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Physico-Chemical Characteristics of Soluble Lignin Fractions Released from Forage Grasses by Ruminant Digestion

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Abstract

To analyze the structural modifications of forage grass lignin during digestion in ruminant animals, dioxane-soluble lignin fractions were isolated from the feces and rumen digesta of ruminants that received forage grasses, without any pretreatments such as ultragrinding, acid and alkali hydrolysis, and their physico-chemical characteristics were compared with those of undigested original lignin preparations of forage grasses. Both dioxane-soluble lignin fractions from the feces and rumen digesta were composed mostly of guaiacyl-syringyl lignin with *p*-hydroxyphenylpropane units and contained only small amounts of non-lignin constituents. When compared with the undigested original lignin preparations, the dioxane-soluble lignin fractions contained a larger amount of syringylpropane units, and a much smaller amount of bound phenolic acids and showed a lower molecular size. Dioxane-soluble lignin fractions from the in vitro rumen-digested residues of forage grass contained a smaller amount of bound phenolic acids and associated carbohydrates and showed a lower molecular size than those from the cellulose-treated residues. These results indicate that soluble lignin fragments, which are likely to be degradation products of grass lignins or lignin-carbohydrate complexes involving bound phenolic acids, are release in the rumen and then excrered in feces.

Discipline: Animal industry/Grassland
Additional key words: lignin structure, phenolic acids, rumen

Introduction

Lignin is a complex macromolecular substance composed of 3 phenylpropanoid residues, guaiacyl-, syringyl- and *p*-hydroxyphenylpropane units, and their ratios vary depending on the plant species, organs, tissues and maturity. It has been widely recognized that lignin in forages is virtually undigested and inhibits rumen fermentation of forage cell wall polysaccharides, thereby reducing the utilization of forage by ruminant animals. The inhibitory effect of lignin is closely related to its structure and thus probably affected by modifications which lignin undergoes in the digestive tract of ruminants[3,11]. Research on the fate of lignin in the digestive tract demonstrated that some part of lignin fed to ruminants is solubilized as a complex with carbohydrates in the rumen[6,7] and, after leaving the rumen, the complex is excreted as solid matter in the feces[18]. Evidence for the solubilization or degradation of grass lignins by rumen microorganisms was provided by *in vitro* experiments[1,16,19]. In addition, rumen digestion has been reported to lead to the decomposition of lignin model dimers into monoaromatic compounds and their metabolites[4,5] and demethylation of the syringyl units of grass lignin[17]. However, little is known about the composition and structure of solubilized or degraded lignin fractions. Characterization of the lignin fractions may provide further information

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not only on the nutritional effect of forage lignin but also on the biodegradation of plant lignins in anaerobic environment.

We conducted a series of studies on the structural characteristics of lignin fractions solubilized or degraded during digestion in ruminant animals[12–15]. We found that an organic solvent-extractable lignin fraction was present in the rumen digesta and feces of ruminants fed on forage grasses, and characterized the soluble lignins by chemical analysis, nitrobenzene oxidation, UV, IR and ^{13}C-NMR spectroscopy and gel permeation chromatography, compared with undigested original lignin preparations of forage grasses.

Isolation and partial characterization of soluble lignin fractions from feces of sheep

1) Isolation procedure of soluble lignin fractions from feces

Orchardgrass (*Dactylis glomerata* L.), timothy (*Phleum pratense* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) were grown as pure swards, harvested at the heading stage of the first growth and, after wilting for a half-day, dried in an air-forced drier to give grass hays. Hay of each type of grasses was offered to 4 sheep at a maintenance level during a 7-day adaptation period and a 7-day collection period. Feces were sampled during the collection period and representative samples were dried at 55°C and ground with a Wiley mill to pass a 1 mm screen.

The fecal samples from 4 different sheep were bulked and pre-extracted with ethyl ether. After air-drying, the pre-extracted residue was extracted with 90% (v/v) aqueous dioxane for 48 h at room temperature, and the dioxane extract was dried under reduced pressure to give a crude dioxane-soluble lignin fraction. The crude lignin fraction was dissolved in 90% (v/v) acetic acid and precipitated in ethyl ether. After drying, the precipitate was suspended in water to remove water-soluble contaminants. The insoluble material was collected by centrifugation and purified by re-dissolution in acetic acid followed by re-precipitation in ethyl ether.

