 1999: 596.5×10^6 ton), wheat (583.6×10^6 ton), maize (600.4×10^6 ton), sugarcane ($1{,}275\times10^6$ ton), cassava (168.1×10^6 ton), barley (130.1×10^6 ton), sweet potato (135.2×10^6 ton) and sorghum (62.8×10^6 ton).

water overnight at 40℃ with shaking (Theander and Westerlund, 1993). The extract free sample is washed well with acetone, air-dried then dried in a vacuum oven at 40℃. The sample should not be dried in an oven at 105℃ to avoid thermal modification. Most of protein free from cell wall polymers and storage carbohydrates such as starch and (1→3)-β-glucan would be removed by the above extraction (Iiyama et al., 1994b). Some parts of protein could not be removed even washing with detergent, because of association with cell wall polymers, especially with lignin. Quantitative determination of cell wall polymers, such as polysaccharides and lignin, should be carried out based on the definition of cell wall polymers.

DEFINITION AND QUANTITATIVE AND STRUCTURAL ANALYSES OF LIGNIN

Lignin is aromatic biopolymer, which is

significantly different from other biopolymers, such as polysaccharides and proteins. At the final stage of biosynthesis of lignin, polymerization step of monolignols, such as hydroxycinnamyl alcohols, breaks away from the control by enzymes. Monolignols are polymerized step-wise by coupling reaction of monolignol radicals (figures 2 and 3) (Iiyama et al., 1993), which are produced by the action of peroxidase or laccase. Thus the term of "Lignin" should be defined as "a dehydrogenated polymer of hydroxycinnamyl alcohols". In addition, it is suggested that hydroxycinnamaldehydes are also involved in the polymerization in special cases such as lignification of brown midrib mutant of forage grasses (Peng and Westermark, 1997; MacKay et al., 1999; Lapierre et al., 2000)

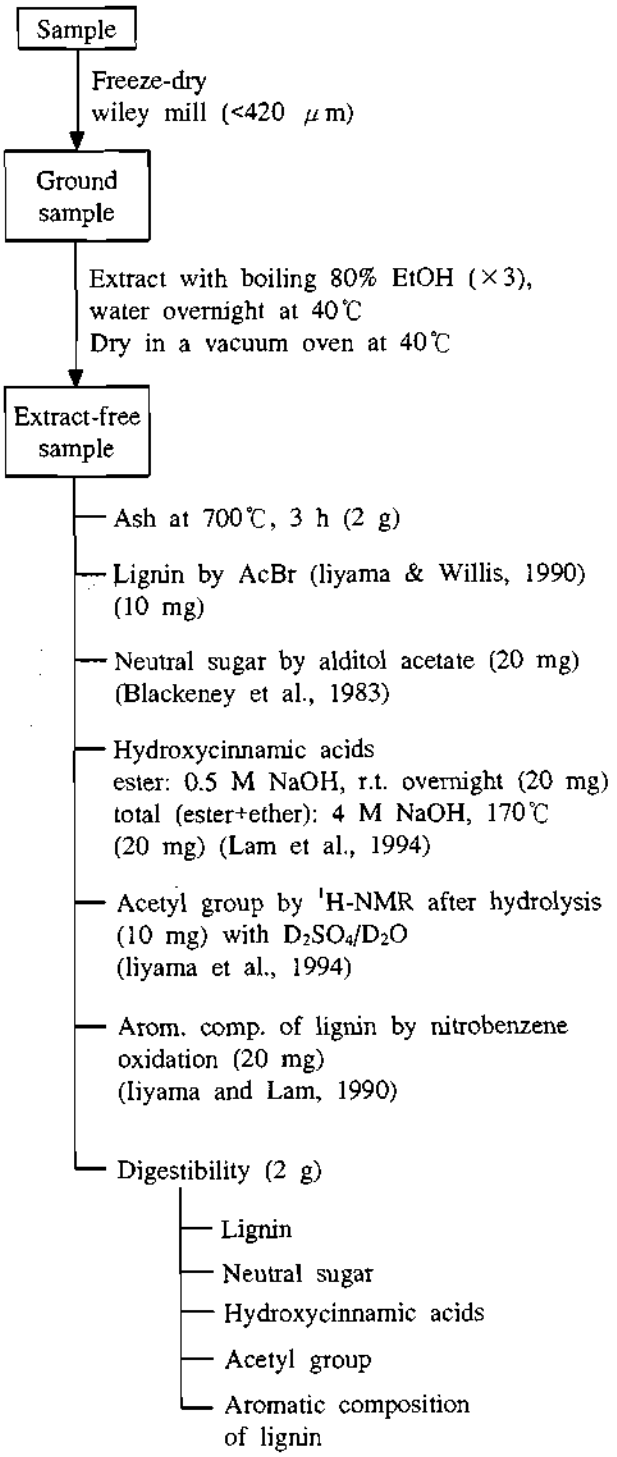

Figure 1. Schematic process for analysis of cell walls of forage grasses

The major intermonomer linkage by the coupling reaction of radicals is arylglycerol-β-aryl ether (figure 4). The presence of this linkage is necessary and sufficient conditions to be defined as lignin. The linkage is easily qualified by ozonolysis (Matsumoto et al., 1984, 1986; Habu et al., 1987, 1988; Iiyama et al., 1991) (figure 5).

Quantitative determination of lignin should be analysed due to the above definition. The combination of Klason procedure and spectrometric determination of acid-soluble lignin (ASL) (Schoning and Johansson, 1965, Iiyama et al., 1994b) would be one of the most suitable procedures. Klason lignin content of forage plants has to be corrected for N content (protein content is estimated as N content×6.25) by a CHN micro-elemental analysis, because some parts of protein are also precipitated together with lignin during Klason treatment (Theander and Westerlund, 1986). Another applicable procedure for quantitative determination of lignin of forage plants is an acetyl bromide procedure (Iiyama and Wallis, 1989, 1990). Lignin content determined by an acetyl bromide procedure is not affected by protein being in forage plants (Iiyama and Wallis, 1989, 1990).

Van Soest (1963, 1982) has proposed an acid detergent procedure for lignin determination, and the procedure is widely used in the fields other than wood science. Ether linkages as major inter-unit linkages of lignin are hydrolysed by strong acidic medium, and significant portion of highly hydrophilic fragments of lignin are dissolved in reaction mixture by the formation of micelle with detergent (figure 6) (Theander and Westerlund, 1993; Lowry et al., 1994; Hatfield et al., 1994). As the result, significantly lower lignin content than that determined by Klason+ASL and acetyl bromide procedures would be given by the acid detergent procedure (table 3) (Lam et al., 1996b).

A procedure to determine lignin content of forage plants using permanganate was also proposed by Van Soest and Wine (1968). It should be noted that some portion of permanganate is consumed by polysaccharides (Iiyama and Pant, 1988). Lignin determination with near infrared reflectance spectroscopy (NIR) should be calibrated using the results by Klason+ASL or acetyl bromide procedures, not by the acid detergent procedure.

