Lignified Materials as Potential Medicinal Resources. III. Diversity of Biological Activity and Possible Molecular Species Involved

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Diverse biological activities of hot-water and alkali extracts of lignified materials were reviewed and the molecular species involved are discussed. Materials tested included pine cone of *Pinus parviflora* SIEB. *et* ZUCC., wood chips of slash pine, Douglas fir, and tallow wood, and two basidiocarps, in addition to their partially degraded preparations and commercial lignins. As a tentative conclusion, the lignin structure of these extracts might be responsible for the potent stimulation of granulocytic cell iodination, inhibition of viral infection and/or proliferation *in vitro*, and inactivation of viral ribonucleic acid (RNA)-dependent RNA polymerase and (adenosine diphosphate-ribose)$_n$ glycohydrolase. Other activities displayed by some of these extracts, such as antibacterial and antitumor activities, induction of hemolytic plaque-forming-cells in mice, and stimulation of deoxyribonucleic acid synthesis of isolated splenocytes, remain to be investigated.

Keywords lignified material; lignin; pine cone; slash pine; Douglas fir; tallow wood; basidiocarp; antitumor; antibacterial antiviral; immunopotentiation

In connection with the noteworthy biological activity of the pine cone extract,[1-11] we have recently tested high-molecular-weight fractions of aqueous extracts obtained from several lignified materials for antimicrobial,[3,12,13] antiviral,[6,9,14,15] antitumor,[1] and antiparasite[10] activities, induction of hemolytic plaque-forming-cell in mice,[16] mitogenic activity toward isolated splenocytes,[5] inhibition of viral ribonucleic acid (RNA) synthesis,[9,14,15] stimulation of granulocytic cell iodination,[4,17] *etc.* The present report describes the correlation among the diverse biological activities displayed by lignified materials and partially degraded preparations. Possible molecular species involved in these biological activities are discussed.

Materials and Methods

Materials Wood chips of slash pine (*Pinus clliotti* ENGELM), Douglas fir (*Pseudotsuga Douglasii* CARR.), and tallow wood (*Pentadesma butyraceae* GUTT.) were the gifts of Mr. Hiroshi Nakatsuka, Head of Seishi-Kogyo Shikenjo, Shizuoka Prefecture, Japan. Two lignified basidiocarps, Reishi (*Ganoderma lucidum* KARST.) and Baikisei (*Elfvingia applanata* KARST.) were the gifts of Mr. Yoshiaki Ikeda, Cancer Research Institute, Kanebo Co., Ltd., Osaka. Pine cone of *Pinus parviflora* SIEB. *et* ZUCC. was supplied by Mr. S. Matsuda and Mr. S. Sato. Alkali-lignin, dealkali-lignin, and tannic acid were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo.

Abbreviations: SP, slash pine; DF, Douglas fir; TW, tallow wood; EA, Baikisei; GL, Reishi; PC, pine cone; Al.Lig., alkali-lignin; Deal. Lig., dealkali-lignin.

Preparation of Hot-Water and Alkali Extracts from Lignified Materials Fractions V and VI of the pine cone extract, PC (fr. V) and PC (fr. VI), were the alkaline-eluate (from a diethylaminoethyl (DEAE) cellulose column) of hot-water extract and the acid-precipitate (pH 5) of 1% NaOH extract, respectively, of pine cone of *Pinus parviflora* SIEB. *et* ZUCC., as previously described.[1]

Each of all the other lignified materials (100 g) was ground into small pieces and extracted with 1.0 l of EtOH at room temperature for two 20 h periods. The residue was extracted with methanol in the same way as for the EtOH-extraction. The residue was then extracted three times with 1.0 l boiling water for 3 h each time. The hot-water extract thus obtained was dialyzed against running water for 12 h, and lyophilized: SP(H_2O), 1.4 g; DF(H_2O), 1.8 g; TW(H_2O), 2.9 g; EA(H_2O), 1.1 g; GL(H_2O), 2.5 g. The residue after hot-water extraction was further extracted two times with 1.0 l of 1% NaOH at room temperature for 3 h each time. The extract was dialyzed and lyophilized in the same way as for the hot-water extraction: SP(1% NaOH), 0.5 g; DF(1% NaOH), 1.3 g; TW(1% NaOH), 1.7 g; EA(1% NaOH), 3.2 g; GL(1% NaOH), 1.1 g. The residue after 1% NaOH-extraction was extracted two times with 1.0 l of 4% NaOH at room temperature for 20 h each time. The extract was treated in the same way as in the 1% NaOH extraction: SP(4% NaOH), 0.6 g; DF(4% NaOH), 1.3 g; TW(4% NaOH), 3.1 g; EA(4% NaOH), 11.3 g; GL(4% NaOH), 8.7 g. When GL(4% NaOH) was acidified to pH 5.0, precipitates came out; GL(4% NaOH-ppt.), 2.4 g. The supernatant was dialyzed and lyophilized; GL(4% NaOH-sup.), 6.3 g.

