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Structural changes in lignin of tropical woods during digestion by termite, *Cryptotermes brevis*

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Abstract Wood samples of apitong (*Dipterocarpus grandiflorua*) and ilang-ilang (*Ilang-Ilang* C. *dadloyi*) and feces of termites [*Cryptotermes brevis* (Walker)] fed on these woods were collected from University of the Philippines, Los Baños. Lignin of each sample was isolated by Björkman's procedure. There was no significant difference in ^{1}H nuclear magnetic resonance (NMR) spectra or in the methoxyl content between Björkman lignins from original woods and termite feces. Differences were detected in the contents of aliphatic and unconjugated phenolic hydroxyl groups, suggesting minor structural changes of lignin during digestion by termites. In addition, the ratio of syringyl to guaiacyl nuclei of Björkman lignin from termite feces determined by ^{1}H NMR spectra was higher than those from the original woods. The molar ratio of syringyl to guaiacyl nuclei of termite feces was higher than those from the original woods as determined by alkaline nitrobenzene oxidation. These results suggest that the structural changes of lignin in the termite gut are due to the insignificant formation of C-C linkages in guaiacyl nuclei. It was concluded that there were minor changes in the structural features of lignin under mostly anaerobic conditions, in contrast to the significant changes that occur through biological modification under aerobic conditions.

Key words *Dipterocarpus grandiflorua* · *Ilang-Ilang* C. *dadloyi* · *Cryptotermes brevis* (Walker) · Termite feces · ^{1}H and ^{13}C NMR spectra

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Introduction

The elucidation of biodegradation and/or biomodification of lignin is important to discuss carbon circulation on the earth. Behavior of biodegradation of plant cell wall components under aerobic conditions is well documented, but this is not the case for lignin under anaerobic conditions. Biomodification of lignin is largely due to peroxidases acting under aerobic conditions.[1,2] Although anaerobic biomodification of lignin is important for carbon circulation, as it is under aerobic conditions, its significance is not clear yet.

Digestion of plant cell wall components in ruminants is a typical anaerobic system. Termite hindguts have long been considered to be simple anoxic fermentors, similar to the rumena of cattle. This concept was challenged by investigations employing microsensor techniques, which demonstrated that the metabolic activity of the termite gut microbiota maintains steep oxygen and hydrogen gradients within the gut lumen.[3] With O_2 microelectrodes, it was confirmed that the H_2 sink below the gut epithelium is located within the microoxic gut periphery.[4] The carbohydrate-utilizing isolates, enumerated on liquid and solid growth media, consisted of aerotolerant lactic acid bacteria and surprisingly large numbers of facultatively aerobic and even strictly aerobic bacteria.[5] Axial oxygen profiles also confirmed that in general, only the paunches were anoxic in their centers, whereas midguts and posterior hindgut regions contained significant amounts of oxygen[6] in the higher termite *Nasutitermes lujae* (Wasmann) and lower termite *Reticulitermes flavipes* (Kollar).

It has been reported that cell wall polysaccharides are digested not only by cellulolytic enzymes of symbiotic flagellates, but also by endogenous cellulases in the midgut of some termites.[7–20] However, the biomodification of lignin by termites has remained unclear. The hindgut flora of the lower termites *Mastotermes darwiniensis* (Froggatt) and *Reticulitermes santonensis* (Feytaud) and the higher termite *Nasutitermes nigriceps* (Haldeman) was tested for its in vivo and in vitro capability to degrade lignin monomers and related aromatic compounds.[21] Most of the tested aromatic

compounds were degraded aerobically by mixed and pure cultures,[21] and the roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites have been reported.[22]

We have discussed biological modifications of lignin during composting,[23] mulching,[24,25] peat formation,[26] mushroom growth,[27] and during burial for long periods underground.[28] It has been found that the carbon–carbon linkage between lignin monomers (condensed structure) was formed, and carboxyl groups were introduced in lignin molecules during such aerobic biomodification of lignin.