Aromatic composition of lignin is analysed by

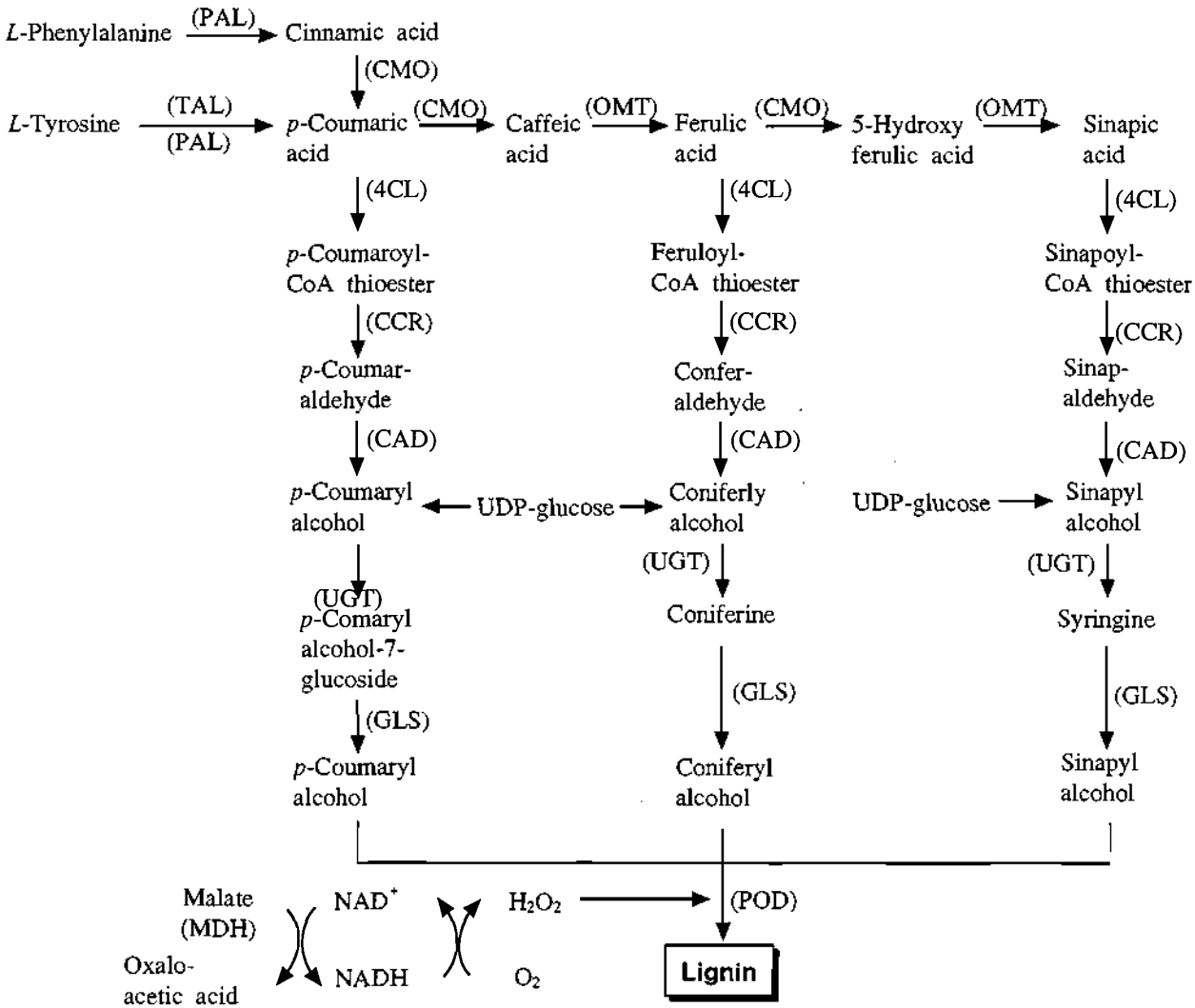

Figure 2. Pathway of lignin biosynthesis

alkaline nitrobenzene oxidation (Iiyama and Lam, 1990) or cupric oxide oxidation. Reaction mechanism of the both oxidation is different, and total yield of oxidation products by cupric oxide oxidation is considerably lower than that by alkaline nitrobenzene oxidation (Iiyama and Lam, 1990; Lam et al., 1990b). In addition to the aromatic composition determined by the oxidation, total yield of the products gives information for condensed structure of lignin.

Lignin of Gymnosperms and Angiosperms are composed of guaiacyl unit together with small amount of p-hydroxyphenyl unit and guaiacyl, syringyl together with small amount of *p*-hydroxyphenyl unit as aromatic composition, respectively. It has been noted that aromatic composition of lignin of monocotyledonous plants is characterised by the presence of significant amounts of *p*-hydroxyphenyl unit. But *p*-hydroxyphenyl unit in lignin of monocotyledonous plants originate from *p*-coumaric acid esterified and etherified to wall polymers, which are not fragments of lignin (Iiyama et al., 1990; Lam et al., 1990c).

DEFINITION AND QUANTITATIVE AND STRUCTURAL ANALYSES OF WALL POLYSACCHARIDES

We have terms of "cellulose" and "hemicellulose". The term of "cellulose" is of course defined scientifically as (1→4)-β-glucan. But the definition of term of "hemicellulose" is significantly specified. Extract free cell wall is treated with acidic $NaClO_2$ at 70℃ to decompose lignin. The residue, which is called as "holocellulose", is extracted with 17.5% (w/w) NaOH or 24.5% (w/w) KOH. Insoluble residue is defined as "α-cellulose", which is composed of "cellulose" and also non-cellulosic polysaccharides having high-molecular weight. Acidified then desalted alkaline soluble fraction was poured into ethanol, the

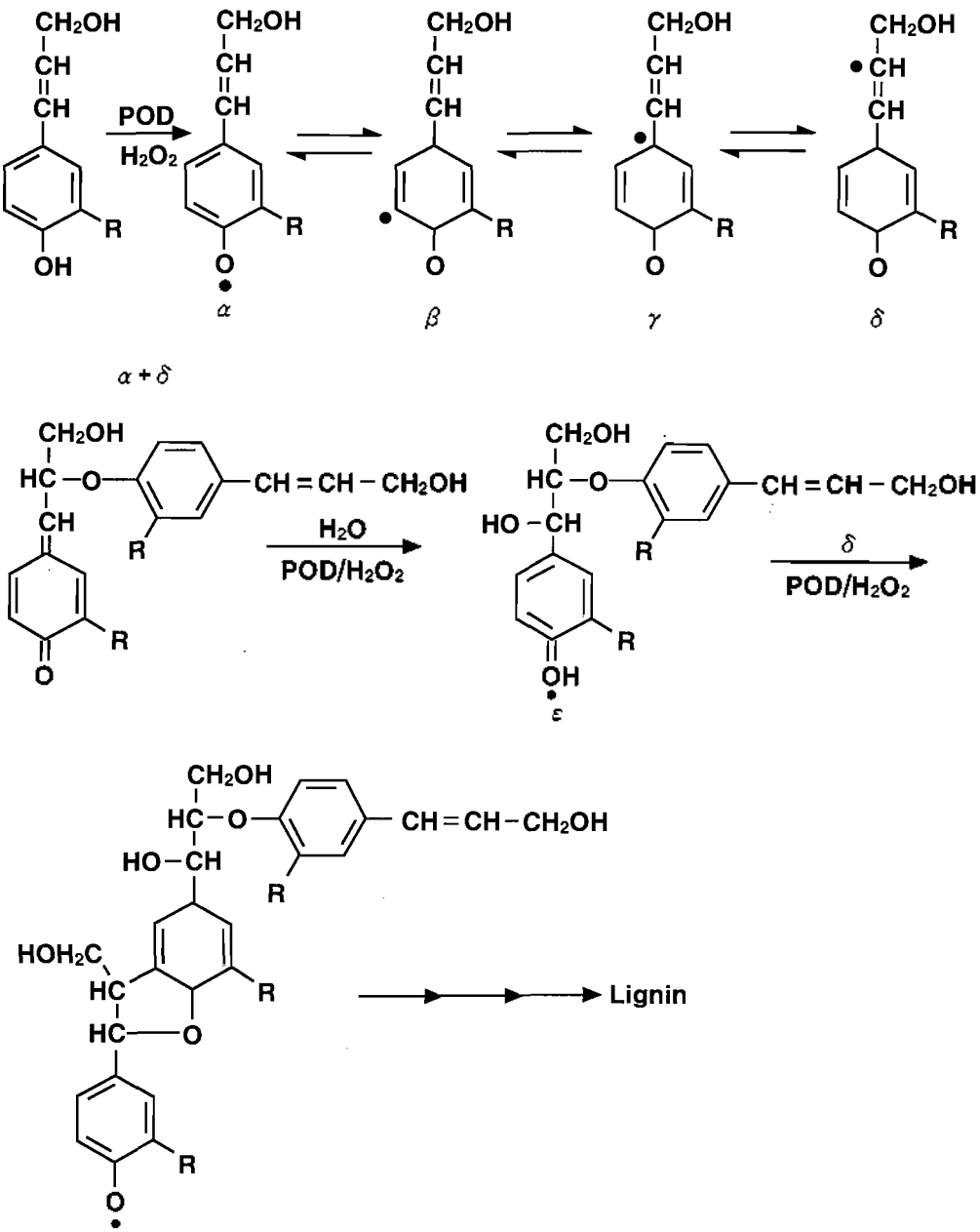

Figure 3. Coupling reaction of monolignol radicals during lignin biosynthesis

precipitate is defined as "hemicellulose", which is mainly composed of low molecular weight fragments of cellulose, in addition to many kinds of non-cellulosic polysaccharides. The soluble fraction in ethanol is called as "γ-cellulose" (figure 7). "Hemicellulose" defined as the above is entirely differing from "polysaccharides other than cellulose", which should be defined as "non-cellulosic cell wall polysaccharides".

It should be noticed that "cellulose" and "hemicellulose" determined by the procedures for neutral (NDF) and acid detergent (ADF) fibers are very far from those scientific definitions.