2) Composition, UV and IR spectra of soluble lignin fractions from feces

The yield of dioxane-soluble lignin from the feces was in the range of 9.5–15.6 g kg^{-1} dry feces (Table 1). The forage grass samples did not contain any significant amount of dioxane-soluble lignin. The dioxane-soluble lignin fractions contained only small amounts of non-lignin constituents such as nitro-

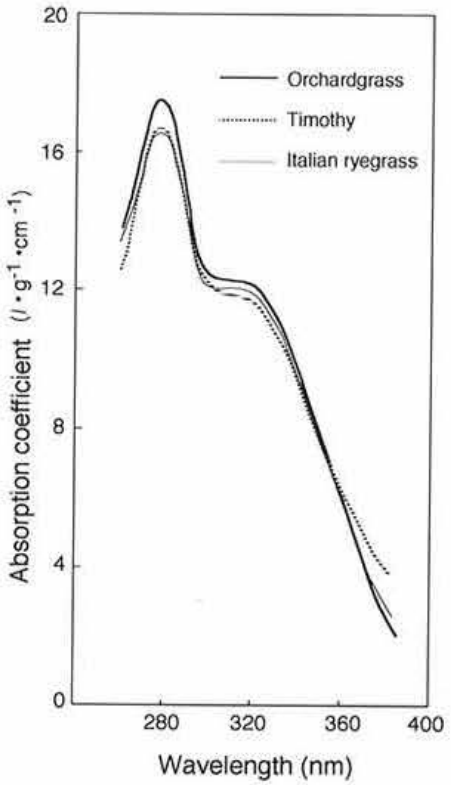

Fig. 1. UV spectra of the dioxane-soluble lignins from feces of sheep fed on forage grasses

Table 1. Yield and composition of the dioxane-soluble lignin fractions isolated from feces of sheep fed on orchardgrass, timothy and Italian ryegrass

	Orchardgrass	Timothy	Italian ryegrass
Yield (g kg^{-1} feces)	12.4	15.6	9.5
Elemental composition (g kg^{-1} soluble lignin)			
C	581	574	585
H	56	59	61
N	8	7	12
Chemical composition (g kg^{-1} soluble lignin)			
Klason lignin	875	844	818
Carbohydrate	18	23	20

genous compounds and carbohydrate, and most part of the lignin was converted into an acid-insoluble residue (Klason lignin) by 72% sulfuric acid hydrolysis for all the grasses. The 3 soluble lignin fractions showed similar UV absorption spectra with a maximum at 278 nm and a shoulder peak at around 320 nm (Fig. 1). The absorption maximum was attributed to hydroxylated aromatic nuclei and the latter shoulder peak to bound phenolic acids. No appreciable differences were found in the absorption coefficient among the 3 soluble lignin fractions. IR spectra showed distinct bands at 1,600, 1,510 and 1,425 cm^{-1} attributable to aromatic nuclei of lignin (Fig. 2). Bands associated with guaiacylpropane units were observed at 1,030 and 1,270 cm^{-1}, and a band assigned to syringylpropane units appeared at 1,330 cm^{-1}.

The present analytical and spectral data revealed the presence of soluble lignin fractions consisting mostly of lignin components in the feces of sheep fed on forage grasses. The dioxane-soluble lignin fractions were insoluble in water but soluble in 80% ethanol, neutral and acid detergents; fibrous residues prepared with these reagents from feces were free of dioxane-soluble lignin. Consequently, the presence of dioxane-soluble lignin in feces of ruminants accounted partly for the incomplete fecal recovery of dietary lignin, which has often been reported.

