

Shuichi Hirosawa · Rui Katahira · Fumiaki Nakatsubo

## Chemical structural elucidation of total lignins in woods I: fractionation of the lignin in residual wood meal after extraction of milled wood lignin

Received: May 25, 2000 / Accepted: February 28, 2001

**Abstract** Residual wood meal after extraction of milled wood lignin (WMEM) of *Eucalyptus globulus* was extracted with alkali and LiCl/*N,N*-Dimethylacetamide (DMAc). These agents dissolve mainly hemicellulose and cellulose, respectively. The extractability of WMEM in alkali solutions was influenced by the degree of swelling of the cellulose. Under good swelling conditions, considerable amounts of cellulose and lignin were extracted with the hemicellulose. Maximum extractability was about 60% of the WMEM under optimum conditions (3M or 5M LiOH or 3M NaOH solution). Some portion of cellulose was extracted with LiCl/DMAc at room temperature. Thus, lignin in *E. globulus* WMEM was divided into three fractions: hemicellulose-lignin fraction, cellulose-lignin fraction, and insoluble-lignin fraction.

**Key words** Wood meal after extraction of MWL · Alkali extraction · Swelling of cellulose · LiCl/DMAc extraction

### Introduction

At present, milled wood lignin (MWL) is used mainly to investigate the chemical structure of lignin, but MWL does not represent the total lignin.<sup>1</sup> Therefore, elucidation of the chemical structure of lignin in wood meal after extraction of MWL (WMEM) is important.

In this study, lignin in WMEM was fractionated by extractions with solvents for polysaccharides (hemicellulose

and cellulose). The purposes of this fractionation was to extract lignin together with polysaccharides using such solvents under mild conditions for further chemical structural analyses. At first, lignin together with hemicellulose was extracted with alkali. Then, lignin together with cellulose was extracted with LiCl/*N,N*-dimethylacetamide (DMAc). Lignin contents assays, nitrobenzene oxidation, and sugar analyses were then carried out to characterize the behaviors of lignin and polysaccharides during extraction.

### Experimental

#### Materials

Wood meals of *Eucalyptus globulus* from Chile was extracted with 95% ethanol/benzene (1:2 v/v) solution for 6h to remove extractives using a Soxhlet apparatus and subsequently with 0.25% potassium acetate solution at 60°C for 13 days to remove pectin with stirring.<sup>2</sup> The resulting extracted wood meal was further ground for 48h. Milled wood lignin (MWL) was prepared by the standard method<sup>3</sup> using dioxane/water (9:1) as the extracting solvent. The yield of MWL was 4% of the extractive-free wood meal.

#### Alkali extraction

Wood meal after extraction of MWL (1 g) was suspended in distilled water (7.5 ml) and stirred overnight at room temperature (r.t.). The suspension was then cooled to 4°C followed by addition of alkali (LiOH, NaOH, KOH) solution (7.5 ml) to prepare the desired concentration (1, 3, or 5M). The suspension was cooled and stirred for 1h at 0°C and then centrifuged (15000rpm × 15min). The precipitate was acidified with excess acetic acid, washed repeatedly with distilled water until the washing solution was pH 6, and then freeze-dried. The extractability with each solvent was expressed by Eq. (1).

$$\text{Extractability (\%)} = (W - W_i) / W \times 100 \quad (1)$$

S. Hirosawa (✉) · R. Katahira · F. Nakatsubo  
Graduate School of Agriculture, Kyoto University, Kyoto 606-8502,  
Japan  
Tel. +81-75-753-6256; Fax +81-75-753-6300  
e-mail: bdbhhia@kais.kyoto-u.ac.jp

Part of this work was presented at the 49th annual meeting of the Japan Wood Research Society, Tokyo, April 1999; and at the 50th annual meeting of the Japan Wood Research Society, Kyoto, April 2000

where  $W$  is the weight before extraction, and  $W_i$  is the weight of the precipitate.