It is not easy to determine separately exact contents of cellulose and non-cellulosic wall polysaccharides. However, the determination of composition of neutral sugar residues of extract free sample (cell wall) can give better information for the contents of cellulose and non-cellulosic wall polysaccharides. Fortunately most of starch and (1→3)-β-glucan are removed from cell walls by the hot water extraction, and xyloglucan should be less than 1% in the secondary walls. Neutral sugar composition should be analyzed by an alditol acetate procedure (Blakeney et al., 1983). If the sample is analysed with partially methylated alditol acetate procedure, we can get much better information for the content and structural feature of cellulose and each non-cellulosic wall

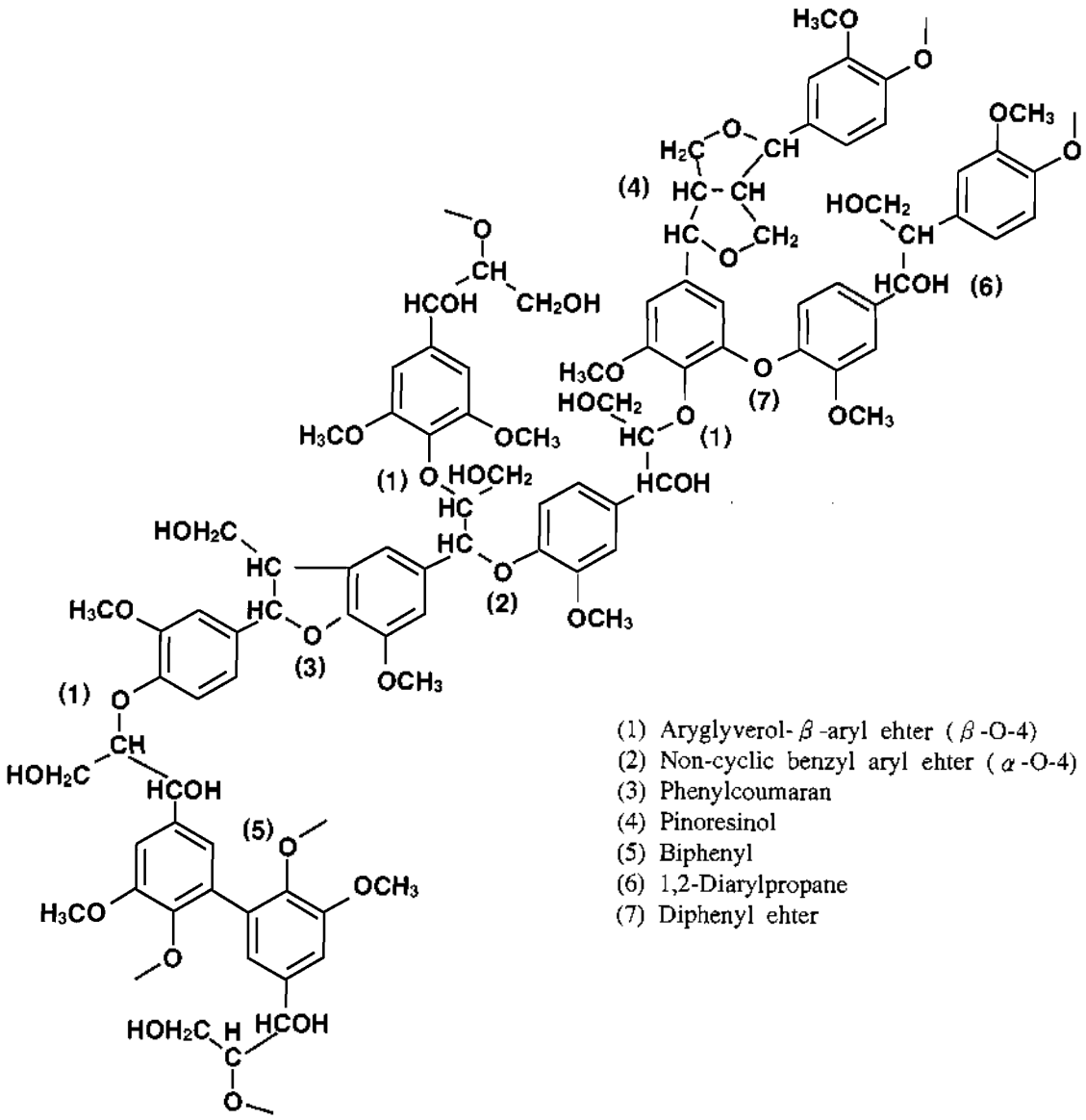

Figure 4. Major intermonomer linkages of lignin

polysaccharide (Harris et al., 1984).

Hydroxyl groups of xylosyl residue of arabinoxylan, which is the most abundant noncellulosic polysaccharide of cell walls of forage grasses, are highly substituted by acetyl group (Iiyama et al., 1994c). These acetyl groups could affect digestion of arabinoxylan. The content of acetyl groups are quantified as 1-acetyl-pyrrolidine by gas-chromatography (Mansson and Samuelsson, 1981), and more easily with ^{1}H-NMR procedure after digestion of plant cell walls with D_2SO_4/D_2O (Iiyama et al., 1994c).

HYDROXYCINNAMIC ACIDS LINKED WITH CELL WALL POLYMERS

It is well recognized that hydroxycinnamic acids, *p*-coumaric, ferulic and sinapic acids (figure 8), are intermediates of lignin biosynthesis (see figure 2) (Iiyama et al., 1993, Terashima et al., 1993). Other derivatives of hydroxycinnamic acids such as caffeic and 5-hydroxyferulic (figure 8) are recently also postulated as intermediates of lignin formation (Suzuki et al., 1997; Chen et al., 1999; Humphreys et al., 1999; Matsui et al., 2000; Franke et al., 2000). These hydroxycinnamic acids are immediately reduced by cinnamoyl CoA-reductase (EC1.2.1.44) and cinnamyl alcohol dehydrogenase (EC1.1.1.195) through hydroxycinnamoyl-CoA thioesters to corresponding hydroxycinnamyl alcohols to be polymerized as lignin, when the formation of secondary walls has started (see figure 2) (Iiyama et al., 1993, Terashima et al., 1993).