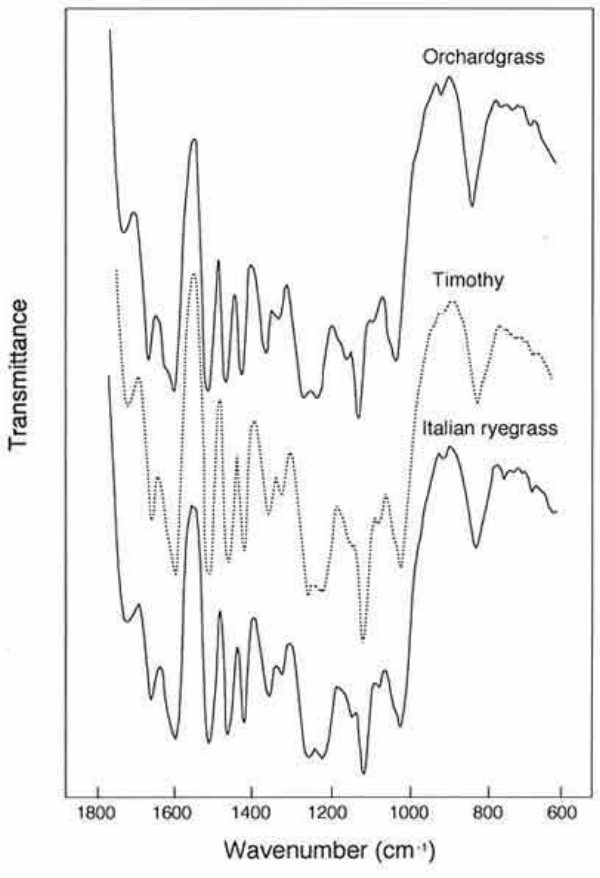

Fig. 2. IR spectra of the dioxane-soluble lignins from feces of sheep fed on forage grasses

Structure of lignin fractions isolated from forage grasses and from feces of sheep fed on them

1) Isolation procedure of lignin fractions from forage grasses and from feces

Two undigested original lignin preparations (ball-milled lignin, BML and ball-milled and cellulase-treated lignin, CTL) were isolated from orchardgrass and timothy hay samples harvested at the heading stage. The grass hay samples were boiled with 80% (v/v) ethanol under reflux for 1 h, the residues collected by filtration and dried. The dried residues were ultraground with a vibratory ball mill in the dry state under N_2 atmosphere with external cooling by water for 24 h. The ultraground sample was extracted with a 90% dioxane solution for 24 h at room temperature, and the extract was dried under reduced pressure to give a crude lignin fraction (BML). The ultraground and dioxane-extracted residue was treated with cellulase at 40°C for 24 h under a toluene atmosphere. The resultant insoluble residue was collected by centrifugation and washed with water. Crude lignin (CTL) in the insoluble residue was obtained by extraction with 90% dioxane. The crude soluble lignin fractions were dissolved in 90% acetic acid and then precipitated in water. The precipitated lignins were further purified by re-dissolution in 90% acetic acid followed by re-precipitation in ethyl ether.

Dioxane-soluble lignin fractions (fecal soluble lignin, FSL) in feces of sheep fed on forage grasses were prepared by the same method as that described above.

2) Characteristics of different soluble lignin fractions

BML and CTL, extracted from the ball-milled grasses, were relatively rich in associated carbohydrates (Table 2). The major neutral sugars of associated carbohydrates in BML and CTL were D-xylose and L-arabinose. In contrast to BML and CTL, FSL from the feces of sheep contained much less carbohydrate, which was poor in D-xylose. The analytical data agreed with the ^{13}C-NMR spectra (Fig. 3). In the spectra of BML and CTL, signals derived from β-1, 4-linked xylan chains were observed

at 63.2–63.7 (C-5), 74.6–75.0 (C-3), 75.5–75.8 (C-4) and 101.4–102.1 (C-1) ppm, together with those of the acetyl group in the xylans at 20.7–21.3 ppm. In the spectra of FSL, on the other hand, NMR signals from the xylan backbone were below the background noise level. These results suggest the occurrence of marked degradation of the lignin-associated xylans by digestion in sheep.

Grass lignins are characterized by high levels of ester-linked *p*-coumaric acid[8]. The undigested original lignin preparations of forage grasses, BML and CTL, contained 10–21 g kg^{-1} ester-linked *p*-coumaric acid while the *p*-coumaric acid ester content of FSL from the feces was very low (Table 2). This difference was confirmed by the ^{13}C-NMR spectra (Fig. 3). A prominent signal at 130.2–130.5 ppm originating from C-2 and C-6 in *p*-coumaric acid ester was observed in the spectra of BML and CTL, whereas the corresponding signal was virtually absent in the spectra of FSL.

The major phenolic aldehydes produced by nitrobenzene oxidation were *p*-hydroxybenzaldehyde, vanillin and syringaldehyde (Table 2), which were derived from *p*-hydroxyphenylpropane, guaiacylpropane and syringylpropane units, respectively. The yield of *p*-hydroxybenzaldehyde decreased in the following order: CTL, BML and FSL. Although no large differences were found in the yields of vanillin and syringaldehyde among the 3 lignins, the molar ratio of syringaldehyde (S) to vanillin (V) was higher for FSL than for BML and CTL. In the ^{13}C-NMR spectra (Fig. 3), FSL showed higher relative intensities of a signal originating from C-2 and C-6 in syringyl units (104.0–104.7 ppm) to those of C-2 (111.3–111.9 ppm) and C-6 (119.1–119.7 ppm) in guaiacyl units than for BML and CDL. The increased molar ratio of S to V in FSL and the ^{13}C-NMR data indicate that the dioxane-soluble lignin fractions from feces of sheep were richer in syringyl units. Based on a microscopic study, plant tissues with syringyl type lignin are more readily degraded by rumen microorganisms than tissues with other types of lignin[2]. A recent study using ^{14}C-lignin cell wall preparations of wheat has revealed a more extensive solubilization of syringyl units by *in vitro* rumen fermentation[17].