Although there are many reports on the digestion of cell wall components of forage grasses,[29] especially for the effects of lignin on digestion of cell wall polysaccharides,[30,31] the knowledge of biomodification of the structural features of lignin under anoxic and/or microoxic conditions remains unclear. Some studies found that some portions of lignin were digested by termites,[16,32–38] but others suggested that degradation of lignin did not occur and any decrease in the mass of lignin was not significant.[38–45] In addition, studies of structural modifications of lignin by microflora in the termite gut[44,46] were focused on aromatic composition,[47] demethylation,[32,48,49] hydrogenation,[6] and cleavage of arylglycerol-β-aryl ether intermonomer linkages.[37,50]

In this study, intact woods of apitong (*Dipterocarpus grandiflorua*) and ilang-ilang (*Ilang-Ilang* C. *dadloyi*), and feces of termite (*Cryptotermes brevis*) fed apitong and ilang-ilang woods were collected at the University of the Philippines, Los Banõs. Cell walls of these tropical woods before and after digestion by termites were analyzed to determine changes in cell wall polysaccharides and lignin. Structural modifications of lignin by microorganisms under anoxic and/or microoxic conditions were studied using lignins that were isolated with Björkman's procedure[51] from intact woods and termite feces.

Materials and methods

Woods and feces

Original woods of apitong (*Dipterocarpus grandiflorua*) and ilang-ilang (*Ilang-Ilang* C. *dadloyi*) were collected from the University of the Philippines, Los Baňos (14°04′ N, 121°10′ E). The feces of termites were supplied by Dr. Ramon A. Razal, Department of Wood Chemical Engineering, University of the Philippines, Los Baňos, who placed the sample woods in concrete tubs and fed wood blocks of apitong and ilang-ilang to termites [*Cryptotermes brevis* (Walker)] for 6 months. The shape of the feces was elliptical globular.

The original wood sample was ground with a Wiley mill to pass a 420-μm sieve, and extracted with ethanol–benzene (1:2, v/v) using a Soxhlet extractor for 8 h. The feces were subjected to following chemical analyses without milling or prior extraction.

Termite feces and extract-free original wood meal (about 40 g each) were dried for 2 nights in a vacuum oven over P_2O_5 at 40°C, and then ground in a vibratory ball mill (VS-2, Irie Shoukai, Tokyo) without any solvent[52] for 72 h with cooling provided by flowing tap water. Lignin was extracted and purified with Björkman's procedure[51] as Björkman lignin.

Chemical analyses

Neutral sugar composition, acidic sugar composition, lignin content, and ash content of the extract-free original wood meal and the feces were analyzed by alditol acetate analysis,[53] spectrometric analysis,[54] acetyl bromide procedure,[55] and combustion for 3 h at 700°C, respectively. Björkman lignin was subjected to CHN microanalysis (Perkin Elmer elemental analyzer: PE 240 CHN), methoxyl group determination by the procedure of Goto et al.,[56] differential ionization analysis ($\Delta\varepsilon_i$) to determine unconjugated phenolic hydroxyl group,[57] and carbonyl content analysis at the C_1 position of the lignin side chain by differential ionization analysis before and after reduction with $NaBH_4$ ($\Delta\varepsilon_r$).[58] Total hydroxyl groups of Björkman lignin were calculated by acetyl group determination by ^{1}H nuclear magnetic resonance (NMR) spectroscopy[59] (Bruker AC300) after acetylation with acetic anhydride and a catalytic amount of pyridine. The Björkman lignin was methylated with freshly prepared diazomethane, and the content of total acidic hydroxyl groups was measured by methoxyl group determination,[57] and the total phenolic hydroxyl groups and carboxyl groups were also calculated by methoxyl content after saponification with 0.5M NaOH overnight at room temperature. All of the determinations were conducted in triplicate.

^{1}H and ^{13}C NMR spectroscopic analyses of Björkman lignin

All NMR spectra were recorded on a Bruker AC 300 spectrometer. After acetylation, ^{1}H spectra of Björkman lignins were recorded using $CDCl_3$ as solvent. ^{13}C spectra of Björkman lignins from the original wood and the feces of apitong species were recorded using dimethyl sulfoxide-d_6 as solvent.