p-Coumaric and ferulic acids were detected by

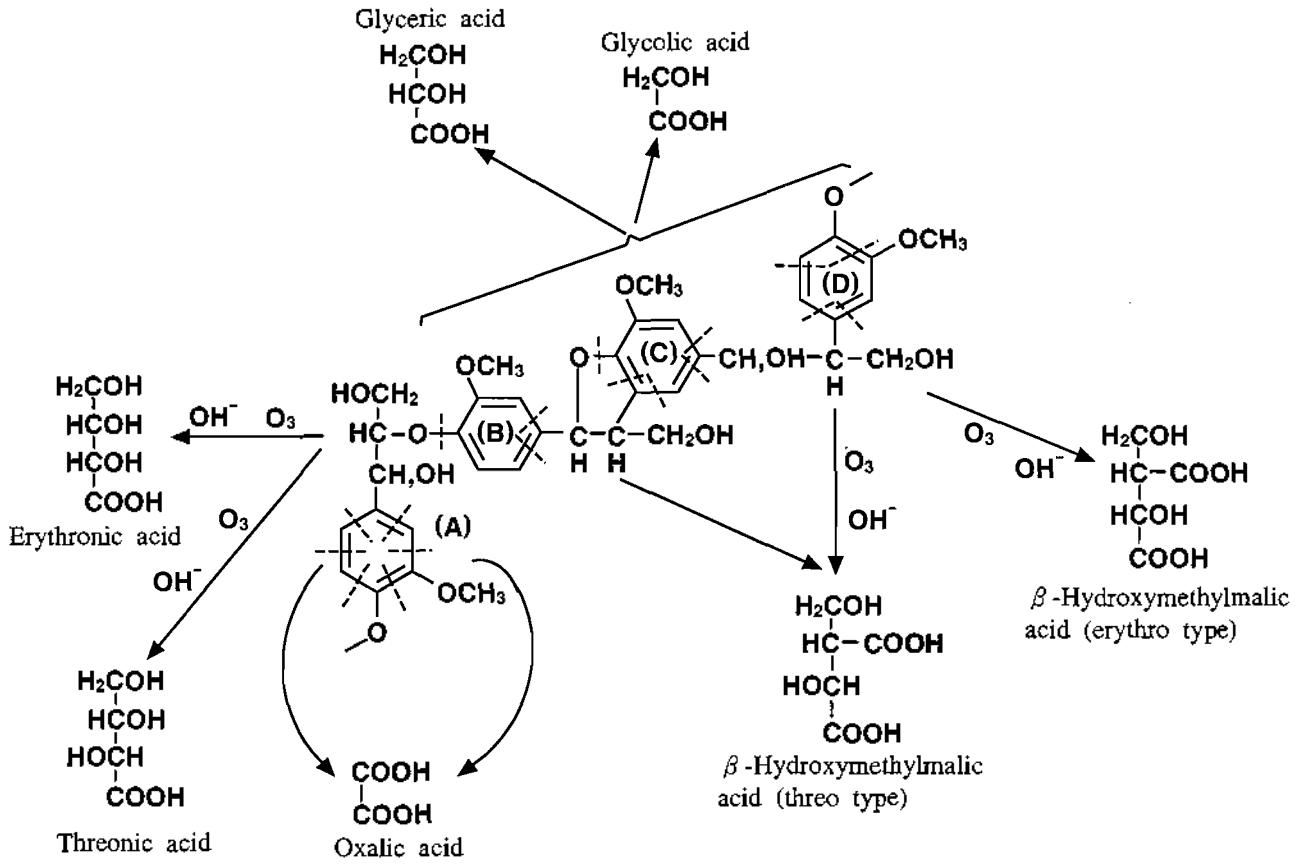

Figure 5. Ozonolysis of lignin to qualify intermonomer linkages

fluorescence in most of monocotyledons (Harris and Hartley, 1980) and few of dicotyledons (Hartley and Harris, 1981). Considerable amounts of these acids are released by alkaline hydrolysis of cell walls of such plants under mild condition, suggesting that these acids are ester-linked to wall polymers, and differ from the definition of lignin as described above. Ferulic acid and *p*-coumaric acid are ester-linked to polysaccharides, mainly arabinoxylan before secondary wall formation, and lignin after lignification start of cell walls of grasses, respectively (Iiyama et al., 1990; Lam et al., 1992a, 1994).

In addition, presence of ether-linked hydroxycinnamic acids has been reported, in wheat (*Triticum aestivum*) stem (Scalbert et al., 1985, 1986b; Iiyama et al., 1990), rice stem (*Oryza sativa*) (Sharma et al., 1986; Lam and Iiyama, 2000), phalaris (*Phalaris aquatica*) (Lam et al., 1992a), and some species of tropical and subtropical forage grasses (Higuchi et al., 1999) internodes. Ether-linked hydroxycinnamic acids are involved in the associations between lignin and polysaccharides in cell walls of grasses (Iiyama et al., 1990, 1994a; Lam et al., 1992a, 1994b; Higuchi et al., 1999) and have important roles for their digestibility (Lam et al., 1993) and mechanical strength of stems of grasses (Lam and Iiyama, 1996) as mentioned later in detail.

DIMERIZATION OF HYDROXYCINNAMIC ACID ESTERS OF POLYSACCHARIDES

Dimerization of hydroxycinnamic acids has also reported (figure 9) (Lam et al., 1990a). Oxidative (peroxidase-catalyzed) reactions between feruloyl residues on arabinoxylans or pectic arabinogalactans to form inter-phenyl, carbon-carbon bonds have been demonstrated *in vitro* (Geissman and Neukom, 1971; Izydorczyk et al., 1990; Rombouts and Thiabaut, 1986; Lam et al., 1992b). The release of small amounts of dehydrodiferulic acid from walls of grasses, some other monocotyledons and a few dicotyledons, by saponification in mild alkali (Harris and Hartley, 1976b, 1980, 1981; Harris and Jones, 1976, 1978; Markwalder and Neukom, 1976; Shibuya, 1984) supports the view that matrix polysaccharides can be bridged in this way. Their formation has been implicated as regulating the mechanical properties of elongating coleoptile walls (Fry, 1979, 1986, 1983; Kamisaka et al., 1990). Since *p*-coumaric acid is known to be esterified to lignins in various grasses, it

Table 3. *In vitro* dry matter digestibility, lignin content determined with different procedures of stems of *bmr* mutants and their normal counterparts

	Extractives (% of ODM)	IVDMD (% of ODM)	Lignin content, % of ODM			
			ADL	Klason lignin	Acid-sol. Lignin	AcBr lignin
Sorghum						
bmr6	40.3	68.3	3.0	6.8	3.3	10.5
normal	31.7	58.8	5.0	8.9	1.8	10.9
Sorghum						
bmr18	47.5	88.2	1.2	4.8	2.8	6.2
normal	43.3	73.1	3.1	7.3	1.7	8.5
Pearl nillet						
bmr	30.7	73.3	2.5	7.2	3.0	10.4
normal	25.0	61.2	5.3	9.1	1.9	10.8
Maize						
bm3	30.8	77.4	3.5	6.5	3.4	10.0
normal	27.9	67.1	6.1	9.5	2.0	11.0

(Lam et al., 1996)

is possible that dimerization with hydroxycinnamic acids esterified to wall polysaccharides could effectively cross-link the two polymers (Ishii, 1991). Dehydrodiferulic acid esterified to wall polysaccharides was released with alkaline hydrolysis under mild condition. In addition, significant amounts of dehydrodiferulic acid etherified to lignin were also detected (figure 9). The values were about 5 times higher than diester of dehydrodiferulic acid in wheat

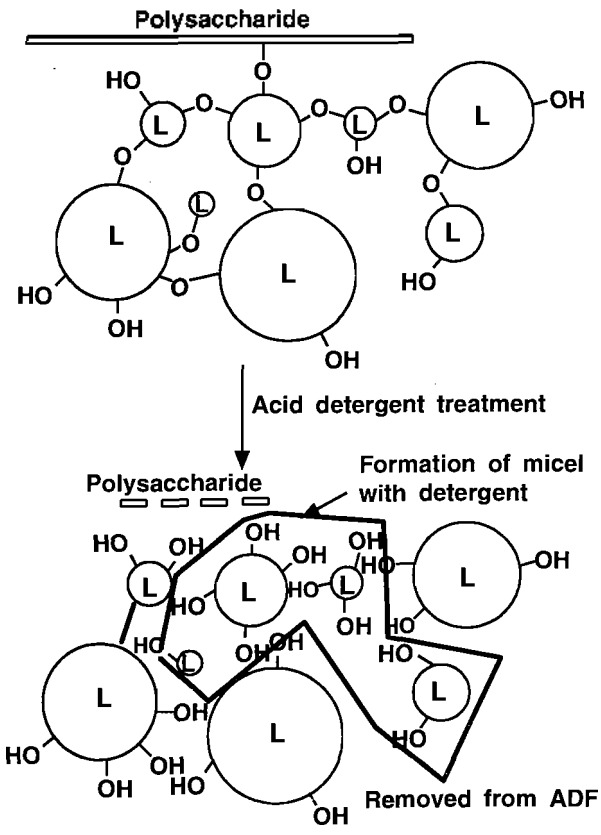

Figure 6. Solubilization of lignin during treatment with acid detergent

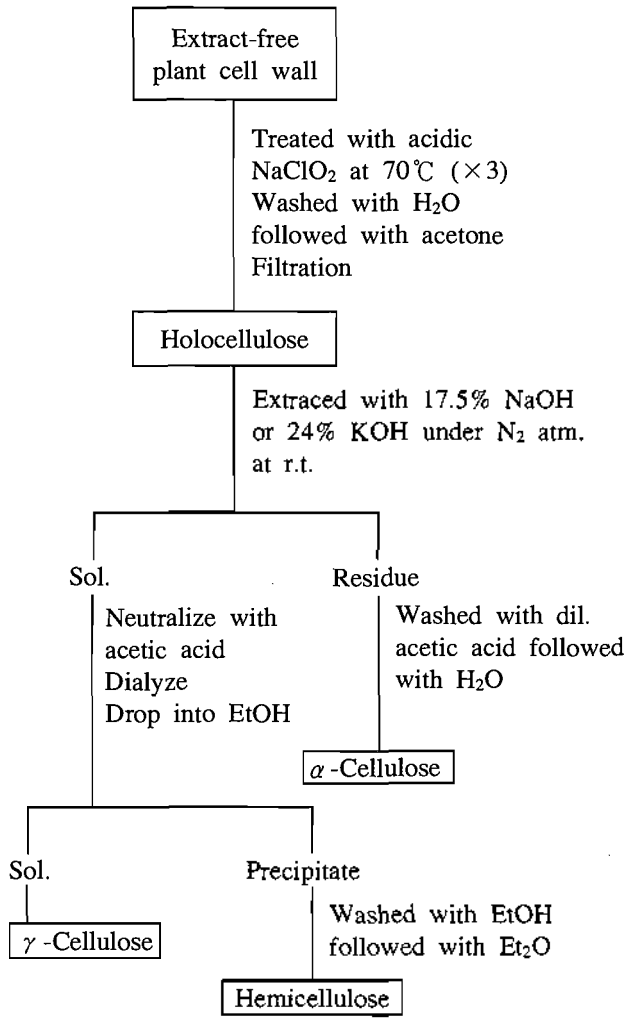

Figure 7. Definition of holocellulose and hemicellulose

and phalaris internodes (Lam et al., 1992b).