#### LiCl/DMAc extraction (heating method)

For the heating method<sup>4</sup> WMEM (1g) was dried in a vacuum oven overnight at 100°C. The sample was transferred to a round-bottom flask (100ml). After DMAc (40ml) was added to the flask, the atmosphere of the mixture was replaced with N<sub>2</sub>. The suspension was stirred for 2h at 120°C and then cooled to 100°C; LiCl (3g) was then added. The suspension was kept for 10min at 100°C and then stirred for 24h at r.t. The suspension was centrifuged (15000rpm × 15min). The supernatant was poured into distilled water (50ml), and the precipitate was suspended in distilled water (50ml). Both fractions (supernatant and precipitate) yielded gel-like substances. They were then centrifuged (3000 × 10min). After removing the supernatant, each fraction was washed five times with distilled water (50ml). The LiCl/DMAc-soluble and LiCl/DMAc-insoluble fractions were freeze-dried. Extractability was calculated the same way (Eq. 1) as for the alkali extraction.

#### LiCl/DMAc extraction (solvent-exchange method)

For the solvent exchange method<sup>4</sup> WMEM (1g) was suspended in distilled water (15ml) for 12h at r.t. The suspension was centrifuged (15000rpm × 10min). The precipitate was washed twice with acetone (15ml) and then suspended in acetone for 24h at r.t. The suspension was centrifuged (15000rpm × 10min). After removing the acetone, the precipitate was washed twice with DMAc (15ml). Dimethylacetamide (40ml) was added subsequently to the precipitate. The suspension was stirred for 4h at r.t., then LiCl (3g) was added. The suspension was stirred for 24h at r.t. followed by centrifugation (15000rpm × 15min). The precipitate was suspended again in DMAc (40ml) and LiCl (3g) for 24h for further extraction at r.t., then centrifuged. This procedure was repeated twice, so the wood meal was ultimately extracted three times with LiCl/DMAc (3g/40ml). The supernatant and the precipitate were treated the same way as described for the heating method.

#### Successive extraction with alkali and LiCl/DMAc

Wood meal after extraction of MWL (1g) was extracted with 3M NaOH at 0°C for 1h. The suspension was centrifuged (15000rpm × 15min). Both the supernatant and precipitate after alkali extraction were acidified with excess acetic acid. The supernatant (i.e., the alkali-soluble fraction) was dialyzed using cellulose ester dialysis membranes (Spectrum Laboratories) (cutoff molecular weight of 500) until the dialyzed solution reached about pH 6. The alkali-insoluble fraction was treated in the same manner as for the alkali extraction.

The alkali-insoluble fraction was suspended in distilled water (7ml) 12h at r.t. The suspension was centrifuged (15000rpm × 10min). The precipitate was washed twice with acetone (7ml), after which acetone (7ml) was added. The suspension was stirred for 24h at r.t. followed by centrifugation (15000rpm × 10min) to remove the acetone. After washing the precipitate with DMAc (20ml) twice, the precipitate was suspended in DMAc (20ml). The suspension was stirred for 4h at r.t. LiCl (1.5g) was then added to the suspension, which was stirred for 24h at r.t. followed by centrifugation (15000rpm × 15min). The extraction was repeated three times with LiCl/DMAc (1.5g/20ml). The collected LiCl/DMAc-soluble fraction and residue were treated in the same manner as described above. The alkali-soluble fraction, LiCl/DMAc-soluble fraction, and extracted residue were freeze-dried.

#### Successive extraction with LiCl/DMAc and alkali

Wood meal after extraction of MWL (1g) was extracted with LiCl/DMAc by the solvent-exchange method three times as described above. The LiCl/DMAc-insoluble fraction was extracted with 3M NaOH at 0°C in the same way as for the alkali extraction. The LiCl/DMAc-soluble fraction, alkali-soluble fraction, and residue were freeze-dried and weighed.