The gel permeation chromatograms of BML, CTL and FSL are shown in Fig. 4. For both grasses, FSL contained a larger amount of low molecular size fractions than CTL and BML; CTL contained higher molecular size fractions that eluted at the void volume. Similar differences were observed with the saponified lignin preparations. Ball milling breaks bonds in the lignin–carbohydrate complex and causes depolymerization of lignin macromolecules due to the cleavage of ether bonds[9] and thereby part of

Table 2. Chemical composition, neutral sugar composition, and nitrobenzene oxidation products of the 3 dioxane-soluble lignin fractions from forage grasses

	Orchardgrass			Timothy		
	BML	CTL	FSL	BML	CTL	FSL
Chemical composition (g kg^{-1} soluble lignin)						
Klason lignin	643	653	875	626	648	844
Carbohydrate	210	191	18	217	175	23
Ester-linked *p*-coumaric acid	10.3	16.8	3.4	12.8	21.1	4.3
Ester-linked ferulic acid	4.9	3.5	1.1	5.4	3.4	tr
Neutral sugar composition (g kg^{-1} total sugars)						
L-Rhamnose	5	tr	61	19	4	85
L-Arabinose	91	144	119	89	182	124
D-Xylose	855	767	383	846	729	389
D-Mannose	2	tr	33	1	tr	21
D-Galactose	17	11	143	9	8	96
D-Glucose	30	78	257	36	77	285
Nitrobenzene oxidation products						
Yield (g kg^{-1} Klason lignin in soluble lignin)						
p-Hydroxybenzaldehyde	8.6	11.9	6.6	8.5	11.2	5.9
Vanillin (V)	85	102	85	108	112	90
Syringaldehyde (S)	65	83	84	80	98	100
Molar ratio of S to V	0.64	0.68	0.83	0.62	0.73	0.92

BML: extracted from ball-milled grass, CTL: extracted from ball-milled and cellulase-treated grass, FSL: extracted from feces of sheep fed on grass, tr: traces.