Results

Chemical composition

The chemical compositions of the original woods and termite feces are summarized in Table 1. Relative content of lignin in the termite feces was quite high for both species. About 86% of neutral polysaccharides in the cell wall was digested by termites on the assumption that lignin was hardly digested by the termites. Weight losses of glucosyl residue of apitong and ilang-ilang woods after passing the termite gut were 93% and 89%, respectively, which were higher than those of xylosyl residue at 85.5% and 83.5%. However, digestion of acidic sugar was only 60%.

Table 1. Chemical composition of original apitong and ilang-ilang woods and their termite feces

Analyte	Apitong			Ilang-ilang		
	Original wood (%)[a]	Termite feces (%)[a]	Digested (%)[b]	Original wood (%)[a]	Termite feces (%)[a]	Digested (%)[b]
Neutral sugars						
Rhamnosyl	0.0	0.4	–	0.0	0.6	–
Arabinosyl	0.0	0.4	–	0.0	0.8	–
Xylosyl	10.2	3.2	85.5	14.6	5.0	83.5
Mannosyl	2.4	1.0	80.7	1.4	1.0	65.5
Glucosyl	45.4	6.7	93.2	44.1	9.8	89.3
Galactosyl	1.3	1.0	64.3	1.1	1.3	43.0
Total	59.3	12.7	90.1	61.2	18.5	85.4
Acidic sugars						
Galacturonic	2.7	2.2	62.2	3.8	3.2	59.4
Gluculonic	0.1	0.2	7.3	0.0	0.2	–
Total	2.8	2.4	60.3	3.8	3.4	56.8
Lignin (AcBr)	31.7	68.4	0.0	33.4	69.2	0.0
Ash	0.7	2.1	–	1.3	4.1	–

All determinations conducted in triplicate
[a] Based on original dry matter
[b] Based on original wood sample, when it was assumed that lignin was not digested by termites

Table 2. Yield of purified Björkman lignin

Species	Original wood	Termite feces
Apitong	21.9	10.3
Ilang-ilang	24.4	9.6

Data given as percent lignin

The yield of Björkman lignin from termite feces was significantly lower than those from the original wood (Table 2). Content of carboxyl groups was very low in the Björkman lignin of termite feces (Table 3). No difference in contents of phenolic hydroxyl groups, unconjugated phenolic hydroxyl groups, and α-carbonyl groups was detected between Björkman lignins from original woods and termite feces. However, the content of aliphatic hydroxyl groups decreased significantly after passing through the termite gut (Table 3). A slight increase in the content of methoxyl groups in samples investigated in this study was detected (Table 3), which was confirmed by the increase of the molar ratio of syringyl nuclei to guaiacyl nuclei (S/V ratio) of the alkaline nitrobenzene oxidation products as shown in Table 4. Slightly higher total yields of alkaline nitrobenzene oxidation products of Björkman lignins from termite feces than those from the original woods were detected (Table 4). Yields of syringyl products were higher, but guaiacyl products were lower in termite feces lignins than those of the original lignins.

Based on elementary and functional group analyses, the molecular formula of the phenylpropane unit of Björkman lignin was calculated as shown in Table 5. Methoxyl contents of Björkman lignins from termite feces of apitong and ilang-ilang increased slightly compared with Björkman lignins from the original woods. All determinations described above were performed in triplicate.

