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Structural Changes of Forage Grass Lignin by Rumen Digestion: Characteristics of Soluble Lignin Released from Timothy (*Phleum pratense* L.) by In Vitro Rumen Digestion

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Abstract

Timothy, harvested at the seed setting stage, was subjected to 3 different treatments: (1) ball milling followed by in *vitro* rumen digestion, (2) ball milling followed by cellulase digestion, (3) digestion by a heifer, and then dioxane-soluble lignins (RDL, CDL and HDL, respectively) were isolated from the 3 resultant residues. RDL contained a larger amount of lignin components and smaller amounts of carbohydrate residues, and bound phenolic acids than CDL and its composition resembled that of HDL. Alkaline nitrobenzene oxidation and IR spectra showed that all the lignins consisted of guaiacyl-syringyl lignins. The molar ratio of syringaldehyde to vanillin was higher for HDL than for CDL and RDL. In gel permeation chromatograms, RDL and HDL were distributed in lower molecular weight regions than CDL. The results suggested that timothy lignin was depolymerized and the phenolic acids bound to timothy lignin disappeared by rumen fermentation.

Discipline: Animal industry/Grassland
Additional key words: carbohydrate, degradation, phenolic acids

Introduction

Although lignin in forages occurs in rather small amounts, it is generally recognized as one of the major chemical components due to its key role in ruminal degradation of forage fibers. The nutritional role of lignin is closely related to its structure and thus probably affected by the modifications that lignin undergoes in the digestive tract[2,10]. Studies on the behavior of lignin in the rumen indicated that water-soluble lignin was released as a complex with carbohydrate residues[6,8,16], for which rumen bacteria and fungi are responsible[1,14]. It has also been found that part of the forage grass lignin fed was released as dioxane-soluble lignin in the rumen and excreted in the feces[11–13]. Rumen digestion has been reported to lead to the decomposition of lignin model dimers into monoaromatic compounds and their metabolites[3,4] and demethylation of the syringyl units of grass lignin[15], but no further evidence is presented showing structural degradation of lignins in plant materials by rumen digestion.

The objective of the present study was to investigate the structural changes of forage grass lignin occurring in the rumen environment. We compared 3 lignin fractions extracted from the rumen-digested residue of timothy hay *in vitro*, from the cellulase-treated residue of timothy hay, and from the feces of a heifer that consumed timothy hay.

Materials and methods

1) Preparation of timothy hay and animal feeding

Timothy (*Phleum pratense* L., cv Climax) was grown in an experimental field of the Tohoku National Agricultural Experiment Station, harvested at the seed setting stage in the first growth and dried in the field for 3 days to obtain timothy hay. The hay (8 kg day^{-1}) supplemented with soybean meal (1 kg day^{-1}) was offered to a heifer (Japanese Black Cattle, 28 months) fitted with a rumen cannula at 09:00 daily during a 7-day adaptation period and a 3-day collection period. Hay and feces were

sampled during the collection period and their representative samples were dried at 55°C. The dried hay and fecal samples were ground with a Wiley mill to pass a 1 mm screen. The chemical composition of the timothy hay (g kg^{-1} DM) was as follows: crude protein 43 g kg^{-1} DM, neutral detergent fiber 735 g kg^{-1} DM, acid detergent fiber 441 g kg^{-1} DM and acid detergent lignin 81 g kg^{-1} DM.

2) *Isolation of dioxane-soluble lignin fractions*

(1) Isolation from timothy

The hay sample was boiled with 80% (v/v) ethanol under reflux for 1 h, filtered on a filter paper and washed with 80% (v/v) ethanol. After drying on silica gel under reduced pressure, the ethanol-extracted residue was finely ground with a porcelain ball-mill for 10 days and then extracted with 90% (v/v) dioxane for 24 h. The dioxane-extracted and air-dried residue was divided into 2 parts; one was subjected to enzymatic hydrolysis and the other to *in vitro* rumen digestion.

One part (15 g) was treated with Cellulase ONOZUKA RS (Yakult Honsha Co., LTD, 50 mg g^{-1} residue) in 50 mM acetate buffer (pH 4.5) at 40°C under toluene atmosphere for 72 h. The insoluble residue after the enzymatic hydrolysis was collected by centrifugation (20,000 × g, 20 min), washed with water and freeze-dried. The dried residue was extracted with 90% (v/v) dioxane at room temperature for 24 h. The dioxane extract, after drying by evaporation, was dissolved in 90% (v/v) acetic acid and then precipitated in water. The precipitated lignin was further purified by dissolution in 90% (v/v) acetic acid followed by precipitation in ethyl ether to give cellulase-digested lignin (CDL).