Cyclobutane-type dimers related to truxillic and truxinic acids have been isolated after treatment of walls of leaves and stems of temperate and tropical grasses with alkali (Ford and Hartley, 1990). The cyclodimers are composed of *p*-coumaric and/or ferulic acid monomers (Ford and Hartley, 1988, 1989, 1990, Hartley et al., 1988). Some of these dimeric compounds have been prepared by photodimerization of the parent hydroxycinnamic acids (Ford and Hartley, 1990). It is proposed (Ford and Hartley, 1990) that the cyclobutane dimers, which are quantitatively more important than dehydrodiferulic acid in the walls, are functional cross-linking units between wall polysaccharides (Ford and Hartley, 1990; Lam et al., 1990a).

The proportions of the two dimeric forms of hydroxycinnamic acids differs between species of grasses as shown by the analyses for truxillic acid and diferulic acid in barley and wheat straws, ryegrass mesophyll and non-mesophyll cell walls using an HPLC procedure. Values obtained were 0.20, 0.00, 0.04 and 0.11 (% dry wt) for truxillic acid and 0.06, 0.29. 0.02 and 0.08 (% dry wt) for dehydrodiferulic acid, respectively (Wallace et al., 1989).

ASSOCIATIONS BETWEEN LIGNIN AND WALL POLYSACCHARIDES IN PLANTS

The associations between lignin and polysaccharides are the subjects of much study in plant science (Lam et al., 1990a; Iiyama et al., 1993, 1994). Björkman (1956) has reported an isolation procedure of

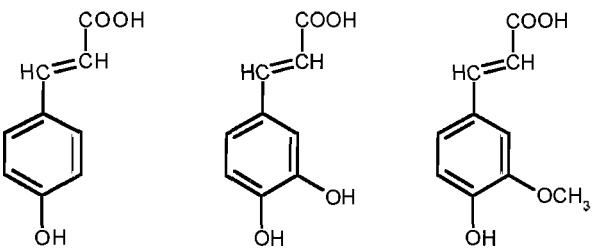

Figure 8. Hydroxycinnamic acids

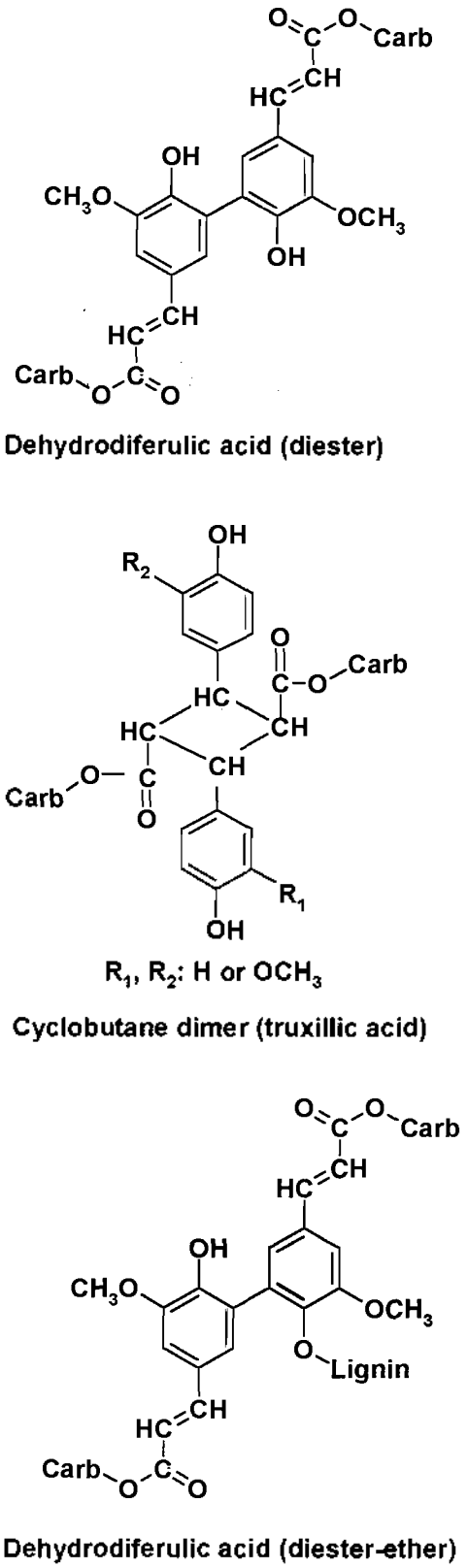

Figure 9. Dimers of hydroxycinnamic acids

lignin-carbohydrate complex (LCC: about 5-10% of cell walls) fraction from finely ground woody materials after separation of Björkman lignin. This fraction is used for structural investigation of LCC. However, most part of lignin (50% for angiosperm wood, 65% of gymnosperm wood) is never extracted as Björkman lignin and LCC. Lignins in the extracted residue would be linked covalently with cellulose (Lam and Iiyama, 1996; Lam et al., 1985; Isogai et al., 1987a, b). The linkage patterns are recognised as benzyl ether-linkage, benzyl ester-linkage and glycosidic linkage (figure 10). A specific oxidant, 2,3-dichloro-5,6-dicyano-1,4-benzoquinene (DDQ) was powerful tool to qualify these linkages (Koshijima et al., 1984; Watanabe et al., 1986, 1989), but not quantitative information at all.

The economic and environmental impacts of the associations between lignin and polysaccharides are also significant. Residual lignins linked firmly to polysaccharides in chemical pulp for papermaking are removed by bleaching using chlorine or chlorine dioxide. Toxic and mutagenic chlorinated chemicals such as dioxin may be released into ecosystems from bleaching system. Lowering of nutritive values of forage plants during maturation is probably due to the formation of associations between lignin and polysaccharides. It is important, therefore, to elucidate the biological, biochemical and chemical features of the associations.

Lignified secondary walls of any vascular plants have associations between lignin and polysaccharides with different extent and with different types of linkages depending on the plant species. The associations visualized by chemical substitution of walls suggest that the associations reinforce the important parts of plant organs mechanically. Lignin macromolecules are flexible, and can cover the surface of polysaccharide fibrils and freely entangle with the polysaccharide matrix, as well as forming covalent linkages. Current understanding of the linkage types between lignin and polysaccharides is reviewed, with particular attention to the chemical and macromolecular features of lignin in cell walls of plants.

ASSOCIATION BETWEEN LIGNIN AND POLYSACCHARIDES IN CELL WALLS OF GRASSES

Growth pattern of grasses is different from dicotyledonous plants. When Björkman lignin is isolated from finely ground samples using a vibratory ball mill, the yield of Björkman lignin is quite lower (3-5% of lignin) than those of other plants (20-30% of lignin for gymnosperms woods, and 35-45% for dicotyledonous plants) (Scalbert and Monties, 1986, Scalbert et al., 1986a, Iiyama et al., 1990). These