#### Determination of Klason lignin

The Klason lignin content of the WMEM was determined by the conventional method.<sup>5</sup>

#### Determination of lignin by the acetyl bromide method

The acetyl bromide technique was carried out according to the conventional method.<sup>6</sup> Ultraviolet (UV) spectra were recorded on a Shimadzu UV-365 UV-VIS-NIR spectrometer. An absorptivity value of 20.091g<sup>-1</sup>cm<sup>-1</sup> was used.<sup>7</sup>

#### Alkali-nitrobenzene oxidation

Samples were subjected to alkaline nitrobenzene oxidation according to the method modified by Katahira and Nakatsubo.<sup>8</sup> Nitrobenzene (0.24ml) and 2M NaOH (4ml) were added to the sample (5mg) in a Teflon reaction tube. The reaction tube was heated at 170°C for 1h in an autoclave. Dioxane solution (100μl) of 5-iodovanillin (0.02M) was added as an internal standard, and the reaction mixture was filtered. The filtrate was acidified with 4M HCl and then extracted with ethyl acetate (15ml). The ethyl acetate layer was dried over Na<sub>2</sub>SO<sub>4</sub> and then concentrated in vacuo.

The acetyl derivatives of nitrobenzene oxidation products were analyzed by a Shimadzu GC-17A gas chromatograph (High Cap-17 capillary column: 30m × 0.25mm i.d.; column temperature 230°C; injector temperature 270°C; detector temperature 270°C; carrier gas He; 98kPa at the column head).

## Neutral sugar analysis (alditol acetate procedure)

The neutral sugars in the hydrolysate during sulfuric acid hydrolysis were analyzed as their alditol acetates by a Shimadzu GC-17A gas chromatograph (SP2330 capillary column: 15m × 0.24mm i.d.; column temperature 220°C; injector temperature 240°C; detector temperature 250°C; carrier gas He; 98kPa at the column head).<sup>9</sup>

## Results and discussion

### Alkali extraction

*Eucalyptus globulus* WMEM was first suspended in distilled water to be dispersed well in alkali solution; then alkali solution was added to the suspension. Figure 1 shows the extractability depending on the concentrations of the various alkali solutions. Maximum extractability was about 60% of the WMEM (3M or 5M LiOH or 3M NaOH). However, about only 37% of the WMEM was extracted with KOH under the optimum concentration (5M). These alkali extractives contain not only hemicellulose but also other wood components.

Zeronian and Cabradilla studied the swelling behavior of cotton cellulose treated with aqueous solutions of LiOH, NaOH, and KOH.<sup>10</sup> Maximum swelling of cotton with KOH solution is lower than that with LiOH or NaOH solution. The swelling in LiOH solution increased initially with the concentration and leveled off at 3N. The swelling of cotton with NaOH reached a maximum at a concentration of 3N and then decreased to some extent. The extractabilities of WMEM (Fig. 1) agreed with the swelling behavior indicated by Zeronian and Cabradilla.<sup>10</sup> Consequently, it was clearly indicated that the extractability of WMEM by alkali solution depends strongly on the swelling of cellulose.

Lignin content, nitrobenzene oxidation, and neutral sugar (alditol acetate procedure) analyses of the fractions are shown in Table 1. The solubility of the wood components in 3M NaOH and 3M KOH calculated from the data in Fig. 1 and Table 1 (nos. 4 and 5) is based on the following assumptions: All of the glucosyl residue and sugar residues other than glucose in Table 1 originated from cellulose and hemicellulose, respectively. For example, the extractability of cellulose in wood meal in 3M NaOH can be calculated to be 54% [ $\{43.1 - (1 - 0.58) \times 47.0\} \times 100/43.1$ ] from the data shown in Fig. 1 and Table 1. Those of hemicellulose and lignin are obtained by the same calculation.

Figure 2 indicates the following results: (1) Both 3M NaOH and 3M KOH solutions are good solvents for hemicellulose. (2) The solubility of cellulose and lignin in 3M NaOH solution is higher than that in 3M KOH solution.