The other part was digested in rumen liquor *in vitro* as described by Goering and Van Soest[9], except that the operation was performed on a large scale (sample size: 15 g, buffer: 1,200 mL, rumen liquor: 300 mL) for 72 h. The rumen liquor used was taken at 14:00 from the fistulated heifer that received the timothy hay and soybean meal. The digested residue was collected by centrifugation (20,000 × g, 20 min), washed with water and freeze-dried. Dioxane-soluble lignin (rumen-digested lignin, RDL) in the residue was extracted and purified as described above.

(2) Isolation from feces

After pre-extraction with ethyl ether, the fecal sample was extracted with 90% dioxane for 48 h at room temperature, and the dioxane extract was dried under reduced pressure to give crude heifer digested lignin (HDL). The crude HDL was dissolved into 90% acetic acid and precipitated into 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 dissolution into acetic acid followed by precipitation into ethyl ether.

3) *Chemical analysis*

Klason lignin content was determined by hydrolysis with 72% (w/w) sulfuric acid followed by hydrolysis with 3% (w/w) sulfuric acid and total carbohydrates were assayed in the 3% sulfuric acid hydrolysate by the phenol-sulfuric acid method[7]. Ester- and ether-linked phenolic acids were extracted by saponification with 1 N NaOH and by subsequent acid hydrolysis with a dioxane-2 N HCl mixture (9:1 v/v)[12], respectively, and analyzed by HPLC[11]. Alkaline nitrobenzene oxidation was performed as previously described[12] and the phenolic aldehydes produced were analyzed by HPLC. Chemical analysis was performed in duplicate.

4) *UV and IR spectroscopy*

UV spectra were recorded for a solution in 90% dioxane on a Shimadzu UV 3000 spectrophotometer. IR spectra were recorded for KBr discs on a Hitachi 270-50 IR spectrophotometer.

5) *Gel permeation chromatography (GPC)*

Molecular size distributions were determined by gel permeation chromatography with a Shimadzu LC-6A GPC system[13]. The lignin preparations were eluted on Shodex GPC KD-802.5 column with dimethylformamide containing 10 mM LiCl at a flow rate of 0.5 mL at 50°C. The column effluent was detected with a UV monitor (280 nm).

Results

The degradability of the ball-milled timothy by cellulase hydrolysis was 65.0% and the digestibility determined by the *in vitro* method was 71.2%. The yields of CDL and RDL were 418 and 200 g kg^{-1} dioxane-extracted residue, respectively. No significant amount of dioxane-soluble lignin could be isolated from the rumen liquor used for the *in vitro* experiment.

The chemical composition of the dioxane-soluble lignins is given in Fig. 1. CDL contained 708 g Klason lignin kg^{-1}, 189 g total carbohydrates kg^{-1},