Partial Degradations of PC(Fr. VI) and Al.Lig. with $NaClO_2$ An established method of isolating holocellulose from lignified materials was used.[18] PC(fr. VI) or Al.Lig. (300 mg) was suspended in 15 ml of distilled water and the pH was adjusted to 4 with acetic acid. The suspension was kept at 75 °C, and 225 mg $NaClO_2$ was added in portions. The mixture was kept at the same temperature for 1 h with occasional stirring. To this, 18 μl of acetic acid and 225 mg of $NaClO_2$ were added and kept at 75 °C for another 1 h. This procedure was repeated once more. The reaction mixture was dialyzed against running water for 12 h, and lyophilized: PC(fr. VI-$NaClO_2$), 110 mg; Al.Lig. ($NaClO_2$), 69 mg.

Preparation of Hemicellulose Fraction of the Pine Cone The pine cone after the hot-water extraction was treated with $NaClO_2$ in the way described above for degradation of PC(fr. VI) and Al.Lig. The pale yellow residue was collected on a filter paper and washed with H_2O and then ethanol. Holocellulose thus obtained in a 45% yield was extracted twice with 4% NaOH for 20 h at room temperature. The extract was dialyzed and lyophilized; PC(hemicell) in 9.5% yield.

Partial Degradations of PC(Fr. VI) and Al.Lig. with Sulfuric Acid A conventional method for quantitative analysis of lignin content in lignified materials was used.[18,19] PC(fr. VI) or Al.Lig. (200 mg) suspended in 12 ml of 72% H_2SO_4 was kept at 20 °C for 2 h with vigorous stirring. Then, the concentration of H_2SO_4 was reduced to 3% by the addition of 450 ml of distilled water and the mixture was heated for 4 h in a boiling water bath.

The precipitates (the so-called Klason lignin[18,19]) were collected on a 0.45 μm-Durapore HV filter (acid-stable Millipore filter), and thoroughly washed with distilled water. The precipitates were suspended in distilled water and lyophilized: PC(fr. VI-H_2SO_4-ppt.), 140 mg; Al.Lig.(H_2SO_4-ppt.), 100 mg. The filtrate was dialyzed and lyophilized: PC(fr. VI-H_2SO_4-sup.) and Al.Lig.(H_2SO_4-sup.).

Partial Degradations of PC(Fr. VI) and Al.Lig. with Trifluoroacetic Acid PC(fr. VI) or Al.Lig. (200 mg) was heated for 4 h in 20 ml of 2 M trifluoroacetic acid (TFA) in a boiling water bath. The resultant precipitates were collected on a Millipore filter, and dried under reduced pressure: PC(fr. VI-TFA-ppt.), 136 mg; Al.Lig.(TFA-ppt.), 102 mg. The filtrates were dialyzed and lyophilized: PC(fr. VI-TFA-sup.), 98 mg; Al.Lig.(TFA-sup.), 98 mg.

Ultraviolet (UV) Absorption Measurements UV Spectra were measured in aqueous solutions at room temperature with a Shimadzu-2100 spectrometer.

Biological Activities All activity data are those reported in our previous papers.[1-17] Some communicated to the editors of journals, and some unpublished data are included. Outlines of each experiment are given in the footnotes of Tables I and II, accompanied by the criteria of activity used for qualitative comparison in the present study.

Results and Discussion

As shown in Table I, a qualitative correlation clearly emerges among the biological activities listed therein, although complete data are not all available; stimulation of iodinating ability of polymorphonuclear cells (PMN), inhibition of virion-associated RNA-dependent RNA polymerase, and inhibition of plaque-formation in cells infected with either influenza or herpes virus. It is noteworthy that capacities of PC(fr. VI) and commercial Al.Lig. were both potentiated in all listed biological activities by acid-treatment which is a quantitative analytical procedure to isolate the lignin component from lignified materials. On the other hand, both PC(fr. VI) and Al.Lig. were completely deprived of all activities listed in Table I by treatment with $NaClO_2$ which is known to chemically modify the lignin portion of the lignified materials, but not their holocellulose portion consisting of cellulose and hemicelluloses.

These results suggest that the biological activities shown in Table I are substantially attributed to the lignin portion regardless of the natural origin, but not to the holocellulose portion included in these extracts. It was confirmed that pine cone hemicellulose fraction, PC(hemicell), prepared from the same pine cone by an authentic method,[18] did not show any of the activities listed in Table I. Taking into account that tannins[17,20,21] and some flavonoids[22] which consist of phenolic moieties as well as lignins, also displayed some of the activities listed in Table I, it may be tentatively concluded that the activities listed in Table I are directly related to lignified or oligomeric phenolic structures, but not to hydrolyzable polysaccharide portions attached to or contaminated with the lignin portions in the extracts.