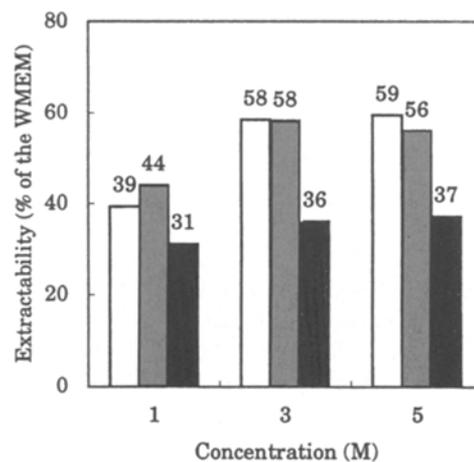


Fig. 1. Extractability with alkali solution. LiOH (open bars), NaOH (gray bars), and KOH (filled bars). WMEM, wood meal after extraction of MWL.

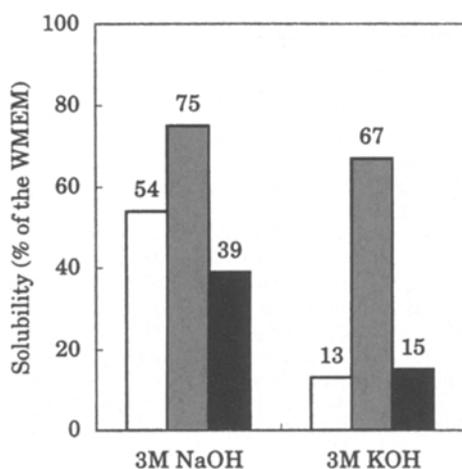
Table 1. Lignin contents; results of nitrobenzene oxidations and sugar compositions

No.	Sample	Quantity (%)	Lignin			Sugar composition (%)				
			Content (%) <sup>a</sup>	s + v (%)	s/v	Ara.	Xyl.	Man.	Gal.	Glc.
1	Extractive free wood meal	100	20.4 <sup>b</sup>	49	4.0	1.4	13.3	1.0	1.4	41.7
2	MWL	4	ND	38	2.5	0.4	4.0	0.2	0.3	0.2
3	WMEM	96	18.6 <sup>b</sup>	51	3.9	1.2	14.5	1.2	1.6	43.1
4	WMEM extracted with 3M NaOH	40	28.0	48	3.5	0.6	7.5	1.2	1.8	47.0
5	WMEM extracted with 3M KOH	61	24.9	49	3.3	0.6	5.9	1.6	1.4	58.5
6	LiCl/DMAc extract 1st	26	6.0	42	2.9	0.3	6.4	2.0	0.8	86.6
7	LiCl/DMAc extract 2nd	5	10.3	42	3.1	0.4	6.5	0.9	0.7	57.7
8	LiCl/DMAc extract 3rd	2	18.5	53	3.3	0.8	10.5	1.3	1.2	46.7
9	Hemicellulose-lignin (HCL)	45	16.4	47	3.4	0.3	10.1	1.6	0.8	48.0
10	Cellulose-lignin (CL)	8	17.8	47	4.0	0.4	7.7	1.3	0.8	66.8
11	Insoluble-lignin (IL) (A-D)	27	37.3	47	3.9	0.5	6.8	1.1	1.8	35.0
12	Insoluble-lignin (IL) (D-A)	29	36.1	48	3.5	0.6	7.1	0.8	1.6	38.3

MWL, milled wood lignin; WMEM, wood meal after extraction of MWL; DMAc, *N,N*-dimethylacetamide; A-D, residual wood meal extracted with at first 3M NaOH, then with LiCl/DMAc; D-A, residual wood meal extracted with at first LiCl/DMAc, then with 3M NaOH; ND, not determined; s, syringaldehyde; v, vanillin in 100mg of lignin

<sup>a</sup>Determined by acetyl bromide method except for nos. 1, 2 and 3

<sup>b</sup>Klason lignin



**Fig. 2.** Solubility of wood components in 3M NaOH and 3M KOH. Cellulose (open bars), hemicellulose (gray bars), and lignin (filled bars)