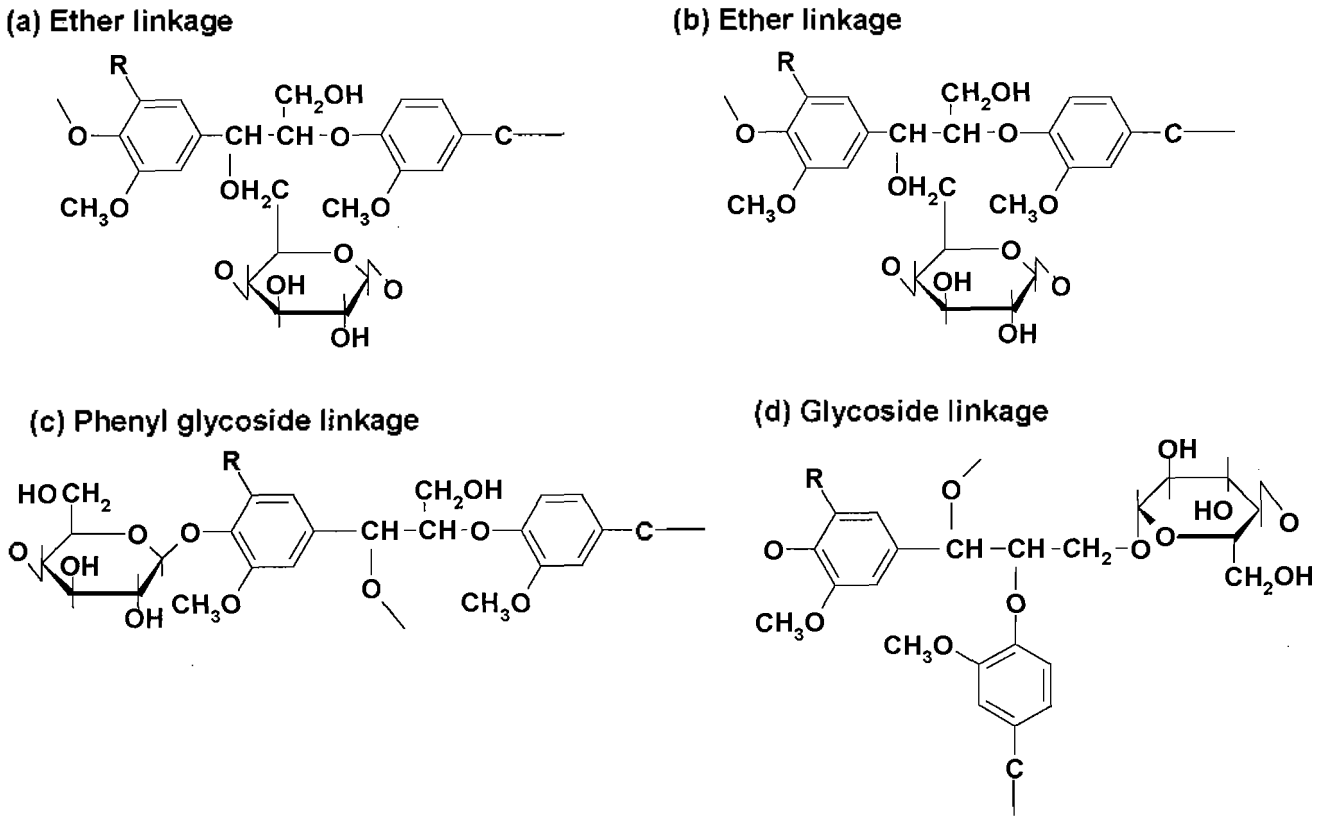

Figure 10. Suggested types of the association between lignin and polysaccharides

results suggest that most of lignin in cell walls of grasses is linked covalently with polysaccharides.

Digestibilities of steam exploded pulps from angiosperm woods were reported, as 65 to 75% of cell walls, suggesting most of polysaccharides were digestible (Akutsu et al., 1986; Saito et al., 1986, 1987). Angiosperm woody plants are composed of vessels and wood fibers. Vessels should be reinforced by the formation of lignin-polysaccharide association, but wood fibers have low quantity of the associations (Lam et al., 1985). When grass cell walls are treated with weak alkaline before extraction of lignin, most of lignin (>90%) is extracted. This fact suggests that the most of linkages between lignin and polysaccharides are alkaline-labile, such as ester-linkages.

Cell walls of grasses compose of hydroxycinnamic acids, such as *p*-coumaric and ferulic acids, but no sinapic acid (Lam et al., 1990c). These hydroxycinnamic acids have two reactive functional groups, -COOH and phenolic OH groups, suggesting the presence of ester-ether bridges between lignin and polysaccharides through hydroxycinnamic acids and their derivatives (figure 11) (Scalbert et al., 1985, 1986b; Iiyama et al., 1990).

To identify these structures, procedure to quantify separately ester-linked and ether-linked hydroxycinnamic acids (Lam et al., 1992a, 1994b) has been developed. The presence of the bridges was quantitatively determined using skilled procedure (table 4, figure 12) (Lam et al., 1992a, 1994b).

These results suggest that all of ferulic acid etherified to lignin is also esterified to polysaccharides to form ester-ether-bridges between lignin and polysaccharides. In addition, the quantity of the association is calculated as the amount of ether-linked ferulic acid in temperate grasses. One lignin phenyl propane unit of each 10 phenylpropane units are involved in the association with polysaccharides, which is about 10 to 15 times higher than those of cell walls of other plants than grasses. Based on these results Lam and coworkers have suggested the biosynthetic pathway of bridges through hydroxycinnamic acids between lignin and polysaccharides (Lam et al., 1994b, 1996a) (figure 13), through nucleophilic addition to quinonemethide intermediate of lignin. Addition of ferulic acid to quinonemethide forms benzyl ether structure (figure 14a). On the other hand, Ralph et al. (1992, 1994:) have suggested using

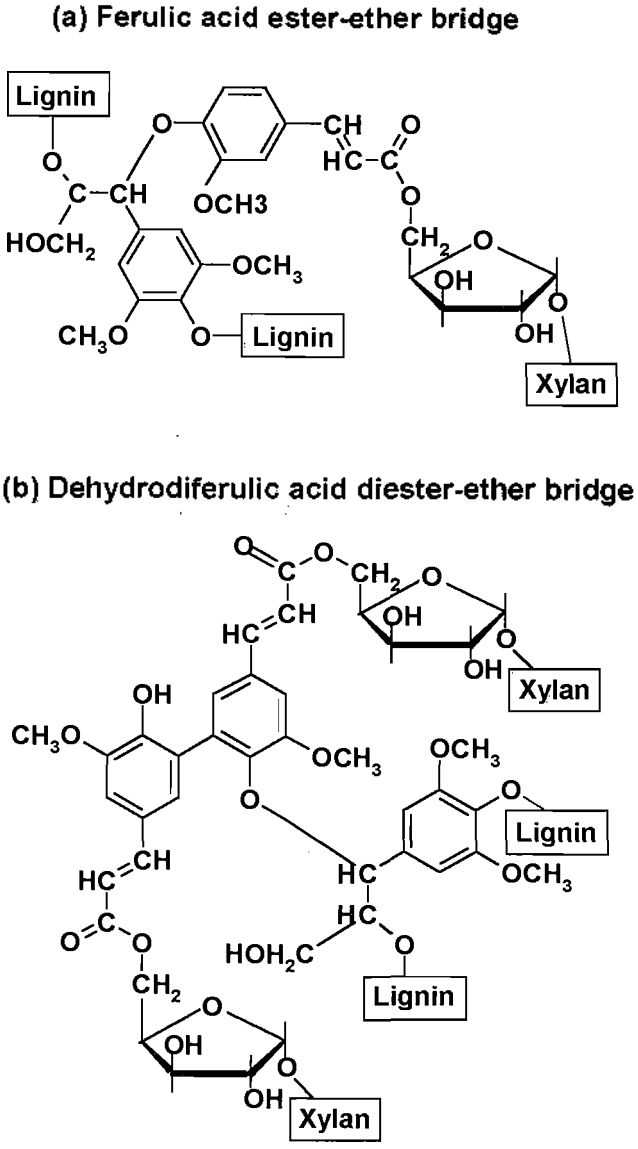

Figure 11. Structures of bridges through ferulic acid (a) and dehydrodiferulic acid (b)

^{13}C-NMR that hydroxycinnamic acids are linked at β -position of lignin side chain through radical coupling reaction (figure 14b), and hydroxycinnamic acids have role as anchor groups of lignification (Terashima, et al. 1993; Jacquet et al., 1995).

BINDING SITE OF HYDROXYCINNAMIC ACIDS ON LIGNIN

Table 4. Linkage types of hydroxycinnamic acids

Sample	*p*-Coumaric acid, wt% of sample			Ferulic acid, wt% of sample		
	Ester only	Ether only	Bridge	Ester only	Ether only	Bridge
Wheat-90%	1.13	0.31	0.00	0.69	0.00	1.41
Phalaris-90%	2.09	0.89	0.00	0.71	0.05	0.60
Phalaris-50%	0.38	0.07	0.00	0.31	0.00	0.51