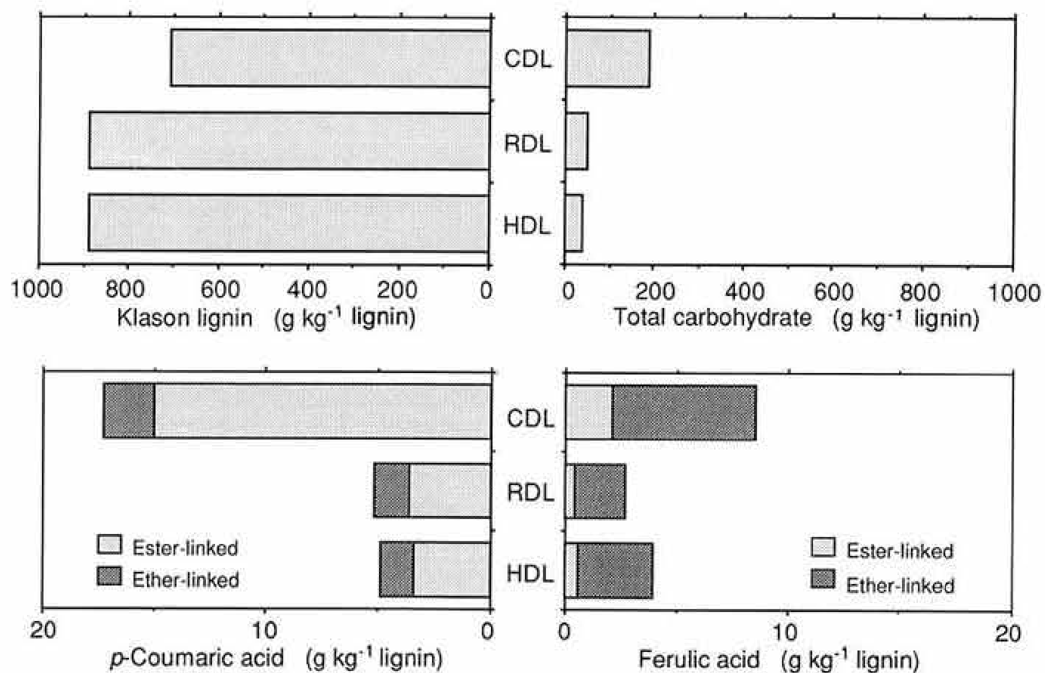

Fig. 1. Chemical composition of cellulase-digested lignin (CDL), rumen-digested lignin (RDL) and heifer-digested lignin (HDL) from timothy

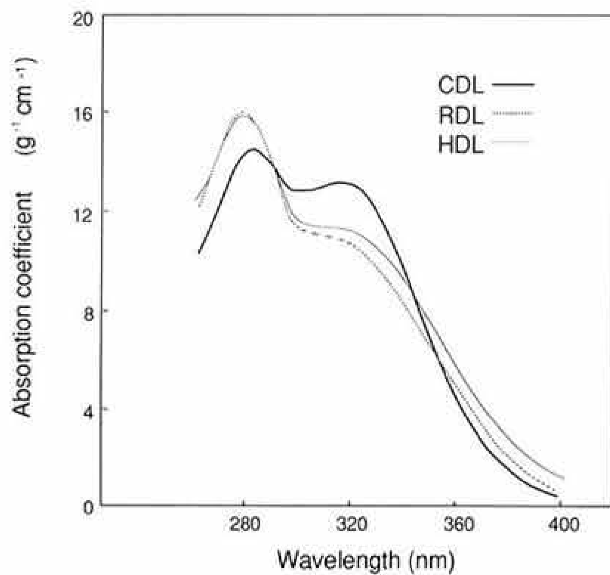

Fig. 2. UV spectra of cellulase-digested lignin (CDL), rumen-digested lignin (RDL) and heifer-digested lignin (HDL) from timothy

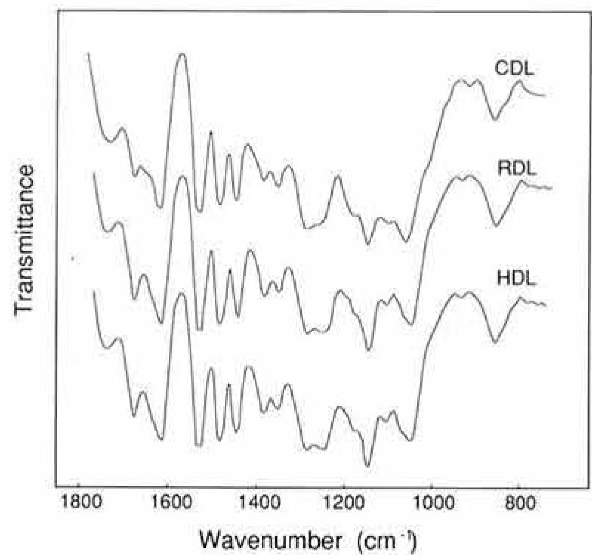

Fig. 3. IR spectra of cellulase-digested lignin (CDL), rumen-digested lignin (RDL) and heifer-digested lignin (HDL) from timothy

and 26 g total bound phenolic acids kg^{-1}. RDL contained a smaller amount of total carbohydrates, and bound phenolic acids than CDL and showed a composition similar to that of HDL. In the UV spectra (Fig. 2), CDL showed a strong peak around 320 nm attributable to bound phenolic acids, which is in agreement with the analytical data. The results of nitrobenzene oxidation (Table 1) and the IR spectra (Fig. 3) indicated that all the dioxane-soluble lignins consisted of guaiacyl-syringyl lignins. Although CDL produced a larger quantity of phenolic aldehydes upon oxidation than RDL, there was no appreciable difference in the molar ratio of syringaldehyde (S) to vanillin (V) between the 2 lignins. HDL showed

Table 1 Nitrobenzene oxidation products of cellulase-digested lignin (CDL), rumen-digested lignin (RDL) and heifer-digested lignin (HDL) from timothy