These data indicate that KOH solution has higher selectivity for extraction of hemicellulose but lower total extractability than 3M NaOH extraction. Schuerch reported that LiOH and NaOH solutions are better solvents for polysaccharides and better swelling agents for cellulose than KOH solution, probably because their hydrated cations are larger than the potassium ion.<sup>11</sup> The selectivity of the KOH solution shown in Fig. 2 supports this previous study. Thus, 3M and 5M LiOH and 3M NaOH solution are appropriate solvents for extracting lignin.

#### LiCl/DMAc extraction

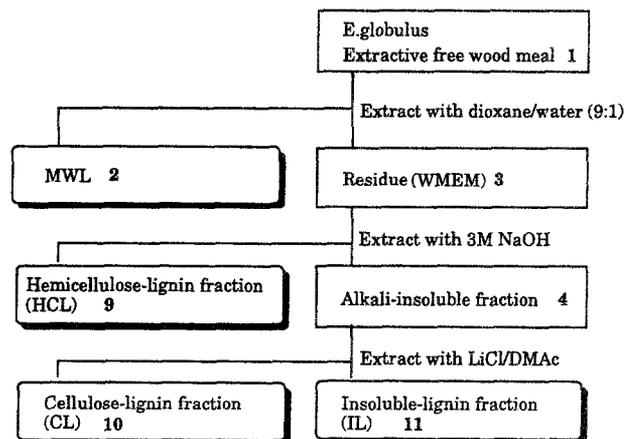
*Eucalyptus globulus* WMEM was extracted with LiCl/DMAc, which is known to be a cellulose solvent.<sup>4</sup> Two essential pretreatments are reported to dissolve cellulose in LiCl/DMAc. The first procedure is to heat a suspension of cellulose fiber in DMAc for 2 h at 120°C, then dissolve it by adding LiCl at 100°C (heating method). Another is the solvent-exchange method.

Both methods were applied to the WMEM. The heating method extracted 34% of the WMEM; and the extractability by the solvent-exchange method was 37% when the extraction procedure was repeated three times. The solvent-exchange method may be better than the heating method for avoiding denaturation of wood components.

Sugar analyses of extracts by solvent-exchange method implied that the major extracted component was cellulose the first time (Table 1, no. 6). The proportion of other components increased, however, after repeated extractions (Table 1, nos. 7 and 8).

#### Successive extraction with alkali and LiCl/DMAc

Wood meal after extraction of MWL was extracted with 3M NaOH and subsequently with LiCl/DMAc by the solvent-exchange method (repeated three times). Wood meal after extraction of MWL was divided into three fractions: the



**Fig. 3.** Fractionation of wood meal. MWL, milled wood lignin; DMAc, *N,N*-dimethylacetamide

3M NaOH-soluble fraction [hemicellulose-lignin (HCL) fraction, no. 9 in Table 1], the LiCl/DMAc-soluble fraction [cellulose-lignin (CL) fraction, no. 10 in Table 1], and the insoluble-lignin (IL) fraction (no. 11 in Table 1), as shown in Fig. 3. There was considerable loss during dialysis after the alkali extraction due to the low molecular weight of part of the HCL fraction. The HCL fraction can be analyzed because of its solubility in solvent. The CL fraction is also expected to be analyzed, although it contains only a small amount of lignin. Quantitatively, the IL fraction is the most important. Further analyses of the three fractions may elucidate the chemical structure of the WMEM in the near future.

The yield of products of alkaline nitrobenzene oxidation suggests no significant difference in the aromatic composition of the lignin in each fraction. The syringaldehyde/vanillin (s/v) ratio of the HCL fraction was lower than those of other fractions. Cellulose remained in significantly large quantities in IL. It seems likely that residual cellulose in the IL fraction is part of the lignin-carbohydrate complex.<sup>12-14</sup>

When the WMEM was first extracted with LiCl/DMAc and subsequently extracted with 3M NaOH, the lignin content of the IL fraction was slightly lower than that first extracted with 3M NaOH and then with LiCl/DMAc. The order of extraction did not significantly affect the solubility of other components.