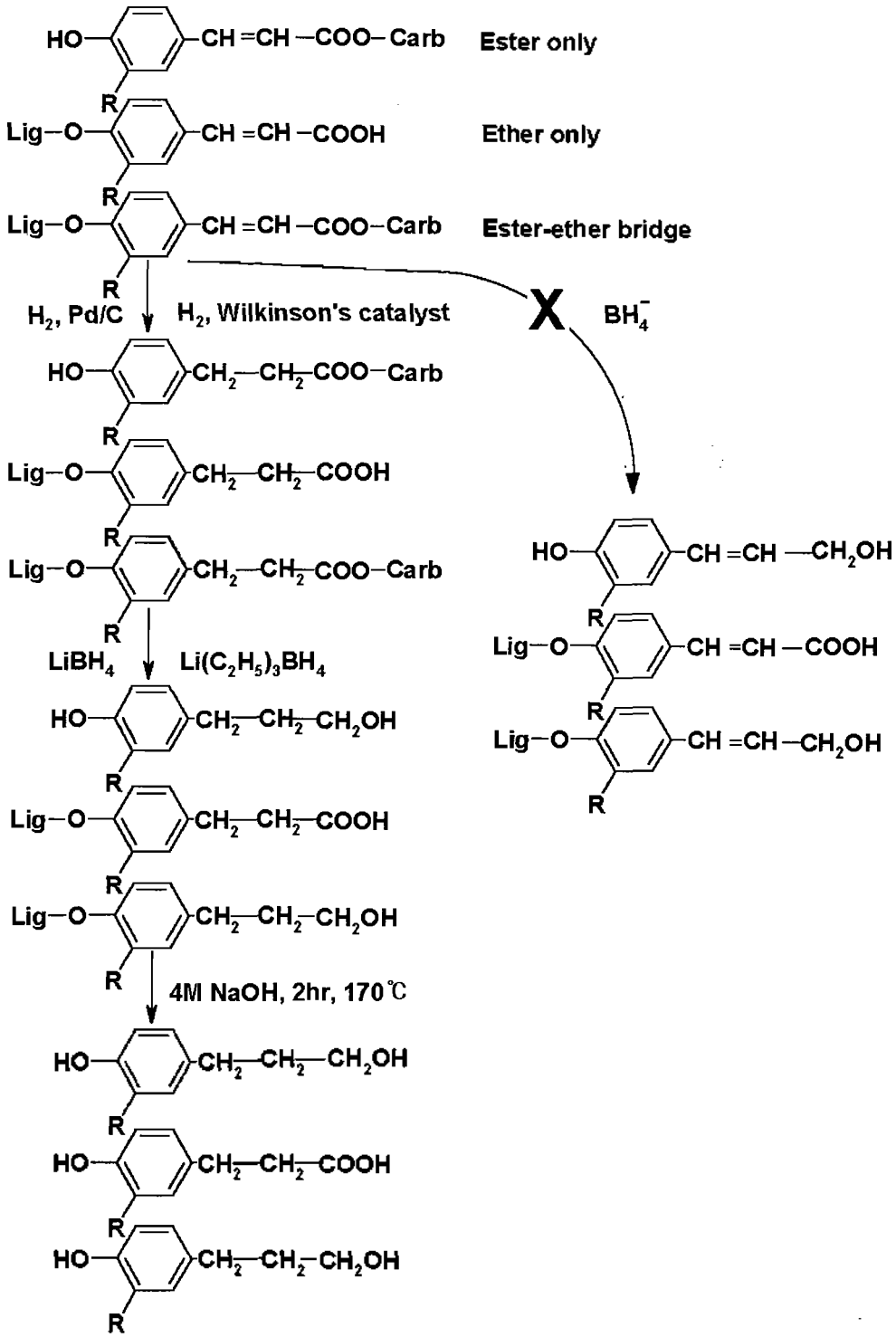

Figure 12. Process to prove the presence of bridges between lignin and polysaccharides through hydroxycinnamic acids

A suspension in dichloromethane-water of various fractions containing hydroxycinnamic acid ester-ether bridges between lignin and polysaccharides prepared from cell walls of matured oat (*Avena sativa* L.) internodes were treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to confirm the binding position of hydroxycinnamic acids. This reagent selectively cleaves benzyl ether and ester linkages of negatively charged aromatic nuclei (figure 15). The sample treated with DDQ was directly hydrolysed either under mild or severe conditions. The hydroxycinnamic acids released in the hydrolysate were

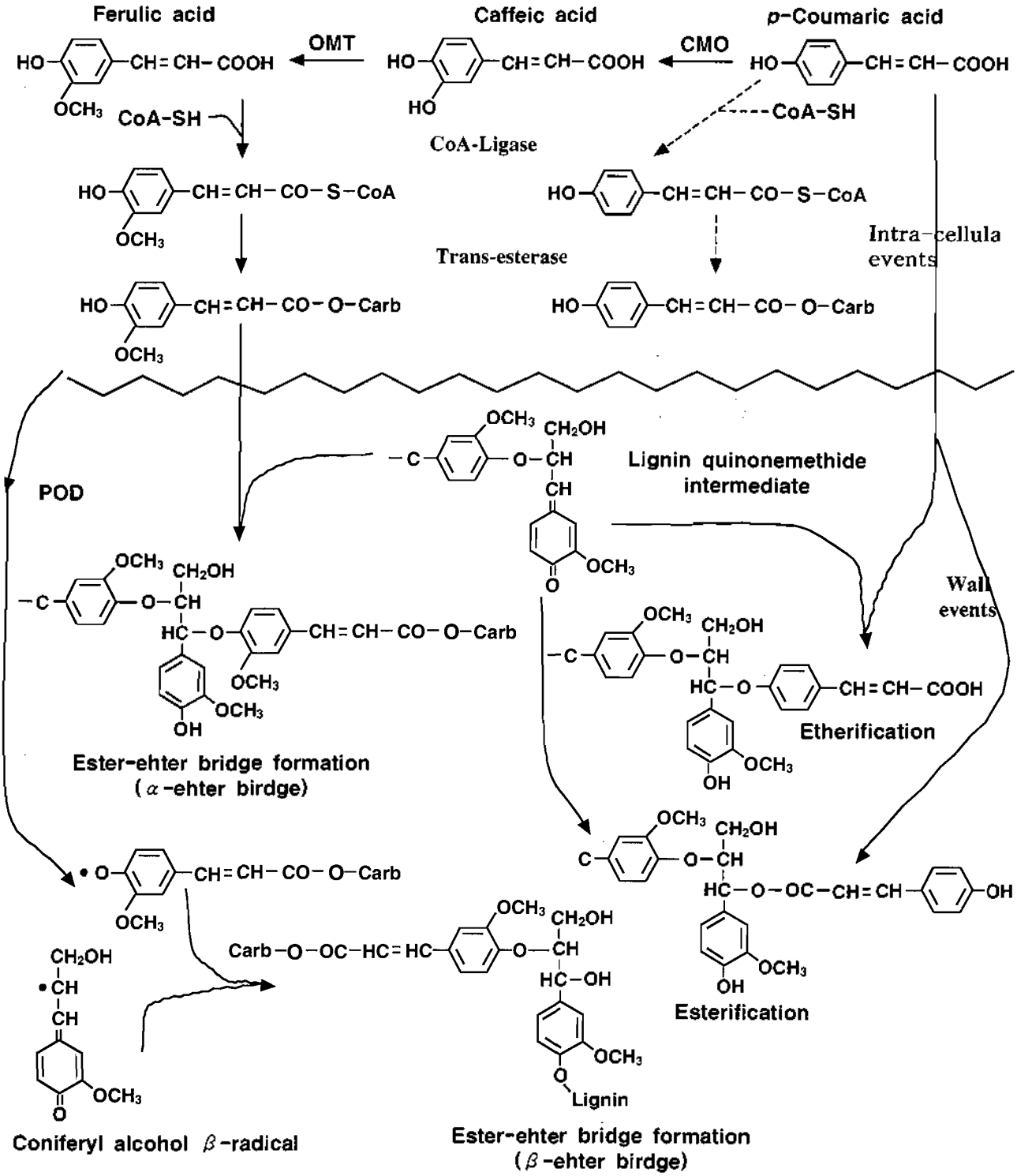

Figure 13. Proposed pathway for biosynthesis of bridges between lignin and polysaccharides through hydroxycinnamic acids

analysed quantitatively. Significant portions of ether linkages between hydroxycinnamic acids and lignin were cleaved with DDQ (table 5), which clearly suggests that most of the hydroxycinnamic acids (>80%) were ether-linked at the benzyl position, and not the β-position, of the lignin side chain (Lam et al., 2001).