All the preparations showed UV absorption with a maximum at *ca.* 280 nm and a minimum at *ca.* 260 nm accompanied by an end-absorption in the visible region. The spectral shape of these preparations coincided with those of typical lignins reported.[19] The intensity at 280 nm of each preparation is listed in Table I. Absorbances of PC(fr. VI) and Al.Lig. are most intense among all the other extracts. Their absorbances were both decreased by the $NaClO_2$ treatment, and increased by the H_2SO_4 treatment. TW(H_2O), which displayed some activity as shown in Table I, had relatively greater UV absorption among the SP, TW, EA, and GL series of extracts. Thus, it seems to be a trend that the more intense the absorption, the more effective the biological activity. The UV intensity–activity relation seems to support the significance of lignin structure in the biological activities listed in Table I.

Looking at other biological activities tested as shown in Table II, one may notice that there is no parallelism between the activities listed in Tables I and II. Antibacterial activity was displayed by most of the extracts examined, most of which were negative in the activities listed in Table I. PC(fr. VI), which was most effective in Table I, was not superior to other lignified extracts in the activities listed in Table II. TW(H_2O), which was also most active in inhibiting virus infection, was rather inferior to the other extracts. With

Table I. Biological Activities Displayed Possibly by Lignin-Related Component of Lignified Materials

	PMN[a] Iodination	Inhibition[b] RNA synthesis	Inhibition plaque formation		Optical density at 280 nm/mg[e]
			Influenza[c]	Herpes[d]	
PC (fr. V)					0.771
PC (fr. VI)	++	+++	+++	++	1.012
($NaClO_2$)	−	−	−		0.331
(H_2SO_4-ppt.)	+++	+++	+++		
(H_2SO_4-sup.)	−	−	−		0.014
(TFA-ppt.)	+++				
(TFA-sup.)	±	−			0.129
PC (hemicell)	−	−	−		0.136
Al. Lig.	++	++	+	+	1.196
($NaClO_2$)	−	−	+		0.694
(H_2SO_4-ppt.)	+++	+++	+++		1.538
(TFA-ppt.)	+++	+++			1.586
(TFA-sup.)	±	−			0.434
Deal. Lig.	++	++			0.606
SP (H_2O)	−	−	−		0.214
SP (1% NaOH)	+	−	±		0.395
SP (4% NaOH)	+	−	±		0.277
TW (H_2O)	++	+++	+++		0.662
TW (1% NaOH)	±	−	−		0.344
TW (4% NaOH)	−	−			0.152
GL (H_2O)		−	±		0.263
GL (4% NaOH-ppt.)		−	−		0.046
GL (4% NaOH-sup.)		±	−		0.286
Tannic acid	+(15 μg, cytotoxic)			+++	1.651

a) Refs. 4, 15, and 17. Multiple of the iodination in control at 50 μg/ml dose: +++, 100; ++, 100—50; +, 49—20; ±, 19—10; −, <9. After the reaction mixture consisting of 1×10^6 PMN, 4 nmol $Na^{125}I$, and a test sample in serum-free RPMI 1640 medium was incubated at 37 °C for 150 min, the cells were collected and washed with 10% trichloroacetic acid. The radioactivity of acid-insoluble fraction was counted. *b*) Refs. 9, 14, and 15. Inhibition % of viral RNA synthesis at 100 μg/ml dose: +++, 100—90; ++, 89—75; +,74—50; ±, 49—25; −, <24. Inhibitory effect of a test sample on the influenza virion-associated RNA-dependent RNA polymerase activity was measured. *c*) Refs. 9, 14, and 15. Inhibition % of plaque-formation at 100 μg/ml dose: +++, 100—90; ++, 89—75; +, 74—50; ±, 49—25; −, <24. MDCK cells were exposed to influenza virus for 30 min. After being washed, the cells were overlaid with agarose (0.2% bovine serum albumin) containing a sample. After 2—3 days' incubation at 34 °C, plaques were counted. *d*) Ref. 20. ED_{50} (μg/ml): +++, <0.1; ++, 0.1—1.0; +, 1.0—10.0. CV-1 cells were exposed to HSV-1 strain HF in the presence of a sample. After being washed, the cells were overlaid with agarose (2% fetal calf serum) containing each sample, and further incubated for 2 d. Then, plaques were counted. Inhibition was displayed only when the sample was present upon viral infection. *e*) UV absorption intensity per milligram of a sample at 280 nm in aqueous solution.