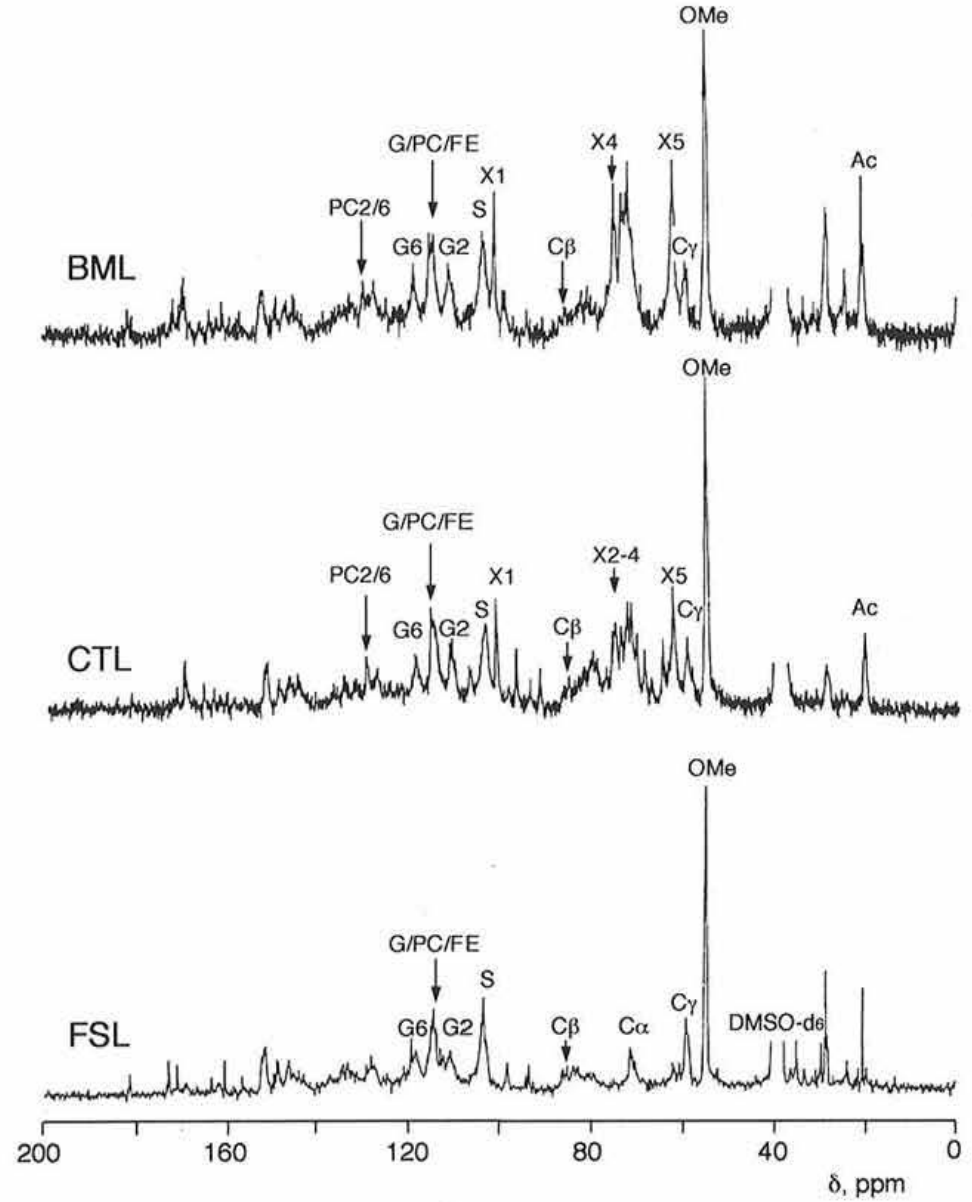

Fig. 3. ^{13}C-NMR spectra of the dioxane-soluble lignins from ball-milled orchardgrass (BML), ball-milled and cellulase-treated orchardgrass (CTL), and feces of sheep fed on orchardgrass (FSL)

Ac: acetyl group in xylans, OMe: methoxyl group in guaiacyl and syringyl units and ferulic acid, Cα: C-α in β-O-4, Cβ: C-β in β-O-4, Cγ: C-γ in β-O-4, X1: C-1 in β-xylose (1→4), X2-4: C2/3/4 in β-xylose (1→4), X5: C-5 in β-xylose (1→4), S: C-2/6 in syringyl unit, G2: C-2 in guaiacyl unit, G6: C-6 in guaiacyl unit, G/PC/FE: C-5 in guaiacyl unit and C-3/5 in *p*-coumaric acid ester and C-β in ferulic acid ester, PC2/6: C-2/6 in *p*-coumaric acid ester.

native lignin is converted to an organic solvent-extractable form. The elution profiles of gel permeation chromatography show that FSL was composed of lignin fragments with lower molecular sizes, compared with BML and CTL, and hence it is likely that the soluble lignin fragments released by digestion in sheep corresponded to depolymerization products of the forage grass lignins.