	CDL	RDL	HDL
Yield ($g\ kg^{-1}$ Klason lignin in lignin fraction)[a]			
p-Hydroxybenzaldehyde	10.9 ± 0.5	6.1 ± 0.1	5.1 ± 0.1
Vanillin (V)	110 ± 4.6	94.7 ± 0.2	81.5 ± 1.0
Syringaldehyde (S)	102 ± 4.4	85.6 ± 0.0	84.6 ± 1.8
Total	223 ± 9.4	186 ± 0.3	171 ± 1.9
Molar ratio of S/V	0.77	0.76	0.87

a): Mean ± SE for duplicate determinations.

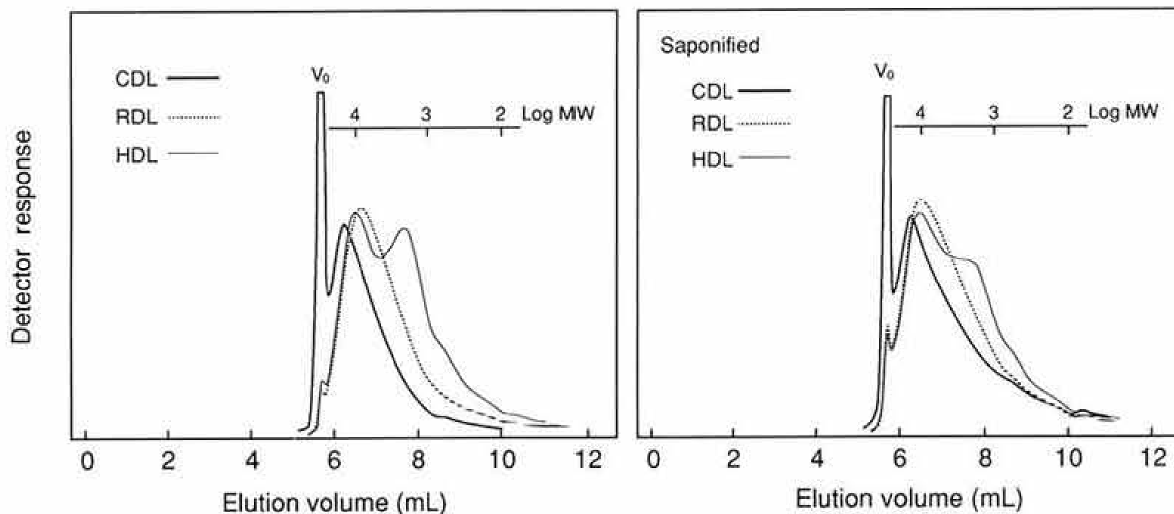

Fig. 4. Gel permeation chromatograms of cellulase-digested lignin (CDL), rumen-digested lignin (RDL) and heifer-digested lignin (HDL) from timothy

a slightly higher molar ratio of S to V than CDL and RDL. Definite differences were observed in the molecular size distribution among the 3 lignin fractions (Fig. 4). CDL was distributed in a higher molecular size region than RDL and HDL. About 30% of CDL, based on the area ratio of the chromatogram, was eluted at the void volume. HDL showed an additional peak in a lower molecular size region. Saponification (1 N NaOH, 25°C, for 16 h) did not substantially affect the differences in molecular size distribution among the 3 lignins, although saponification reduced the total amount of carbohydrates from 189 to 39 $g\ kg^{-1}$ for CDL, from 50 to 21 $g\ kg^{-1}$ for RDL and from 37 to 18 $g\ kg^{-1}$ for HDL.

Discussion

Lignin modifications occurring in the rumen environment have been often reported and the major modification observed was solubilization of lignin. Release of lignin due to solubilization was considered to result from the degradation of surrounding polysaccharides in the rumen[5,6]. Although CDL and RDL were released from the same timothy sample, the 2 lignin fractions differed in chemical and physical properties. The lower levels of phenolic acids in RDL than in CDL suggest that phenolic acids are removed or degraded from timothy lignin by rumen fermentation. This assumption is consistent with the similarity in the composition of RDL and HDL. RDL was composed of lower molecular size fragments than CDL. Furthermore, a similar difference in molecular size distribution was observed for the saponified lignins with fewer associated carbohydrate residues. Consequently, RDL probably consisted of lignin fragments with lower molecular sizes than CDL, suggesting that timothy lignin was depolymerized by rumen digestion.

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