Table II. Some Other Biological Activities Displayed by Lignified Materials

	Antibacterial[a]		PFC[b] production	Mitog.[c] splenocyte
	E. coli	*P. aerug.*		
PC (fr. V)			+++	+++
PC (fr. VI)	++	+	−	±
Al. Lig.	−		−	−
SP (H_2O)	++	++	+++	
SP (1% NaOH)	++	++	−	
SP (4% NaOH)	++	++		
DF (H_2O)	++	++	−	
DF (1% NaOH)	++	++	−	
DF (4% NaOH)	++	++		
TW (H_2O)	+	+	−	
TW (1% NaOH)	++	++	−	
TW (4% NaOH)	++	++		
EA (H_2O)	++	++	+	
EA (1% NaOH)	++	++	−	
EA (4% NaOH)	++	++		
GL (H_2O)	++	++	−	
GL (4% NaOH-ppt.)	+	++		
GL (4% NaOH-sup.)	++	++		
Tannic acid			−	−

a) Refs. 3, 12, and 13. Survivors out of 10 mice treated with 8 mg/kg of a test sample 2 d before virual inoculation. Survivors in control group were 0—2/10—12 mice: ++, 10—8; +, 7—5; −, <4. *b*) Refs. 16 and 23. Hemolytic PFC assay at a dose of 20 mg/kg mouse (% of control): +++, 400; ++, 399—300; +, 299—150; −, <144. *c*) Ref. 5. Mitogenic activity in isolated splenocytes at 100 μg/ml dose (% of control): +++, 900; ++, 899—500; +, 499—200; ± 199—150; −, <149.

regard to the hemolytic plaque-forming-cell induction, SP(H_2O) was most active, as was PC(fr. V), but it did not show any other activity except antibacterial. As another discrepancy found in relative biological capacities of the extracts, PC(fr. V) was superior to PC(fr. VI) in either hemolytic PFC induction or mitogenicity of isolated splenocytes, as seen in Table II, whereas the former was less effective in antitumor activity than the latter, as previously reported.[1] Tannic acid, which was effective in the activities listed in Table I, displayed no activity in mitogenesis of splenocytes[5] and hemolytic plaque-forming-cell induction.[23] These results suggest that, in addition to the lignin-related structure and tannin-like substances responsible for the biological activities listed in Table I, the extracts might include other active components, each of which might be responsible for its specific target of biological activities listed in Table II. It remains to be determined which molecular species is involved in the diverse biological activities listed in Table II, which are related to immunopotentiation leading probably to antitumor and antiviral activities *in vivo*.

Perspective of Medicinal Utility of Lignified Materials

Little attention[24] has until recently been given to the biological and medicinal utility of lignified materials including lignins themselves, which have long been regarded as cumbersome waste from the pulp industry. Our serial study on lignified materials as a potential medicinal resource, starting with antitumor and anti-human immunodeficiency virus (HIV) pine cone extract, has shed light on the diverse noteworthy biological activities of lignified materials that we recently reported.[1-17] The biological activities listed in Table I were all demonstrated in *in vitro* assay systems including cellular experiments, suggesting that these activities might be achieved through direct interaction of the active principles, probably lignin-related substances, with biopolymers such as capsular proteins of viruses and cellular surface-constituent(s). Tannins, which display activity in some of these assay systems[17,20] including $(adenosine\ diphosphate\text{-}ribose)_n$ glycohydrolase inhibition,[21] are well known for their strong interaction with protein molecules. In fact, when an increased amount of serum was present in the assay system, significant suppression appeared in biological activities concerned. Even so, however, lignin fractions must be capable of serving no less than as medicines for external application to protect the host from viral infections,[6,9,14] *etc.* It is worth mentioning that the alkaline extract of pine cone, which stimulates PMN iodination, *i.e.*, probably activates bactericidal neutrophil leukocytes, protected host mice from various fatal bacterial infections except for *Salmonella enteritidis* which can proliferate even if engulfed in phagocytic leukocytes.[25] This might indicate that the capacity to activate granulocytic cells, as we demonstrated,[4,17] takes place in antibacterial activity *in vivo*.[3,12,13] In addition, it is a fact that lignified extracts contain active principle(s) responsible for immunopotentiation of the host leading to antitumor and antiviral activity, no matter which molecular species is concerned; lignin-related component(s) or some others covalently bound to or associated with the lignin portion in the extract. From other laboratories, several interesting papers were recently published on anti-HIV activity of lignin-related materials; water-soluble lignin from the culture medium of *Lentinus edodes* mycelia[26] and lignin-sulfonate as a pulp-processing waste.[27] In conclusion, lignified materials widely distributed in the environment should be more highly considered as potential resources. In the plant kingdom, long-lived arbores might be endowed by lignification with both mechanical strength toward environmental dynamic forces and biological resistance to microbial and viral infection.

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