Comparison of soluble lignin fractions from rumen digesta and feces of heifer

1) Isolation procedure of soluble lignin fractions

Orchardgrass hay harvested at the pre-heading

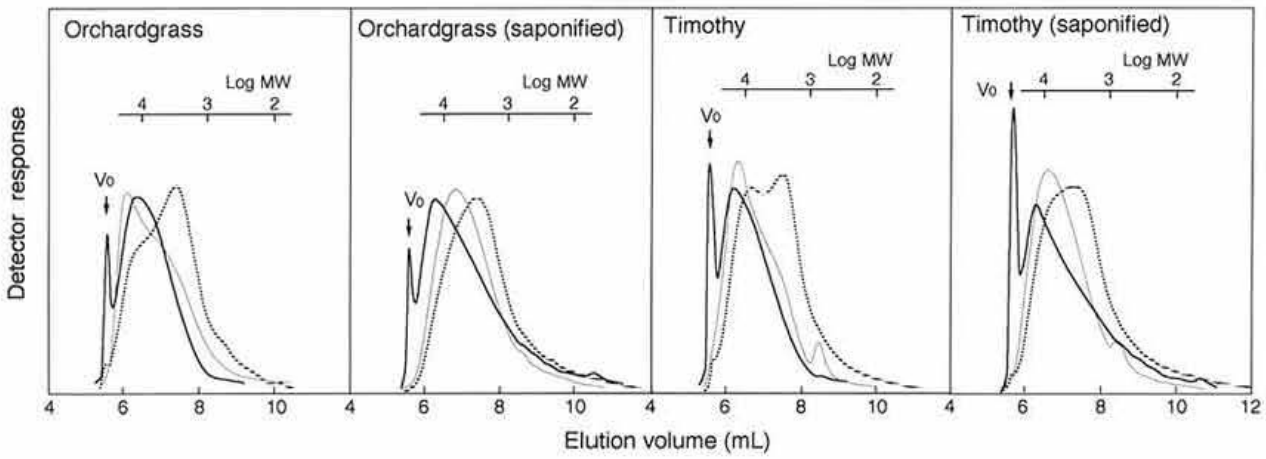

Fig. 4. Gel permeation chromatograms of the dioxane-soluble lignin fractions from ball-milled grasses (BML, —), ball-milled and cellulase-treated grasses (CTL, —), and feces of sheep fed on grasses (FSL, ····)

stage was offered to a heifer (Japanese Black Cattle) fitted with a rumen fistula during a 14-day adaptation period and a 2-day collection period. A rumen digesta sample was taken prior to 09:00 feed for 2 days, freeze-dried and bulked. Feces collected during the 2-day period were mixed thoroughly and a representative sample of the feces was freeze-dried. Soluble lignin fractions were extracted from the rumen digesta and fecal sample with 90% dioxane and purified as described above.

2) Physical and chemical characteristics of soluble lignin fractions

The yields of the dioxane-soluble lignin were 3.3 g kg^{-1} for the rumen digesta and 8.2 g kg^{-1} for the feces. It was preliminarily confirmed that the orchardgrass hay sample did not contain any significant amounts of dioxane-soluble lignin. Most of the dioxane-soluble lignin was recovered as Klason lignin by 72% sulfuric acid hydrolysis (Table 3). There were no considerable differences in the levels of

Table 3. Chemical composition, nitrobenzene oxidation products and molecular size of the dioxane-soluble lignin fractions from rumen digesta and feces of heifer fed on orchardgrass

	Rumen digesta	Feces
Chemical composition (g kg^{-1} soluble lignin)		
Klason lignin	821	856
Carbohydrate	29	31
Nitrogen	25	18
p-Coumaric acid		
Ester-linked	3.0	2.8
Ether-linked	1.4	1.6
Ferulic acid		
Ester-linked	1.3	1.3
Ether-linked	1.8	2.1
Yields of nitrobenzene oxidation products (g kg^{-1} soluble lignin)		
p-Hydroxybenzaldehyde	5	5
Vanillin	55	58
Syringaldehyde	44	47
Molecular size		
Number average (M_n)	690	650
Weight average (M_w)	1,480	1,450
Polydispersity (M_n/M_w)	2.14	2.23

bound phenolic acids and the yields of phenolic aldehydes produced by nitrobenzene oxidation between the rumen digesta and feces samples (Table 3). Both dioxane-soluble lignin fractions showed a wide molecular size distribution ranging from monomer up to 15,000. Elution maxima appeared at a molecular weight of 1,500 for the rumen digesta and at a molecular weight of 1,480 for the feces. The rumen and fecal dioxane-soluble lignin fractions had similar number-average molecular weights (M_n) and weight-average molecular weights (M_w) (Table 3).

The presence of dioxane-soluble lignin fractions in the rumen digesta indicates that, part of the orchardgrass lignin fed was released in the rumen as soluble lignin fragments. The chemical and physical properties of the fecal dioxane-soluble lignin were similar to those of the rumen dioxane-soluble lignin, suggesting that the soluble lignin fragments did not undergo any significant modifications after leaving the rumen.

Characteristics of soluble lignin fractions formed by *in vitro* rumen digestion of forage grass

1) Isolation procedure of soluble lignin fractions

Timothy hay harvested at the seed setting stage, supplemented with soybean meal, was offered to a heifer (Japanese Black Cattle) fitted with a rumen cannula during a 7-day adaptation and a 3-day collection period. Hay and feces were sampled during the collection period and their representative samples were dried, ground to pass a 1 mm screen. The hay sample was boiled with 80% ethanol under reflux for 1 h. The ethanol-extracted and dried residue was ultraground with a porcelain ball-mill for 10 days and then pre-extracted with 90% dioxane. The dioxane-extracted and air-dried residue was divided into 2 parts: one was subjected to enzymatic treatment and the other subjected to *in vitro* rumen digestion.