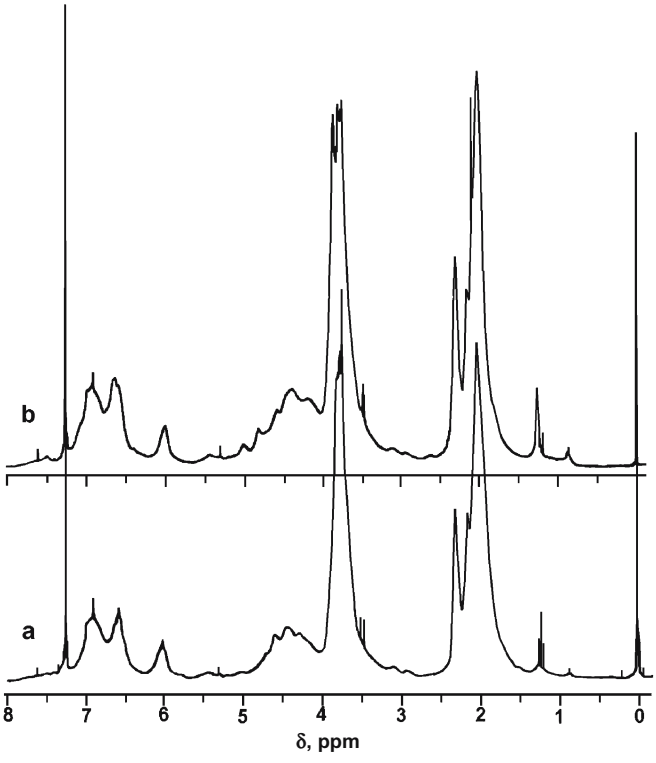

Fig. 1a, b. ^{1}H Nuclear magnetic resonance (NMR) spectra of Björkman lignins of **a** original wood and **b** termite feces of apitong

^{1}H and ^{13}C NMR spectroscopy

No clear difference in ^{1}H NMR spectra was detected between Björkman lignins from original woods and termite feces for both apitong (Fig. 1) and ilang-ilang (Fig. 2). In ^{1}H NMR spectra of lignin, signals assigned to aromatic protons are present at about δ 6.90 (guaiacyl protons) in gymno-

Table 3. Hydroxyl groups of Björkman lignin

	Apitong		Ilang-ilang	
	Original wood	Termite feces	Original wood	Termite feces
Phenolic-OH[a]	0.29	0.27	0.28	0.27
Unconjugated phenolic-OH[b]	0.13	0.13	0.13	0.12
Carboxyl group[a]	<0.01	<0.01	<0.01	<0.01
Aliphatic-OH[d]	1.79	1.26	1.61	1.25
Total-OH[c]	2.21	1.66	2.02	1.64
α-Carbonyl group[e]	0.14	0.12	0.13	0.13
Methoxyl group	1.07	1.10	1.06	1.13

Data given as mol/200 g lignin. All determinations were conducted in triplicate
[a] Calculated from content of methoxyl groups after diazomethane methylation followed by saponification
[b] Determined by differential ionization spectroscopy ($\Delta\varepsilon_i$ method)
[c] Calculated from acetyl group content determined by ^{1}H NMR after acetylation
[d] Calculated as the difference between total and phenolic hydroxyl groups
[e] Determined by differential ionization spectroscopy before and after reduction with $NaBH_4$ ($\Delta\varepsilon_r$ method)

Table 4. Alkaline nitrobenzene oxidation

	Wood meal				Björkman lignin			
	Apitong		Ilang-ilang		Apitong		Ilang-ilang	
	Original wood	Termite feces	Original wood	Termite feces	Original wood	Termite feces	Original wood	Termite feces
H	7	6	7	5	6	6	6	5
HA	0	0	0	0	0	0	0	0
V	199	184	215	170	196	179	210	172
VA	14	13	15	12	14	13	19	21
S	231	258	240	246	202	237	182	234
SA	30	25	21	32	26	32	19	21
Total yield	481	486	498	465	444	465	436	453
S/V	1.23	1.43	1.14	1.53	1.09	1.40	0.88	1.32

Data given as mmol/200 g lignin. All determinations conducted in triplicate
H, *p*-Hydroxybenzaldehyde; HA, *p*-hydroxybenzoic acid; V, vanillin; VA, vanillic acid; S, syringaldehyde; SA, syringic acid; S/V, molar ratio of vanillin and vanillic acid to syringaldehyde and syringic acid