RELATIONSHIP BETWEEN LIGNIN AND DIGESTIBILITY

It has been believed that lignin is the most

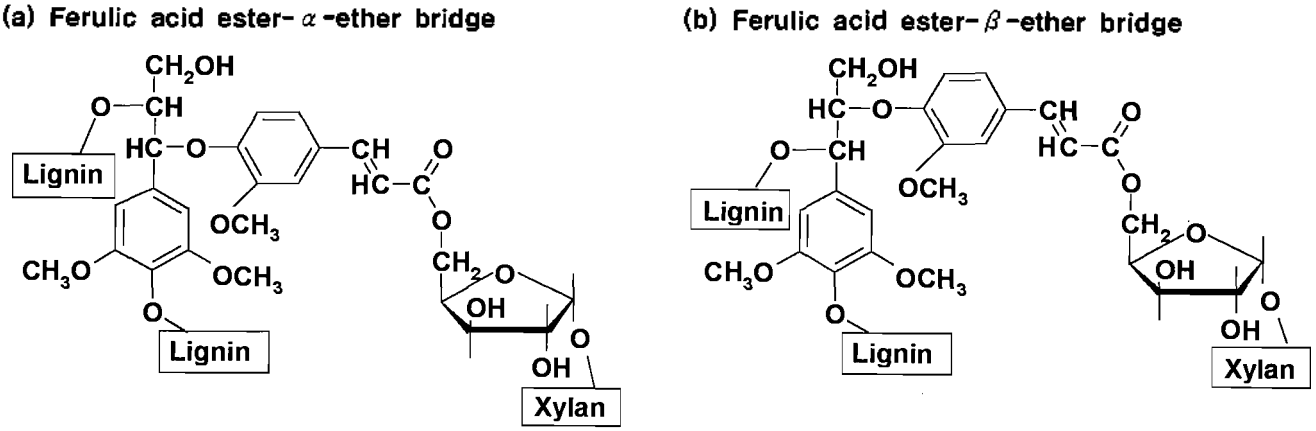

Figure 14. Proposed structures of bridges through hydroxycinnamic acids. (a) α-bridge, (b) β-bridge

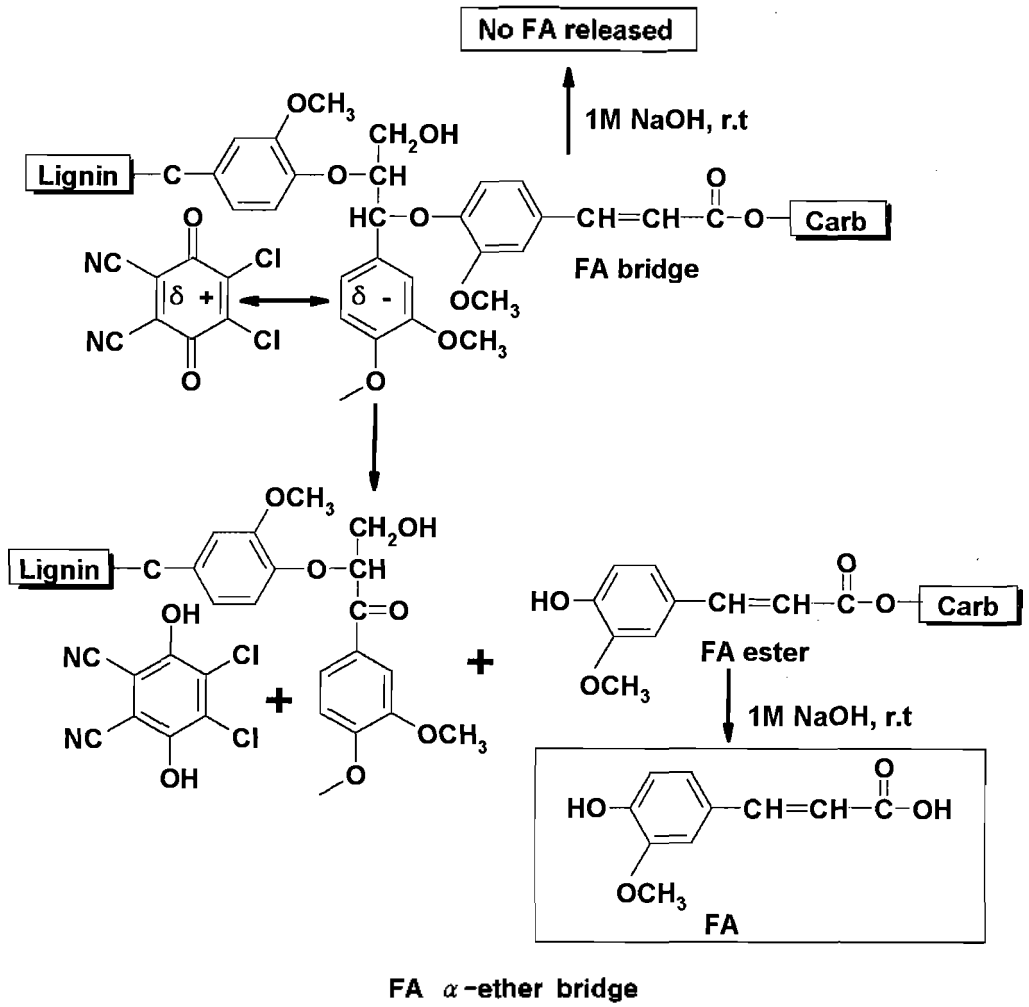

Figure 15. Oxidative cleavage of α-bridge between lignin and polysaccharides through ferulic acid

important secondary cell wall component, which limits digestibility (Van Soest, 1982, 1993). During maturation of plants, lignin content increases gradually, and digestibility of cell walls rapidly decreases. So that, it has been speculated that digestibility of cell walls is deeply dependent with deposition of lignin. The effect of lignin on digestibility should be discussed using samples of the same stage of

Table 5. Released hydroxycinnamic acids by alkaline hydrolysis before and after DDQ oxidation (% of sample)

Sample	Treatment	Alkaline hydrolysis			
		Ester linked		Ether linked	
		PCA	FA	PCA	FA
Oat cell wall	Untreated	0.40	0.35	0.17	0.90
90% Dioxane sol.	Untreated	1.26	0.73	0.63	0.91
	DDQ	1.87	1.54	0.13	0.16
50% Dioxane sol.	Untreated	0.92	0.82	0.52	0.89
	DDQ	1.44	1.62	0.08	0.12
DMSO sol.	Untreated	0.63	0.81	0.32	1.21
	DDQ	1.07	2.00	0.06	0.10
DMSO residue	Untreated	0.45	0.31	0.31	1.31
	DDQ	0.68	0.95	0.11	0.30

DDQ: DDQ oxidation without acetylation, PCA: *p*-coumaric acid, FA: ferulic acid, DMSO: dimethylsulfoxide.

maturation of plants. About 100 varieties of ryegrass and also phalaris were examined at the same stage of maturation. No relationship between lignin content and *in vitro* dry matter digestibility was detected (figure 16).

EFFECT OF FERULIC ACID BRIDGES BETWEEN LIGNIN AND POLYSACCHARIDES ON CELL WALL DIGESTIBILITY

As mentioned above, all of ferulic acid ether-linked to lignin in cell walls of temperate grasses is also esterified to wall polysaccharides (Lam et al., 1992a, 1994b) to form bridges between lignin and polysaccharides. Thus the association between lignin and polysaccharides could be quantified by the determination of ether-linked ferulic acid. The relationship between content of bridge, that is, content of ether-linked ferulic acid, and *in vitro* dry matter digestibility was examined statistically for about 100

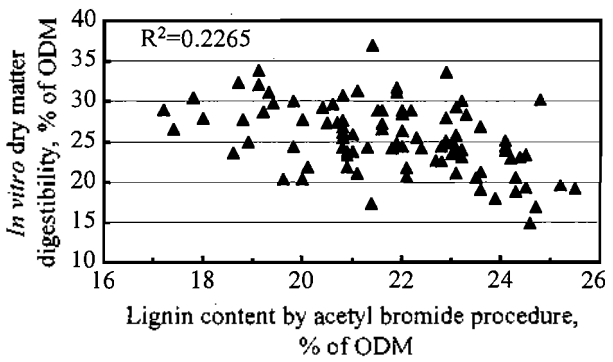

Figure 16. Relationship between lignin content and *in vitro* dry matter digestibility of rye grass

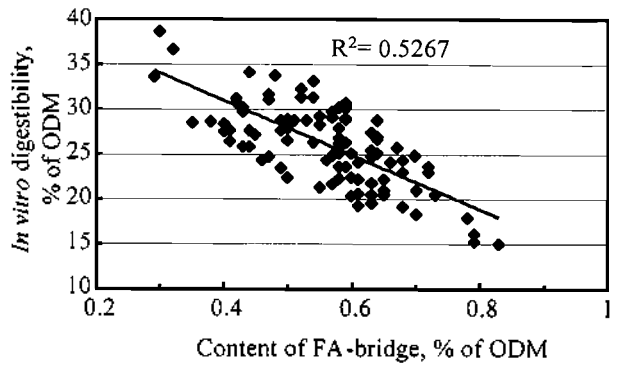

Figure 17. Relationship between content of bridge through ferulic acid and *in vitro* dry matter digestibility of rye grass

varieties of ryegrass and also phalaris. Digestibility of grasses is negatively correlate with the content of associations between lignin and polysaccharides (figure 17).

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