One part was treated with cellulase at 40°C under a toluene atmosphere for 72 h. The insoluble residue after the enzymatic hydrolysis was collected by centrifugation, washed with water and freeze-dried. The dried residue was extracted with 90% dioxane. The dioxane extract, after drying by evaporation, was dissolved in 90% acetic acid and then precipitated in water. The precipitated lignin was further purified by dissolution in 90% acetic acid followed by precipitation in ethyl ether to give cellulase-treated lignin (CTL).

The other part was digested in rumen liquor *in vitro* for 72 h. The rumen liquor used was taken from the fistulated heifer that received the timothy hay and soybean meal. The *in vitro* rumen-digested residue was collected by centrifugation, washed with water and freeze-dried. A soluble lignin fraction in the residue (rumen-digested lignin, RDL) was extracted with 90% dioxane and purified by re-dissolution and re-precipitation.

Dioxane-soluble lignin (heifer-digested lignin, HDL) in the fecal sample of the heifer was extracted and purified in the same way as described above.

2) Physical and chemical characteristics of soluble lignin fractions

Although CTL and RDL were prepared from the same timothy sample, the 2 lignin fractions differed considerably in chemical and physical properties. RDL contained less carbohydrate, ester- and ether-linked *p*-coumaric and ferulic acids than CTL (Table 4). The lower levels of the bound phenolic acids in RDL than in CTL suggest the removal or degradation of the bound phenolic acids from timothy lignin by rumen fermentation. This assumption does not conflict with the similarity in the composition between RDL and HDL. Phenolic acid ethers are reported to form a cross-linking unit between lignin and polysaccharides in grass cell walls[10,20]. The decrease in the carbohydrate and phenolic acid ether contents in RDL may be indicative of ruminal degradation of a lignin–carbohydrate complex involving cross-linking phenolic acid ethers.

Appreciable differences were observed in the molecular size distribution among the 3 lignin fractions (Fig. 5). CTL was distributed in a a higher molecular size region than RDL and HDL. About

Table 4. Chemical composition of the 3 dioxane-soluble lignin fractions from timothy

	CTL	RDL	HDL
	(g kg^{-1} soluble lignin)		
Klason lignin	708	887	889
Carbohydrate	189	50	37
p-Coumaric acid			
Ester-linked	15.0	3.7	3.4
Ether-linked	2.3	1.5	1.5
Ferulic acid			
Ester-linked	2.1	0.4	0.6
Ether-linked	6.5	2.3	3.4

CTL: extracted from ball-milled and cellulase-treated timothy, RDL: extracted from ball-milled and rumen-digested timothy, HDL: extracted from feces of heifer fed on timothy.

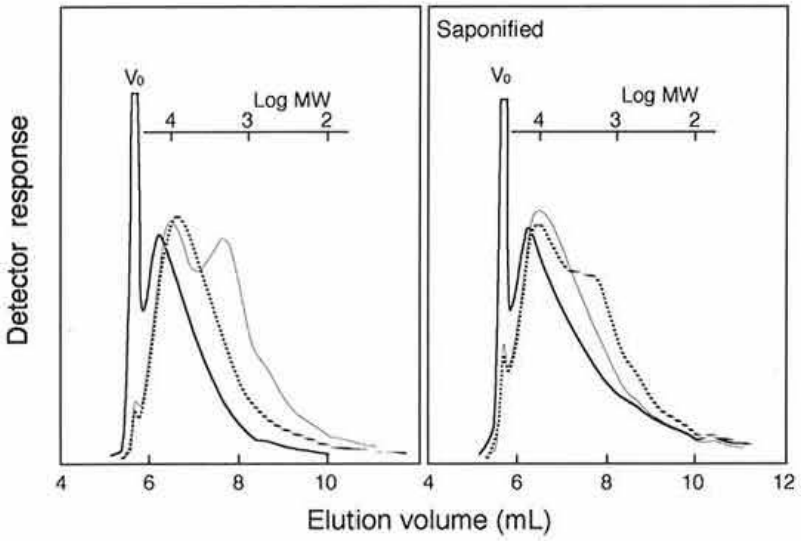

Fig. 5. Gel permeation chromatograms of the dioxane-soluble lignin fractions from ball-milled and cellulase-treated timothy (CDL, ——), ball-milled and rumen-digested timothy (RDL, ·····), and feces of heifer fed on timothy (HDL, ——)

30% of CTL, based on the area ratio of the chromatogram, was eluted at the void volume. Saponification did not substantially affect the differences in molecular size distribution among the 3 lignin fractions (Fig. 5), although saponification reduced the carbohydrate content of all the soluble lignin fractions extensively and liberated ester-linked phenolic acids. Consequently, it is considered that RDL consisted of lignin fragments with lower molecular sizes than CTL. This finding suggests that rumen microorganisms are able to modify the macromolecular structure of forage grass lignin.