Table 5. Molecular formula of phenylpropane unit of Björkman lignin

Species	Sample	Molecular formula
Apitong	Original wood	$C_9H_{6.71}O_{1.28}(R\text{-}OH)_{1.82}(Ph\text{-}OH)_{0.29}(\alpha\text{-}CO)_{0.14}(COOH)_{0.01}(OCH_3)_{1.12}$
	Termite feces	$C_9H_{7.71}O_{1.77}(R\text{-}OH)_{1.28}(Ph\text{-}OH)_{0.27}(\alpha\text{-}CO)_{0.12}(COOH)_{0.01}(OCH_3)_{1.16}$
Ilang-ilang	Original wood	$C_9H_{7.24}O_{1.60}(R\text{-}OH)_{1.63}(Ph\text{-}OH)_{0.28}(\alpha\text{-}CO)_{0.13}(COOH)_{0.01}(OCH_3)_{1.12}$
	Termite feces	$C_9H_{7.68}O_{1.67}(R\text{-}OH)_{1.27}(Ph\text{-}OH)_{0.27}(\alpha\text{-}CO)_{0.13}(COOH)_{0.01}(OCH_3)_{1.18}$

R-OH, Aliphatic hydroxyl group; Ph-OH, phenolic hydroxyl group; α-CO, α-carbonyl group; COOH, carboxyl group; OCH_3, methoxyl group

sperm lignin, and at δ 6.65 (syringyl protons) and δ 6.90 for angiosperm lignin.[60] Björkman lignins from termite feces of both apitong and ilang-ilang exhibited a relatively stronger signal at δ 6.65 than those from original woods (Figs. 1 and 2). The molar ratio of syringyl moieties to guaiacyl moieties (S/G ratio) determined from ^{1}H NMR spectra of Björkman lignins from termite feces was higher than those from the original woods. There was no clear difference in the ^{13}C NMR spectra of Björkman lignins from original woods and termite feces of apitong (Fig. 3).

Discussion

Chemical composition

The relative content of lignin in the termite feces was quite high for both apitong and ilang-ilang, suggesting that cell wall polysaccharides were markedly digested by both symbiont and endogenous cellulases during residence of the termite gut.[8–10,12–15,48] About 86% of neutral polysaccharides in the cell wall was digested by termites on the as-

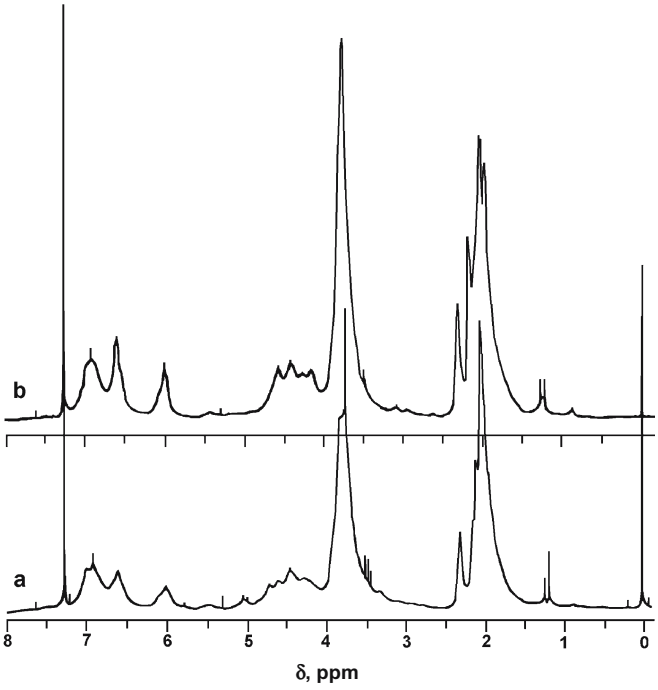

Fig. 2a, b. ^{1}H NMR spectra of Björkman lignins of **a** original wood and **b** termite feces of ilang-ilang