## Conclusions

When *Eucalyptus globulus* WMEM was extracted with alkali and LiCl/DMAc the following results were obtained. (1) Swelling of cellulose was a dominant factor and affected the extractability of *E. globulus* WMEM in alkali solution. (2) Aqueous 3M NaOH and 3M or 5M LiOH solutions were appropriate solvents for extracting polysaccharides and some lignins. About 60% of the WMEM was extracted under those conditions. (3) LiCl/DMAc extraction dis-

solved 37% of the WMEM at r.t. (4) *E. globulus* WMEM was divided into hemicellulose-lignin, cellulose-lignin, and insoluble lignin fractions.

**Acknowledgments** The authors thank Dr. T. Koshijima, Emeritus Professor of Kyoto University, for his invaluable suggestions about extraction of hemicellulose. The authors also thank Mr. D. Tatsumi and Mr. N. Tamai, Graduate School of Agriculture, Kyoto University, for their helpful suggestions about dissolution of cellulose in LiCl/DMAc.

---

## References

1. Koshijima T (1990) Isolation of lignin. In: Nakano J (ed) Chemistry of lignin – basic and application (in Japanese). Uni Press, Tokyo, pp 41–42
2. Watanabe R, Azuma J, Koshijima T (1987) A convenient method for preparing lignin-carbohydrate complex from *Pinus densiflora* wood. Mokuzaï Gakkaishi 33:793–803
3. Bjorkman A (1956) Studies on finely divided wood. Part I. Extraction of lignin with neutral solvents. Svensk Papperstidn 59:477–485
4. Turbek AF, El-Kafrawy A, Snyder FW, Auerbach AB (1981) Solvent system for cellulose. US patent 4,302,252
5. Dence CW (1992) Determination of acid-insoluble (klason) lignin in wood and pulp. In: Lin SY, Dence CW (eds) Methods in Lignin Chemistry. Springer-Verlag, Berlin, pp 34–39
6. Dence CW (1992) Determination of lignin in wood and pulp by acetyl bromide method. In: Lin SY, Dence CW (ed) Methods in Lignin Chemistry. Springer-Verlag, Berlin, pp 44–48
7. Iiyama K, Wallis AFA (1988) An improved acetyl bromide procedure for determining lignin in woods and pulps. Wood Sci Technol 22:271–280
8. Katahira R, Nakatsubo F (1997) Chemical characterization of *Eucalyptus globulus* lignin: analysis by a modified nitrobenzene oxidation (in Japanese). In: Proceedings of the 42nd lignin symposium, Sapporo, pp 17–20
9. Borchardt LG, Piper CV (1970) A gas chromatographic method for carbohydrates as alditol-acetates. TAPPI 53:257–260
10. Zeronian SH, Cabradilla KE (1973) Further studies on the action of alkali metal hydroxides on cotton. J Appl Polym Sci 17:539–552
11. Schuerch C (1975) The hemicelluloses. In: Browning BL (ed) The chemistry of wood. Robert E. Krieger, Huntington, p 195
12. Tuyet LTB, Iiyama K, Nakano J (1985) Preparation of carboxymethyl cellulose from refiner mechanical pulp. V. Physical and chemical associations among cellulose, hemicellulose and lignin in wood cell walls. Mokuzaï Gakkaishi 31:475–482
13. Isogai A, Ishizu A, Nakano J (1987) Residual lignin in unbleached kraft pulp. Part I. Alkali-extraction of residual lignin from unbleached kraft pulp solution. J Wood Chem Technol 7:311–324
14. Isogai A, Ishizu A, Nakano J (1987) Residual lignin in unbleached kraft pulp. Part II. Analysis of unbleached kraft pulp by new permethylation method. J Wood Chem Technol 7:463–484