References

1) Akin, D. E. & Benner, R. (1988): Degradation of polysaccharide and lignin by ruminal bacteria and fungi. *Appl. Environ. Microbiol.*, **54**, 1117–1125.
2) Akin, D. E. & Chesson, A. (1989): Lignification as the major factor limiting forage feeding value especially in warm conditions. *In* Proc. XVIth Int. Grassl. Congr. Nice. ed. Desroches, R. Association Française pour Productions Fourragères, INRA, Versailles, France, 1753–1760.
3) Belse, J-M., Cornu, A. & Jouany, J-P. (1994): Roles of structural phenylpropanoids in forage cell wall digestion. *J. Sci. Food Agric.*, **64**, 171–190.
4) Chen, W. et al. (1985): Anaerobic degradation of vetratrylglycerol-β-guaiacyl ether and guaiacoxyacetic acid by mixed rumen bacteria. *Appl. Environ. Microbiol.*, **50**, 1451–1456.
5) Chen, W. et al. (1987): Anaerobic degradation of dehydrodiisoeugenol by rumen bacteria. *J. Ferment. Technol.*, **65**, 221–224.
6) Conchie, J., Hay, A. J. & Lomax, J. A. (1988): Soluble lignin-carbohydrate complexes from sheep rumen fluid: their composition and structural features. *Carbohydr. Res.*, **177**, 127–151.
7) Gaillard, B. D. E. & Richards, G. N. (1975): Presence of soluble lignin-carbohydrate complexes in the bovine rumen. *Carbohydr. Res.*, **42**, 135–145.
8) Higuchi, T. et al. (1967): Chemical properties of milled wood lignin of grasses. *Phytochemistry*, **6**, 1551–1556.
9) Hon, D. N. S. (1983): Mechanochemical reactions of lignocellulosic materials. *J. Appl. Polymer Sci.*, **37**, 461–481.
10) Iiyama, K., Lam, T. B. T. & Stone, B. A. (1990): Phenolic acid bridges between polysaccharides and lignin in wheat straw internodes. *Phytochemistry*, **29**, 733–737.
11) Jung, H. G. & Fahey, G. C. (1983): Nutritional implications of phenolic monomers and lignin: a review. *J. Anim. Sci.*, **57**, 206–219.
12) Kondo, T., Ohshita, T. & Kyuma, T. (1993): Isolation and characterization of dioxane-soluble lignins from faeces of sheep fed on orchardgrass hay and silage. *Anim. Feed Sci. Technol.*, **41**, 213–221.
13) Kondo, T., Ohshita, T. & Kyuma, T. (1994): Release of soluble lignin fragments from orchardgrass during its passage through the rumen. *J. Sci. Food Agric.*, **65**, 429–431.
14) Kondo, T. et al. (1995): Comparative characterisation of dioxane-soluble lignins released by ball milling and by sheep digestion from forage grasses. *J. Sci. Food Agric.*, **68**, 383–388.
15) Kondo, T., Ohshita, T. & Kyuma, T. (1997): Structural changes of forage grass lignin by rumen digestion: Characteristics of soluble lignin released from

timothy (*Phleum pratense* L.) by *in vitro* rumen digestion. *JARQ*, **31**, 49–54.
16) McSweeny, C. S. et al. (1994): Solubilization of lignin by ruminal anaerobic fungus *Neocallimastix pattriciarum*. *Appl. Environ. Microbiol.*, **60**, 2985–2989.
17) Mosoni, P. et al. (1994): Transformations of (^{14}C-lignin) cell walls of wheat by rumen microorganisms. *J. Sci. Food Agric.*, **64**, 379–387.
18) Neilson, M. J. & Richards, G. N. (1978): The fate of the soluble lignin-carbohydrate complex produced in the bovine rumen. *J. Sci. Food Agric.*, **29**, 513–519.
19) Nordkvist, E., Graham, H., & Aman, P. (1989): Soluble lignin complexes isolated from wheat straw (*Triticum arvense*) and red clover (*Trifolium pratense*) stems by an in-vitro method. *J. Sci. Food Agric.*, **48**, 311–321.
20) Scalbert, A. et al. (1985): Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochemistry*, **24**, 1359–1362.

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