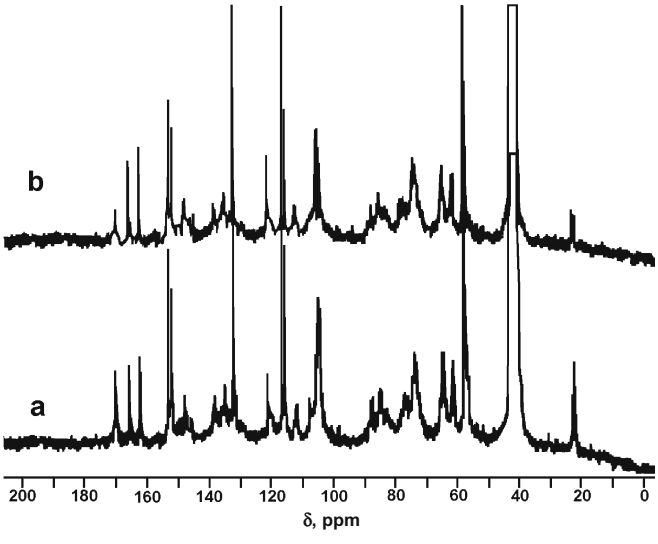

Fig. 3a, b. ^{13}C NMR spectra of Björkman lignins of **a** original wood and **b** termite feces of apitong

sumption that lignin was hardly digested by the termites (Table 1).[16,39–45] Glucosyl and xylosyl residues, which are the major neutral sugar residues originating from cellulose and heteroxylan in tropical woody angiosperms, were digested markedly, but digestion of acidic sugars was about 60%. Weight loss of glucosyl residue (85.5%) of apitong and ilang-ilang woods after passing the termite gut was higher than that of xylosyl residue (83.5%). These results were consistent with the study of Kovoor,[43] which found that cellulose was digested more than hemicellulose by termites. Holdaway[39] reported that the weight ratio of cellulose to lignin of 2:1 in wood changed to 1:4 in the feces of termites, which was calculated as about 87% cellulose loss. Hyodo et al.[38] estimated the ratio of carbohydrate to lignin in the sound earlywood to be about 2.28:1 and that in the termite feces to be about 1:1.76, suggesting that about 75% of cell wall polysaccharides were digested by termites. Values reported by Holdaway,[39] agreed very well with the results for apitong and ilang-ilang woods examined in this study.

Since Hogan et al.[61] suggested the presence of endogenous cellulases in termites, the characterization of endogenous cellulase in the midgut of termites, which help digestion of cellulose, has been documented by many scientists.[7–20] Digestion of cell wall polysaccharides during passage through the termite gut was evidently higher than the digestion of forage grasses by rumen microorganisms of ruminants, which was in range of 30%–40%.[31] This is due to the higher activity of polysaccharide hydrolases of the both symbiotic flagellates and endogenous cellulases in the midgut of some termites compared with those in the rumen. In addition, differences in the covalent linkages of cell wall polysaccharides with lignin between tropical woody angiosperms and forage grasses would influence their digestion.[29,30,62] Lower yield of Björkman lignin for feces than from original woods (Table 2) and remarkable swelling of the sample in 90% dioxane during preparation of Björkman lignin from feces suggested that covalent linkages between polysaccharides and lignin were concentrated in feces due to the digestion of polysaccharides of apitong and ilang-ilang during passage through the termite gut.

Although carboxyl groups are introduced significantly in lignin during biological modification of lignin under aerobic conditions,[23,26,27] the content of carboxyl groups was very low in the Björkman lignin of termite feces. No difference in the contents of phenolic hydroxyl groups, unconjugated phenolic hydroxyl groups, and α-carbonyl groups was detected between Björkman lignins from original wood and termite feces (Table 3). On the other hand, content of aliphatic hydroxyl groups decreased significantly after passing through the termite gut. These results also suggest that a considerable proportion of aliphatic hydroxyl groups at the benzyl position of Björkman lignin from termite feces may be substituted markedly with cell wall polysaccharides to form lignin–polysaccharide complexes. The fraction involving the complexes was extracted as Björkman lignin because the fragments of polysaccharide were small after digestion in the termite gut.

It was reported by Kato et al.[37] that when lignin model compounds were treated with microorganisms under aerobic conditions, the number of methoxyl groups was decreased by the cleavage of aromatic nuclei. Kuhnigk et al.[21] documented that methoxyl groups were also released even under anaerobic conditions. However, a slight increase in the content of methoxyl groups was detected in samples investigated in this study (Table 3), which was confirmed by the increase of the molar ratio of syringyl nuclei to guaiacyl nuclei (S/V ratio) in the alkaline nitrobenzene oxidation products. The total yields of alkaline nitrobenzene oxidation products of Björkman lignins from termite feces were slightly higher than those from the original woods (Table

4). Yields of syringyl products were higher, but those of guaiacyl products were lower in termite feces lignins than those of the original lignins. These results suggest that the structural changes in lignin under anoxic and microoxic conditions in the termite gut were due to polymerization of guaiacyl units of lignin through C-C linkages.

^{1}H and ^{13}C NMR spectroscopy

Small differences in ^{1}H NMR spectra were detected between Björkman lignins from original woods and termite feces in both apitong (Fig. 1) and ilang-ilang (Fig. 2). In ^{1}H NMR spectra of lignin, signals assigned to aromatic protons were at δ6.90 in gymnosperm lignin (guaiacyl protons), and at δ6.90 and 6.65 (syringyl protons) for angiosperm lignin.[60] Björkman lignins from termite feces of both apitong and ilang-ilang exhibited a relatively stronger signal at δ 6.65 than those from original woods (Figs. 1 and 2). In contrast to previous studies[32,47–49] that reported that syringyl-rich lignin fractions seemed to break down faster, the molar ratio of syringyl moieties to guaiacyl moieties (S/G ratio) determined by ^{1}H NMR spectra of Björkman lignins from termite feces was higher than those from the original woods. The results of ^{1}H NMR spectra clearly suggested that syringyl-rich lignin was resistant against digestion under anoxic and microoxic conditions. These results also support the results of chemical analysis (Table 4). Although it is documented that microorganisms in the termite gut cleave the arylglycerol-β-aryl ether intermonomer linkage of lignin,[37,50] no significant difference was detected at δ 6.0 of ^{1}H NMR spectra of Björkman lignins, which was assigned to the proton at the C_{α} of the arylglycerol-β-aryl ether intermonomer linkage,[60] from original woods and termite feces for the both apitong and ilang-ilang.

Based on elementary and functional group analyses, the molecular formulas of Björkman lignins suggest that methoxyl contents of Björkman lignins from termite feces of apitong and ilang-ilang increased slightly when compared with Björkman lignins from original woods. These results were also contrary to findings that found demethylation of lignin occurred during digestion by termites[49] and that methoxyl groups were lost.[48]

There was no clear difference in ^{13}C NMR of Björkman lignins from original woods and termite feces of apitong (Fig. 3), particularly for signals at δ 74, 87, and 62 assigned to carbons at the α, β, and γ positions of the arylglycerol-β-aryl ether intermonomer linkage, respectively. This was also the case for signals at δ 119 and 149 assigned to the C_5 position of guaiacyl and syringyl nuclei, respectively.[63–65]

Conclusions

Cell wall polysaccharides in apitong and ilang-ilang wood meals decreased markedly after digestion by termites. Glucosyl residues were 90% digested. Lignin was hardly decomposed by termites. Björkman lignin was prepared from each sample to investigate structural changes during digestion by termites. Slightly higher methoxy contents of Björkman lignin from termite feces than those from original woods were observed. These results were supported by S/G ratios of Björkman lignins obtained from ^{1}H NMR spectra and S/V ratios from alkaline nitrobenzene oxidation products. The total yields of alkaline nitrobenzene oxidation products of Björkman lignins from termite feces were lower than those from the original woods. These results suggest that guaiacyl units of lignin were polymerized through C-C linkages during passage through the termite gut. Small differences in the contents of aliphatic hydroxyl and unconjugated phenolic hydroxyl groups in lignins were detected between original woods and termite feces, suggesting minor structural changes in side chains of lignin, such as cleavage of α-ether linkages during digestion by termites.

It is concluded that there were some changes in the structural features of lignin under anoxic and microoxic conditions in the termite gut, although the changes were minor in comparison with biological modifications that occur under aerobic conditions.

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